NOVEL STRATEGIES FOR BIOANALYSIS OF HYDROPHOBIC ANTIPSYCHOTIC DRUGS AND HYDROPHILIC OLIGONUCLEOTIDE MACROMOLECULES

by

GUODONG ZHANG

(Under the Direction of Michael G. Bartlett)

ABSTRACT

Schizophrenia is a severe psychiatric disorder affecting approximately 1.5% of the world's population. It has long been known that chronic exposure to certain antipsychotics, such as haloperidol, often results in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Given that cognition is now recognized as a key factor that influences long term functional outcome in schizophrenia, it is important to determine if there is a correlation between antipsychotic plasma or brain tissue levels and cognitive function. Since antipsychotic drugs are very active, they are usually administered at low daily dosages. They are extensively metabolized in the body. At steady state, these doses result in plasma levels in the low ng/ml range. In order to carry out pharmacology and toxicology studies, highly sensitive, selective and accurate bioanalytical methods are necessary to determine antipsychotic drugs in biological samples. Most of the antipsychotics are hydrophobic chemicals. It is a challenge to extract them from biological samples, especially from brain tissue. Chapter 1 is the introduction and describes the layout of the dissertation. Chapter 2 reviews the literature for analytical methods for determination of antipsychotic drugs in biological samples. A HPLC-UV method to simultaneously quantitate five antipsychotic drugs including the active metabolite 9-hydroxyrisperidone in rat plasma is included in

Chapter 3. LC-MS/MS methods for the simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat plasma and brain tissue are described in Chapters 4 and 5. In addition, LC-MS/MS methods developed and validated for the determination of highly hydrophobic antipsychotic drugs (chlorpromazine and ziprasidone) in plasma and brain tissue are presented in Chapters 6 and 7.

Phosphorothiate oligonucleotides (PS-ODNs) as therapeutical antisense oligonucleotides are attracting more and more attention. PS-ODNs pose unique analytical challenges. First, PS-ODNs are very hydrophilic macromolecules and highly bound to the biological matrix. It is a challenge to extract them from biological samples. Secondly, it is a challenge to develop analytical methods that enable the baseline separation and quantitation of an intact oligonucleotide, as well as putative metabolites which may differ by one or two nucleotides. A capillary gel electrophoresis method for the determination of a 24-mer oligonucleotide and its chain-shortened metabolites is described in Chapter 8. Sample preparation was performed using a combined phenol/chloroform liquid-liquid extraction, strong anion-exchange column solid-phase extraction and ion-pair HLB solid-phase extraction method.

INDEX WORDS: High performance liquid chromatography, tandem mass spectrometry, LC-MS/MS, HPLC-UV, capillary gel electrophoresis, CGE, solid-phase extraction, liquid-liquid extraction, quantitation, antipsychotics, oligonucleotides.

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GUODONG ZHANG

B.S., Anhui College of Traditional Chinese Medicine, Hefei, China, 1995

M.S., Peking University, Beijing, China, 2001

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GUODONG ZHANG

Major Professor: Michael G. Bartlett

Committee: Warren Beach
Brain Cummings
Alvin Terry Jr
Catherine White

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I would like to dedicate this dissertation to my wife (Meilan), my lovely children (little Charles and Victoria), Mom and Dad, because of their strong encouragement, perseverance and confidence I am able to realize my great dream in life. Thank you for your sacrifice, your support and your unconditional love.

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Chapter 1

Introduction and Literature Review

In routine clinical practice, first-generation antipsychotics (FGAs) (such as haloperidol (HAL) and chlorpromazine (CPZ)) and second-generation antipsychotics (SGAs) (such as risperidone (RISP), olanzapine (OLZ), clozapine (CLOZ) and ziprasidone (ZIP)) are popular for the treatment of schizophrenia and other psychoses. It has long been known that chronic exposure to FGAs such as haloperidol often results in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Furthermore, given that cognition is now recognized as a key factor that influences long-term functional outcome in schizophrenia, it is important to determine if there is a correlation between antipsychotic plasma and brain tissue levels (particularly in association with chronic drug exposure) and cognitive function. In general, oral doses of antipsychotics are on the order of a few milligrams per day. They are also widely metabolized in the body. Therefore, the concentration of these drugs in plasma is very low (pg-ng/ml levels). In addition, therapeutic drug monitoring (TDM) of antipsychotic drugs has proven to be of notable value due to poor compliance of patients and the considerable genetic variability in their metabolism. In order to carry out pharmacology and toxicology studies, clinical TDM, polypharmacy treatment, and drug metabolism of antipsychotics, highly sensitive, selective and accurate bioanalytical methods are necessary to determine these analytes. Most of the antipsychotics are hydrophobic chemicals. It is a challenge to extract them from biological samples, especially from brain tissue. Chapter 2 reviews the most recent bioanalytical methods for the determination of antipsychotics including separation techniques, sample pretreatment, detectors and validated methods. Chapter 3 presents a HPLC-UV method to

simultaneously quantitate five antipsychotic drugs including the active metabolite 9-hydroxyrisperidone in rat plasma with a LLOQ of 2.0 ng/ml. A single step liquid-liquid acid solution back extraction technique with wash procedure was optimized to provide very clear baseline from blank plasma extracts with high recoveries for all of the analytes. Chapter 4 and Chapter 5 present highly sensitive and selective LC-MS/MS methods for the simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat plasma and brain tissue which uses a simple liquid-liquid extraction for sample clean-up. In chapter 6, a LC-MS/MS method was validated for the determination of highly hydrophobic antipsychotic drug chlorpromazine in plasma and brain tissue. A simple liquid-liquid extraction was used to extract chlorpromazine from plasma and brain tissue with high recovery. Gradient elution condition and reconstitution solution were optimized to reduce the matrix effect and improve the chromatographic peak shape. Ziprasidone was another highly lipophilic antipsychotic drug, which was difficult to extract from brain tissue without producing high matrix effects (shown as Chapter 5). Chapter 7 presents a validated method for the determination of ziprasidone in plasma and brain tissue using LC-MS/MS with low matrix effect. Extraction glass vials were siliconized to reduce the adsorption of ziprasidone on the surface.

The development of therapeutic antisense oligonucleotides is moving forward rapidly. They are predominantly phosphorothiate oligonucleotides (PS-ODNs) where one oxygen is replaced by sulfur at a nonbridging position of the phosphodiester linkage. PS-ODNs pose unique analytical challenges. For example, one expected route of metabolism would likely involve the sequential removal of nucleotides from either the 3'

or 5' terminus. The resulting family of oligonucleotides, shortened by one to several nucleotides, may still possess pharmacological activity. It is therefore critical to develop analytical methods that enable the detection and quantitation of the intact oligonucleotide, as well as putative metabolic products which may differ by only one or two nucleotides. In addition, PS-ODNs are very hydrophilic macromolecules and highly bound to the biological matrix. Therefore, it is a challenge to extract them from biological samples. Chapter 8 presents a capillary gel electrophoresis method for the determination of a 24-mer oligonucleotide and its chain-shortened metabolites. Sample preparation was performed using liquid-liquid extraction followed by two SPE methods, which produced a cleaner extraction solution and improved the sensitivity. The methods presented in the following chapters were validated for specificity, linearity, accuracy and precision. The results were acceptable according to the current FDA bioanalytical validation requirements.

Chapter 2

BIOANALYTICAL METHODS FOR THE DETERMINATION OF ANTIPSYCHOTIC DRUGS IN BIOLOGICAL SAMPLES

Zhang G, Terry AV Jr and Bartlett, MG. To be submitted to ***Biomedical Chromatography***.

ABSTRACT: Antipsychotic drugs are popular for the treatment of schizophrenia and other psychoses. In general, oral doses of antipsychotics are on the order of a few milligrams per day. They are widely metabolized in the body. Therefore, the concentration of these drugs in plasma is very low (pg-ng/ml levels). In addition, therapeutic drug monitoring (TDM) of antipsychotic drugs has proven to be of notable value for the poor compliance of patients and the considerable genetic variability in their metabolism. In order to carry out pharmacology and toxicology studies, clinical TDM, polypharmacy treatment, and drug metabolism of antipsychotics, highly sensitive, selective and accurate bioanalytical methods are necessary to determine the analytes. The most recent studies on the determination of antipsychotics will be reviewed including separation techniques, sample pretreatment, detectors and validation methods.

INTRODUCTION

About 20% of prescriptions currently written in the United States are for the treatment of mental process (Gilman *et al.,* 1996). Schizophrenia is a severe psychiatric disorder affecting approximately 1.5% of the world's population (Freedman, 2003). Clinical symptoms of schizophrenia include positive symptoms (auditory hallucinations, disorganized thoughts and delusions), negative symptoms (social withdrawal, lack of motivation) and cognitive dysfunction (disorganized thinking, memory impairments). The high rate of suicide in patients affected by the disease has recently attracted more attention (Siris, 2001). Antipsychotic agents are used in psychiatric patients for the management of psychotic episodes as well as for other behavioral symptoms such as

agitation. Other uses include schizophrenia, mania and delusional disorders. Antipsychotics may also be used in mood disorders (e.g. bipolar disorder) even when no signs of psychosis are present. In routine clinical practice, first-generation antipsychotics (FGAs) (such as haloperidol (HAL) and chlorpromazine (CPZ)) and second-generation antipsychotics (SGAs) (such as risperidone (RISP), olanzapine (OLZ), clozapine (CLOZ), ziprasidone (ZIP), quetiapine (QTP) and aripiprazole (APZ) are popular for the treatment of schizophrenia and other psychoses (Titer *et al.* 2003). SGAs are effective in the treatment of both positive and negative symptoms of schizophrenia and are less likely to produce extrapramidals symptoms (EPS) and tardive dyskinesia (TD) when compared with FGAs such as HAL and CPZ (Ereshefsky, 1996; Reynolds, 2002). SGAs also have many other advantages over FGAs, including greater improvements in negative symptoms, preventions of relapse, increased functional capacity, fewer movement-related side effects, and superior effects on cognition (Miyamoto *et al.*, 2005; Terry *et al.*, 2007). Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. Oral doses of antipsychotics for the treatment of chronic schizophrenia are on the order of a few milligrams per day. In addition, they are widely metabolized in the body. The hepatic metabolizing system of cytochrome P450 (CYP450) plays a key role in the biotransformation of antipsychotics. The major isozymes of CYP450 involved for each antipsychotic are CYP1A2 and CYP2D6 for OLZ; CYP1A2 and CYP3A4 for CLOZ; CYP3A4 and CYP2D6 for RISP; CYP3A4 for QTP; CYP3A4 and aldehyde reductase for

ZIP; CYP1A2 and CYP2D6 for CPZ and CYP3A for HAL. Therefore, the concentration of these drugs in plasma is very low (pg-ng/ml levels). For example, the therapeutic plasma levels of OLZ are in the range of 8-80 ng/ml (Olesen *et al.*, 1999; Gex-Fabry *et al.*, 2003). In the case of HAL levels as low as 2.0 ng/ml have also been described as within the therapeutic range, though most references indicate that 5.0 ng/ml represents the lower end of the therapeutic range. The upper end of the therapeutic range of HAL is 20.0 ng/ml (Volavka *et al.*, 1993; Baldessarini *et al.*, 1988). QTP concentrations between 1.5 and 350.0 ng/ml in human plasma have been observed following administration of a 100.0 mg QTP dose (Thyrum *et al.*, 2000; Diletti *et al.*, 1991). Plasma levels of psychoactive drugs resulting under a given dose are highly variable between individual patients. This is primarily due to inter-individual variations in compliance and in the activities of the various drug metabolizing enzymes. This leads to poor predictability of drug concentrations at a given dose. The mean half-life of OLZ is about 30 hrs and the drug is widely metabolized by the liver, CYP1A2 isoform is responsible for the formation of 4'-N-desmethylolanzapine, while the CYP2D6 isoform is related to the formation of 2-hydroxymethylolanzapine (Callaghan *et al.*, 1999). In patients treated with inhibitors of the CYP1A2 isoform of CYP450 such as fluvoxamine (a selective serotorin reuptake inhibitor) (Raggi *et al.* 2004), OLZ plasma levels appear to be increased, therefore recent clinical trials suggest the coadministration of a subtherapeutic dose of fluvoxamine to reduce the OLZ daily dose (Albers *et al.*, 2005). The need for clinical monitoring of patients undergoing therapy is still evident because the onset of side effects is often related to high plasma concentrations of the drugs. The clinical monitoring of patients can significantly improve the knowledge of

pharmacological interactions among different antipsychotic drugs, as well as enhance the compliance of the patients thus leading to higher treatment efficacy (Raggi, 2002). In the last few years, therapeutic drug monitoring (TDM) of antipsychotic drugs has proven to be of notable value for the poor compliance of patients and the considerable genetic variability in their metabolism. Moreover, TDM has demonstrated to be particularly useful in avoiding severe adverse effects caused by high dosages of the drugs or their interaction with other medications (Mitchell, 2001).

From above mentioned information, reliable bioanalytical methods are needed to determine the analytes at very low concentrations to carry out efficient clinical TDM and drug metabolism studies. In addition, recently, bioanalysis of multiple antipsychotics are attracting more and more attention in pharmacology, toxicology and new SGAs drug discovery and development (Zhang *et al.*, 2007b; Mercolini et al. 2007; Srinivas 2006). Approximately 30 antipsychotic drugs are currently available in the clinic. The pharmacological treatment of schizophrenia is often carried out with the simultaneous use of two or more antipsychotic agents to achieve sufficient control of psychotic symptom. The analytical field as a whole has shown significant technological advancement not only in the field of separation science but also in the availability of sensitive and selective means of detection, such as fluorimetric, electrochemical or mass spectrometry detectors. Also chromatographic columns, novel stationary phases, column switching options, automation tools and improvement in extraction procedures are becoming more frequent in this area. The scope of this review mainly focuses on bioanalytical methods for FGAs and SGAs in biological samples. Different aspects of analytical methods that are addressed include separation techniques, sample

preparation methods, detectors, method validation and future directions in the bioanalysis of FGAs or SGAs and their main metabolites. The main published techniques are summarized in Table 1.

CHROMATOGRAPHIC SEPARATION TECHNIQUES

Separation techniques are typically combined with some type of detection for the analysis of FGAs and SGAs. This section discusses the main types of separation procedures including HPLC, GC and capillary electrophoresis (CE) used in the analysis of antipsychotics and their metabolites in biological samples.

High-performance liquid chromatography

Based on the chemical and physical properties of antipsychotics, most of them are hydrophobic and involatile compounds. As would be expected, they are good candidates for separation using reversed phase HPLC (RP-HPLC). Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. For toxicological purpose, Titer *et al.* (2002) developed a HPLC assay for the simultaneous determination of RISP and 9-OH RISP in human plasma. 0.2 ml of plasma was extracted using a single liquid-liquid extraction and both compounds were separated on a C_{18} column and measured at 280 nm. The absolute recovery was above 77.0%. A good inter-assay accuracy (116%) was achieved with inter-assay precision less than 12%. The LLOQ were 10 ng/ ml. Raggi *et*

al. (2005) developed a HPLC-DAD method for determination of RISP and its main metabolite 9-OH RISP in 0.5 ml of plasma. Separation was obtained on a C8 (150×4.6 mm, 5 µm) column, using a mobile phase composed of acetonitrile (27%) and a pH 3.0 phosphate buffer (73%). A sample clean-up procedure was carried out by using C8 solid-phase extraction cartridges. The extraction yield was about 95%. The LLOQs were 4.0 ng/ml. The method was applied to plasma samples from a patient who had tried to poison himself with 150 mg of RISP, and was undergoing polypharmacy. Llerena *et al.* (2003) reported a HPLC UV method for quantitation of RISP and 9-OH RISP. One ml of plasma was extracted using 4.0 ml of isoamyl alcohol in isopropyl ether (3: 97, v/v). The mean recovery was above 96.8%. The method was used for the determination of the plasma concentrations of a schizophrenic patient treated daily with an oral dose of 4.5 mg RISP. The patient suffered severe extrapyramidal side-effects after adding RISP to his previous medication of HAL and levomepromazine. The RISP plasma concentration was well above the average (75 ng/ml), which suggests that a pharmacokinetic interaction occurred, presumably due to inhibition of the enzyme CYP2D6.

Dusci *et al.* (2002) measured OLZ in plasma using a HPLC-UV method. Back extraction LLE method was developed to extract the OLZ from 1.0 ml of plasma. Although the recovery was not reported, the LLOQ was approximately 1.5 ng/ml. Separation was achieved on a RP Select B C_{18} column and commonly administered drugs did not interfere with the assay. The inter-day and intra-day relative standard deviations were less than 10%. OLZ was shown to be stable in plasma for up to 7 days when stored at 4ºC. The method has been used to measure OLZ concentrations in patients treated with various doses of the drug varying from 5 to 40 mg/day. Gervasini *et*

al. (2003) developed a sensitive method for OLZ using HPLC-MS method (LLOQ: 0.1 ng/ml). 0.5 ml of plasma was extracted with 2 ml of ethyl acetate for 10 min. The organic phase was evaporated to dryness at 37 °C under a stream of nitrogen. After reconstitution of the residue in 75 µL of mobile phase, 30 µL was injected on an Hypersil BDS C18 [125 × 3.0 mm (i.d.); 3 µm particle size] reversed-phase column (Agilent Technologies) and eluted isocratically with a mobile phase containing 780 mL/L formic acid (1 g/L) and 220 mL/L acetonitrile at a flow rate of 0.4 mL/min. The HPLC eluant was introduced in the source via an electrospray ionization interface, generating a positively charged pseudomolecular ion at $[M + H]^+$ (*m/z* 313). The linear range was from 0.1 to 200.0 ng/ml and the recovery was above 94.0%. Midha *et al.* (1981) used HPLC-UV method for determination of CPZ after extracting from 2.0 ml of plasma. The recovery was above 34.5% and LLOQ was 1.0 ng/ml. Recently, Zhang et al. (2007a) report a sensitive HPLC-MS/MS method for determination of CPZ in plasma and brain tissue using a single step LLE. CPZ is a high lipophilic antipsychotic drug and was highly bound to brain tissue (lipids and protein). It was a challenge to extract CPZ from brain homogenate. Gradient elution and reconstitution solution were optimized to improve sensitivity and reduce matrix effectS. The LLOQ was 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue.

Monolithic columns combine the high resolving power of nonporous media under a wide range of flow rate conditions with the high capacity of conventional porous media. In addition, the low backpressure offered by the columns allows the use of high flow rates for faster chromatographic separations. Aboul-Enein *et al.* (2006) carried out the quantitation of HAL and its main metabolite RHAL using a monolithic silica column

(Chromolith Performance RP-18e, 100 × 4.6 mm). The authors compared the direct protein precipitation (method A) and protein precipitation followed by SPE (method B) for sample preparation (0.9 ml of plasma). The results showed the HAL recovery of method A (85.0%) was much higher than that of method B. HAL is a relative hydrophobic drug and direct protein precipitation for HAL was not suitable. The mobile phase consisted of sodium phosphate (0.1 M, pH 3.5)-acetonitrile (80:20, v/v) at a flow rate of 2.0 mL/min. UV detection at 230 nm was used, with the LLOQ 3.0 ng/ml for HAL and 2.0 ng/ml for RHAL.

The technique of column switching can increase the versatility of the liquid chromatograph significantly. The first column is the clean-up column which separates the antipsychotics from lipids, proteins, and other impurities. The antipsychotics will be retained and concentrated on the first column. After clean-up, a six-port valve is switched to connect the clean-up column with the analytical column. Using column switching with an on-line sample clean up may be even more advantageous, since it enables automated sample analysis for routing clinical samples (Weigmann *et al.*, 2001; Sachse *et al.*, 2003, 2006; Yasui-Furukori *et al*., 2004; Kollroser *et al*., 2002; Olesen *et al.*, 1993). Weigmann *et al.* (2001) used a cyanopropyl (CPS) coated column (10 µm; 10 x 2.0 mm I.D.) for clean-up. Interfering serum constituents were washed off to waste, and the antipsychotics were next separated on a C18 ODS Hypersil reversed phase material (5 µm; 250 x 4.6 mm I.D.) using acetonitrile-water-tetramethylethylenediamine (37:62.6:0.4, v/v/v) adjusted to pH 6.5 with concentrated acetic acid. UV-detection was performed at 254 nm. The LLOQ was 10-20 ng/ml. Relative day to day standard variations ranged between 4.5 and 13.5%. The method was suitable for routine

monitoring of OLZ and CLOZ including their demethylated metabolites. Yasui-Furukori *et al.* (2004) describes a method for determination of HAL and RHAL using automated column-switching HPLC. The test compounds were extracted from 1ml of plasma using chloroform-hexane (30:70 (v/v)), and the extract was injected into a hydrophilic metaacrylate polymer column for clean-up and a C_{18} analytical column for separation. The mobile phases consisted of a phosphate buffer (0.02M, pH 4.6), perchloric acid (60%) and acetonitrile (54:1:45 (v/v)) and was delivered at a flow-rate of 0.6ml/min. The peak was detected using a UV detector set at 215nm. The method was validated over the concentration range from 1-100ng/ml, and good linearity (r >0.999) was observed. A specific and sensitive direct-injection HPLC/ESI-MS/MS method was developed by Kollroser *et al.* (2002). Plasma samples were directly injected into the LC/MS/MS system. Proteins and other large biomolecules were removed during an online sample cleanup using an extraction column (1 x 50 mm i.d., 30 µm) with a 100% aqueous mobile phase at a flow rate of 4 mL/min. The extraction column was subsequently brought inline with the analytical column by automatic valve switching. Analytes were separated on a 5 µm Symmetry C18 (Waters) analytical column (3.0 x 150 mm) with a mobile phase of acetonitrile/0.1% formic acid (20:80, v/v) at a flow rate of 0.5 mL/min. The total analysis time was 6 min per sample. By eliminating the need for extensive sample preparation, the proposed method offers very large savings in total analysis time.

Gas chromatography

GC is used far less often than HPLC in the analysis of FGAs and SGAs. Antipsychotics are involatile and therefore much better suited for HPLC analysis.

Bianchetti *et al.* (1978) described a sensitive gas-chromatographic method for quantitative analysis of HAL in human plasma. 2.0 ml of plasma was extracted using back extraction LLE method. The use of nitrogen-phosphorus selective detection (NPD) reduces the time required for analysis. The LLOQ was 1.0 ng/ml. Azaperone is used as the internal standard. The method is suitable for the determination of HAL plasma levels in patients treated with doses ranging from 1.2 to 200 mg/day. No interference from drugs needed in the associated antipsychotic therapy have been found. The simplicity, specificity and sensitivity of the method make it suitable for routine analysis of HAL plasma levels in psychotic patients undergoing chronic treatment. Hattori *et al.* (1986) report a GC-MS method for quantitation of HAL in plasma or urine (1.0 ml). Positive-ion electron-ionization (EI), positive-ion chemical ionization (CI) and medium-pressure negative-ion CI mass spectra of HAL were presented. A procedure for the extraction of CPZ from human urine and plasma was developed to serve for their identification in forensic science.

McKay *et al.* (1982) used GC-MS for the quantitation of chlorpromazine in plasma and compared the result with a HPLC assay using electrochemical detection (ED). Measurements were made after extraction of chlorpromazine and the internal standard, prochlorperazine, from basified plasma with an isopropanol--pentane solvent mixture for single-dose studies of CPZ. Following evaporation of the organic solvents the residue was reconstituted in a small volume of methanol and subjected to GC-MS. The recovery was 86.0%. The specific method displayed excellent correlation for plasma concentration determinations in the range of 0.25-10 ng/ml and applied for the study of the pharmacokinetics of chlorpromazine following single low doses of the drug.

Recently, de la Torre *et al.* (2005) reported a simple and reliable GC-NPD method without derivatization for determination of CPZ, OLZ, CLOZ and HAL in whole blood and brain tissue. The SPE method was optimized for 0.1 ml of whole blood or brain homogenate to improve the recovery and LLOQ. Linearity was observed in the studied range for all compounds with correlation coefficient (R^2) values of >0.999. Jenkins *et al.* (1998) described the determination of OLZ in biological specimens including blood, bile, gastric contents, cerebrospinal fluid and urine specimens using GC-MS. However, no detailed validation data was reported.

Capillary electrophoresis

CE, a recent analytical technique, is another technique not as widely used as HPLC for the analysis of antipsychotics and their metabolites. CE is a powerful tool which offers very high resolution capability and high efficiency in a short time. Furthermore the use of very low volumes of buffer (μL) and samples (nL) make this new technique very interesting for rapid and practical analysis also in the biomedical field (Desiderio *et al.*, 1994, 1999; Strickmann *et al.* 2000; Ramseier *et al.* 1999). The main drawback when using CE is a high LLOQ.

Raggi et al. (2001) developed a CE-UV method for the determination of CLOZ and DMC in human plasma. The separation of the two analytes was carried out in an untreated fused-silica capillary [33 cm (8.5 cm effective length) x 50 μm I.D.] filled with a background electrolyte at pH 2.5 containing β-cyclodextrin. Baseline separation of CLOZ and DMC was recorded in less than 3 min. Oasis HLB SPE cartridges were used to extract the analytes from 0.5 ml of plasma. The method showed good precision (mean RSD = 4.0%) as well as satisfactory extraction yields (approximately 88%) with a

LLOQ of 100.0 ng/ml. Ho *et al.* (2004) report a field-amplified sample stacking capillary electrophoresis (FASS CE) method for the simultaneous determination of CLOZ and its metabolites, CNO and DMC in human plasma. FASS CE is based on the idea that ions electrophoretically migrating through a low-conductivity solution into a high-conductivity solution slow down dramatically at the boundary of the two buffers and stack into a narrow zone. This method could result in up to a 100-fold sensitivity enhancement and can be applied to biological materials. Plasma (0.2 mL) was extracted with organic solvents (ethyl acetate/*n*-hexane/isopropyl alcohol, 8/1/1 by volume) and centrifuged. An aliquot of supernatant was evaporated and suitably reconstituted with water for CE analysis. An untreated fused-silica capillary was used (31.2 cm; effective length, 20 cm; 50 μm i.d.) for the analysis. The LLOQ was 50.0 ng/ml for CLOZ, 25.0 ng/ml for DMC and 30.0 ng/ml for CNO.

Lara *et al.* (2005) described a validated of CE-UV method for determination of CPZ in human urine with high sensitivity using field-amplified sample injection (FASI). Separation was obtained on a 64.5 cm x 75 μm bubble cell capillary using a buffer containing 150 mM tris(hydroxymethyl)aminomethane and 25% acetonitrile at pH 8.2, with temperature and voltage of 25 degrees C and 20 kV, respectively. Oasis HLB SPE procedure was developed to extract CPZ from 5.0 ml of urine. The recovery was 94.5% and LLOQ was as low as 8.0 ng/ml. In addition, Li *et al.* (2006) used CE-ECL method to determine CPZ in urine. ECL is a sensitive detection method, which has advantages of high sensitivity, good selectivity, a wide dynamic linear range, simple and inexpensive instrumentation (Xu *et al.* (2000)). 0.2 ml of urine was extracted using 90:10

heptane/ethyl acetate (v/v) to eliminate the influence of ionic strength in the sample. The recovery was above 83.0% and LLOQ was 8.0 ng/ml.

SAMPLE PRETREATMENT TECHNIQUES

Antipsychotic drugs are widely distributed in the human body. Before analyzing for antipsychotics and their metabolites, it is often necessary to extract the compounds from the biological matrix. Sample pretreatment is a critical step in the analysis of drugs from biological fluids (Raggi, 2002). There are four main methods used for extraction of these compounds. These include: LLE, SPE, Protein precipitation, and direct injection of biological samples without sample preparation. Each of these methods has advantages as well as disadvantages. How each type of extraction works and in which situation each works best will be discussed in this section.

Liquid-liquid extraction

LLE, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. Hydrophilic compounds will have an affinity for the aqueous phase and hydrophobic compounds will have affinity for the organic phase. Therefore, the analyte will partition into the solvent that offers the greatest solubility. The two solvents should be immiscible to allow for facile isolation. Disadvantages to using LLE include emulsion formation and the mutual solubility of the analytes in the two phases. Advantages of LLE include the solvents being usually readily available and inexpensive. Although LLE is a very traditional extraction means, it is particularly useful for extraction of the antipsychotics, which are lipophilic compounds from biological samples.

LLE was used to extract RISP and 9-OH RISP from plasma (Titer *et al.*, 2002; LLerena *et al*., 2003; Zhang *et al*., 2005). Aravagiri *et al.* (2000) used LC-MS/MS for the quantitation of RISP and 9-OH RISP from plasma. A simple LLE was described to get high recovery. 0.5 mL of plasma was extracted with 7 mL of 15% methylene chloride in pentane to isolate the compounds. The LLOQ was 0.1 ng/ml and recovery was about 84.0%. Some LLE methods for OLZ, HAL, CLOZ and CPZ were reported (Gervasini *et al.*, 2003; Nirogi *et al.,* 2006; Hattori *et al.,* 1986; Yasui-Furukori *et al*., 2004; McKay *et al*., 1982; Li *et al*., 2006;, Midha *et al*., 1981; Zhang *et al*., 2007; Garcia *et al*., 2003; Ho *et al*., 2004; Chung *et al*., 1993). Berna *et al*. (2002) described a LC-MS/MS method for the determination of OLZ from whole blood. Liquid-liquid extraction, using n-butanol:cyclohexane (3:47, v/v), was used to isolate OLZ from 250 µL of whole blood instead of using a more traditional SPE approach. Detection occurred using a Perkin-Elmer Sciex API III Plus triple quadrupole mass spectrometer using positive ion APCI and multiple reaction monitoring (MRM). The linear dynamic range was from 5 to 500 ng/ml in blood. The inter-day precision (%RSD) and accuracy (%RE) ranged from 3.65 to 10.64 and from -2.14 to 3.07, respectively. The recovery was above 73.6% and LLOQ was 5.0ng/ml.

Garcia *et al.* (2003) used a single LLE step to extract CLOZ from plasma. 1.0 ml of plasma was extracted with 2.0 ml of diethyl ether. The analysis was performed on a XTerra MS C18 column with UV linear in the range 50-1000 ng/ml. This isocratic and rapid method (run time<10 min) is useful for the management of acute detection. The LLOQ was 15.0 ng/ml. Chung *et al.* (1993) report a method using RP HPLC for the simultaneous determination of CLOZ and its DMC metabolite in human plasma. CLOZ

and DMC were extracted with n-hexane-isoamyl alcohol (98.5:1.5, v/v). The LLOQ is 2.0 ng/ml. The sensitivity and precision of this method can be utilized for pharmacokinetic studies and therapeutic drug monitoring regimens. Among the antipsychotics, ZIP and APZ are among the most hydrophobic compounds. It is not easy to use SPE to extract them from biological samples. Therefore, it is not surprising that several LLE methods have been reported for them (Sucknow *et al*., 2004; Al-Dirbashi *et al*., 2006; Aravagiri *et al*., 2007; Shimokawa *et al*., 2005; Kubo *et al*., 2005). An accurate, rapid and simple LC-MS/MS assay method was developed for the determination of ZIP in the plasma of schizophrenia patients (Aravagiri et al., 2007). 0.5 ml of plasma was extracted using a simple one step LLE with 20% methylene dichloride in pentane. The absolute extraction efficiency was 82%. The ZIP standard calibration curve was linear over the range of 0.25-500 ng/ml.

Shimokawa *et al*. (2005) described a HPLC-UV method using LLE to extract APZ from 0.5 ml of plasma and 1.0 ml of brain homogenate. The recovery of APZ was above 87.2% for plasma and only 41.3% for brain. The LLOQ was 10.0 ng/ml for plasma and 30.0 ng/g for brain tissue. Validated HPLC methods were successfully applied to pharmacokinetic study APZ rats, demonstrating brain concentrations after oral administration five times higher than plasma concentrations. A LC-ESI-MS/MS method combined with a simple LLE has been developed for the measurement of QTP in human plasma and in human liver microsomes (HLM) (Lin *et al*., 2004). 0.1 ml of plasma was extracted with 3.0 ml *n*-butyl chloride. The recovery was above 62.0% and LLOQ for QTP was 1.0 ng/ml. In addition, Shen *et al*. (2002) report a HPLC-UV method for determination of multiple antipsychotcs (CLOZ, DMC, CNO, RISP and 9-OH RISP).

1.0 ml of plasma was extracted with LLE two times. The recovery was above 93.0% and the LLOQ was 5.0 ng/ml. Recently, Zhang *et al*. (2007c) developed a simple and sensitive HPLC-UV method for simultaneous quantification of OLZ, HAL, CPZ, ZIP, RISP and its active metabolite 9-OH RISP in rat plasma using imipramine as an internal standard (I.S.). The analytes were extracted from rat plasma (1.0 ml) using a single step liquid-liquid acid solution back extraction technique with wash procedure, which provided a very clear baseline from plasma. The compounds were separated on an Agilent Eclipse XDB C8 (150 mm x 4.6mm i.d., 5μm) column using a mobile phase of acetonitrile/30mM ammonium acetate including 0.05% triethylamine (pH 5.86 adjusted with acetic acid) with gradient elution. All of the analytes were monitored using UV detection. The method was validated and the linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries, selectivity and stability were determined. The LLOQ was 2.0 ng/ml and the recovery was above 74.8% for all of the analytes. This validated method has been successfully used to quantify the plasma concentration of the analytes for pharmacological and toxicological studies following chronic treatment with antipsychotic drugs in the rat.

Solid-phase extraction

As a sample preparation procedure, solid phase extraction (SPE) is growing in use as an effective method for sample pre-treatment and clean-up of biological samples in all types of drug testing laboratories. SPE offers several advantages over traditional LLE, including better specificity, the ability to obtain cleaner extracts, good reproducibility, avoidance of emulsion formation, the ability to automate the extraction procedure, and a substantial reduction in the volume of solvents required. SPE is an isolation technique in

which the analyte is (usually) retained on a cartridge, while undesirable chemical entities in the sample matrix either pass through or are irreversibly bound to the cartridge. The analyte is subsequently eluted from the cartridge and is commonly evaporated to dryness and reconstituted. SPE cartridges are essentially small, disposable HPLC columns. Several different sorbents (packing material to which analyte adsorbs) and cartridge sizes are available with SPE. Sorbents available include C_{18}, C_8, C_2, phenyl, amino, HLB, polymer, WAX, SAX, WCX and SCX. SPE methods are very popular for the determination of antipsychotics in biological samples (Raggi *et al*., 2005; Berna *et al*., 1998; Bogusz *et al*., 1999; Catlow *et al*., 1995; Lara *et al*., 2005; Olesen *et al*., 1993; Gupta, 1995; Barrett *et al*., 2007; Josefsson *et al*., 2003; Frahnert *et al*., 2003; Mercolini *et al*., 2007; Hasselstrom *et al*., 2003).

Remmerie *et al*. (2003) used LC-MS/MS method for the determination of RISP and 9-OH RISP in human plasma using a mixed-mode phase SPE cartridge (Bond Elut Certify). SPE with a mixed-mode phase, containing both weak ion-exchange and reversed-phase functionalities, was applied at pH 6 in order to affect the retention of the positively charged analytes based on both cation exchange and hydrophobic interactions. Similar and almost complete extraction recoveries were found for both RISP and its more polar 9-OH RISP metabolite, indicating that the analyte extraction is mainly due to ion-exchange effects. This means that the selectivity of sample preparation is complementary to the (reversed-phase) selectivity of the subsequent chromatographic separation, which is advantageous for the robustness of the total analytical procedure. The recovery was above 90.0%. The LLOQ was 0.1 ng/ml using 0.5 ml of plasma. de la Torre *et al.* (2005) also optimized different SPE cartridges for

extraction of CPZ, OLZ, CLOZ and HAL from 0.1 ml of blood using Bond Elut Certify cartridges. The use of the mixed-mode bonded-silica Bond Elut Certify columns showed advantages when compared with Chem Elut columns for the screening of antipsychotic agents such as higher recoveries, cleaner extracts, better sensitivity, better precision and less solvent consumption and subsequent disposal.

The characteristics of automated on-line solid phase extraction with liquid chromatography-mass spectrometry (SPE-LC-MS) are flexibility and high throughput in therapeutic drug monitoring (TDM). Recently Niederlander and co-workers developed a flexible, automated, on-line SPE-LC-MS for the analysis of CLOZ and its main metabolites (DMC and NOX) in serum (Niederlander *et al*., 2006). This method was based on an automated cartridge exchange SPE system coupled to LC-MS. Optimization of chromatographic and SPE conditions for increased throughput resulted in a SPE-LC-MS cycle time of only 2.2 min, demonstrating the great potential of automated on-line SPE-LC-MS for TDM. The new method was shown to be clearly favorable, in particular in terms of ease of sample handling, throughput and detection limits. 50.0 µL of serum was injected onto the SPE cartridges. Recovery was essentially quantitative (above 90.0%) and the LLOQ was 50.0 ng/ml.

Janiszewski *et al.* (1997) reported an automated sample preparation method using membrane microtiter extraction for bioanalysis of ZIP in serum. The development and application of membrane SPE in 96-well microtiter plate format was described for the automated analysis of drugs in biological fluids. The small bed volume of the membrane allows elution of the analyte in to a very small solvent volume, permitting direct HPLC injection and negating the need for the time consuming solvent evaporation step. A

programmable liquid handling station (Quadra 96) was modified to automate all SPE steps. To avoid drying of the SPE bed and to enhance the analytical precision a novel protocol for performing the conditioning, loading and washing steps in rapid succession was utilized. A block of 96 samples could be extracted in 10 min., about 30 times faster than manual solvent extraction or single cartridge SPE methods. This sample processing method complements the high-throughput speed of contemporary HPLC/MS analysis. The quantitative analysis of ZIP in plasma demonstrates the utility and throughput of membrane SPE in combination with HPLC/MS. The recovery was up to 76.0% and LLOQ was 1.0 ng/ml from 0.3 ml of serum.

An original HPLC-UV method (Saracino *et al.* 2006) was developed for the determination of QTP in human plasma using mixed-mode cation exchange (DSC-MCAX) SPE cartridges. 0.3 ml of plasma was loaded onto the conditioned SPE cartridge. The cartridge was sequentially washed with: 1mL of a pH 6.0, 25mM phosphate buffer, then 1mL of methanol and finally 50 µL of a mixture of methanol (95%, v/v) and 7.6 M aqueous ammonia (5%, v/v). The analytes were subsequently eluted with 1mL of the same methanol/ammonia mixture. Extraction yield values were always higher than 93%. The method LLOQ was 5.0 ng/ml.

SPE methods have also been used for the determination of multiple antipsychotics from biological samples. Frahnert *et al.* (2003) used SPE to extract CLOZ, DMC, CNO, RISP, 9-OH RISP and QTP from 1.0 ml of whole blood. The recovery was above 91.2%. The LLOQ was 5.0 ng/ml for QTP, RISP and 9-OH RISP, 10.0 ng/ml for CLOZ and 100.0 ng/ml for DMC with HPLC-UV detection.

Recently, Mercolini *et al.* (2007) developed a HPLC-UV method for the simultaneous determination of CLOZ, DMC, CNO, RISP, 9-OH RISP, HAL, OLZ, DMO, ZIP and CPZ using special cyanopropyl cartridges, which gave a high extraction yield (above 93%). Such a suitably selective sample pre-treatment procedure is usually needed when using a technique, such as HPLC-UV, which is highly reliable and has wide applicability, but is susceptible to interference from endogenous and exogenous interference. The authors optimized different SPE cartridges. The first sorbent tested was an HLB (hydrophilic-lipophilic balance) resin, owing to its characteristics of versatility and to the different physicochemical properties of the analytes. However, the HLB sorbent did not confer sufficient selectivity to the method and severe interference was detected. Lipophilic sorbents such as C_8, C_2 and C_1 provided low yields of some analytes. Since all analytes had at least one aromatic ring in their structure, the phenyl (PH) sorbent was also tested; however, it provided poor results, with low yields and numerous interference peaks. Cyanopropyl (CN) cartridges, however, possessed an interesting mixture of hydrophilic, lipophilic and hydrogen-bond-forming properties. Preliminary assays with this sorbent provided promising results in terms of both the extraction yields and selectivity. The LLOQ was 2.6 ng/ml.

Protein precipitation

Protein precipitation is a type of extraction in which either a solvent (typically ice-cold acetonitrile or methanol) or an acid (typically trichloroacetic acid or perchloric acid) is added to a sample to denature the proteins. Typically, a volume of protein precipitation reagent equal to three or four times the sample volume is added to each biological sample. Protein precipitation may be performed alone or in conjunction with another

extraction technique. For example, protein precipitation is commonly performed prior to SPE.

Flarakos *et al*. (2004) developed a robust LC-MS/MS for the determination of RISP and 9-OH RISP in plasma and saliva using protein precipitation followed by column switching. The method used 25 µL of sample precipitated with 75 µL of acetonitrile containing the internal standard (R068808). Analyses were conducted on a PE Sciex API-III+ triple quadrupole mass spectrometer fitted with a Turbo IonSpray source. The method was validated for human plasma using EDTA as the anticoagulant and cross-validated to heparinized human plasma and saliva. The recoveries of RISP and 9-OH RISP were 90-93 and 89-93%, respectively. The validated method was applied to clinical samples to study RISP and 9-OH RISP concentrations in plasma and saliva.

Bhatt *et al.* (2006) described another simple protein precipitation method for RISP and 9-OH RISP using LC-MS/MS. A 0.1mL aliquot of human plasma sample was mixed with 10µL of the internal standard working solution. Then, 250µL of acetonitrile was added and the mixture was vortexed-mixed. The sample was centrifuged at 12000 rpm for 5 min. The supernatant layer was removed and a volume of 10µL was injected into the LC-MS/MS system. The overall recoveries for RISP and 9-OH RISP were 82.1% and 83.2%, respectively. Aboul-Enein *et al.* (2006) described a protein precipitation followed by SPE method to extract HAL and its metabolites from human plasma. This method greatly improved the recovery of HAL and its metabolites.

Recently, Kirchherr *et al.* (2006) developed a LC-MS/MS method for the determination of CLOZ, RISP, 9-OH RISP, QTP, HAL, APZ, CPZ, OLZ and ZIP in human serum using direct protein precipitation. Chromatographic separation was necessary for isobaric

mass fragments and performed on a monolithic C18 column (50mmx4.6mm) with a methanol gradient and 5mM acetate buffer at pH 3.9. The injection interval was 8 min. The recovery was above 95.0%. The LLOQ was 1.0 ng/ml for HAL and RISP, 5.0 ng/ml for 9-OH RISP, 10.0 ng/ml for OLZ, APZ, QTP, ZIP and CPZ, and 100.0 ng/ml for CLOZ.

Direct injection of crude biological samples without pretreatment

The classic methods of plasma sample pretreatment such as liquid–liquid extraction or solid phase extraction are still favored by many analysts and allow excellent analyte recovery and cleaner samples. However, they require additional labor and are often time-consuming. Recently, a new HPLC polymer stationary phase (MSpak GF-310), which enables direct injection of crude biological samples into the HPLC column, has been developed for use in HPLC–MS in Japan. Arinobu *et al.* (2002) present a rapid HPLC–MS analysis for haloperidol and its two metabolites, RHAL and CPHP by direct injection of human plasma and urine using the MSpak GF-310 column without sample pretreatment and without using column switching. The separation by the GF-310 column is based on the size exclusion chromatography (SEC) principle associated with slight action of partition and adsorption, and this column is suited to eliminate proteins, nucleic acids and polysaccharides from biological samples, because their molecular size is too large to enter the pores of the stationary phase, whereas drugs with small molecular masses can enter the pores and be retained on the polyvinyl alcohol phase; the principle has enabled the direct injection of crude biological samples. The recovery was above 50.0%. The LLOQ was 10.0 ng/ml for HAL, 15.0 ng/ml for RHAL and 400.0 ng/ml for CPHP. Pistos and Stewart (2003) have published a direct injection HPLC-UV method for the determination of CPZ in plasma using a Hisep column. Hisep columns use

specially designed stationary phases to restrict the interaction of serum proteins with partitioning of the analyte. 20 µL of plasma was injected onto the column. The recovery was 70.0% and the LLOQ was 100.0 ng/ml.

DETECTORS

Several types of detectors have been used for the determination of antipsychotics in biological samples. The bioanalysis of antipsychotics and their metabolites requires high sensitivity, specificity, stability and dynamic range. The detectors discussed in this section mainly include mass spectrometers (MS), ultraviolet detection (UV), fluorescence detection (FD), electrochemical detection (ED) and nitrogen phosphorous detection (NPD). Mass spectrometers are commonly used with GC, HPLC and less frequently with CE. UV, ED, and FD detectors are commonly used in conjunction with HPLC or CE analysis. NPD detector is used almost exclusively with GC.

Mass spectrometers

The mass spectrometer is the leading detector for antipsychotics. This detector has outstanding sensitivity, stability, specificity and dynamic range. Mass spectrometry has been widely used in identification, quantification and structure elucidation. Quadrupole mass spectrometers are the most commonly used mass spectrometers in quantitative analysis. The main disadvantage of quadrupole mass spectrometers is poor mass resolution (typically 1 Da). This, however, is more of a drawback when analyzing compounds with larger molecular weights and multiply-charged compounds (such as proteins, peptides and oligonucleotides) and is not typically an issue with small

molecules. When coupled with chromatography and run in SIM mode, single quadrupole instruments yield chromatograms containing peaks that are only of the mass(es) of interest. Triple quadrupole mass spectrometers have the added advantages of even greater specificity and sensitivity. The selection of specific analyte ion(s) with the first quadrupole and diagnostic abundant fragment ion(s) with the third quadrupole results in a technique called multiple reaction monitoring (MRM). Many sensitive GC-MS, LC-MS or LC-MS/MS methods for bioanalysis of antipsychotics have been published (Niederlander *et al*., 2006; Zhang *et al*., 2007a, 2007b, 2007d; Janiszewski *et al*., 1997; Al-Dirbashi et al., 2006; Aravagiri *et al*., 2000, 2007; Lin *et al*., 2004; Barrett *et al*., 2007; Kubo *et al*., 2005; Josefsson *et al*., 2003; Kollroser *et al*., 2002; Zhou *et al*., 2004; Kirchherr *et al*., 2006; Li *et al*., 2007; Remmerie *et al*., 2003; Bhatt *et al*., 2006; Flarakos *et al*., 2004; Bera *et al*., 1998, 2002; Bogusz *et al*., 1999; Gervasini *et al*., 2003; Nirogi *et al*., 2006; Hattori *et al*., 1986; Arinobu *et al*., 2002; McKay *et al*., 1982). Zhang *et al.* (2005) described a LC-MS method for the quantitation of RISP and 9-OH RISP in human plasma. 0.5 ml of plasma was extracted using LLE with a recovery of 74.9%. Zhou *et al.* (2004) developed a LC-MS method for simultaneous determination of CLOZ, OLZ, RISP and QTP in plasma using LLE extraction. The recovery was above 80.7% and the LLOQ was 20.0 ng/ml for QTP and CLOZ and 1.0 ng/ml for OLZ and RISP. Bhatt *et al.* (2006), Flarakos *et al.* (2004) and Kirchherr *et al.* (2006) developed several rapid and high throughput LC-MS/MS methods for the quantitation of antipsychotics in biological samples just using simple protein precipitation or column-switching methods for sample pretreatment. Kubo et al. (2005) described an accurate, sensitive, reproducible, and selective liquid

chromatography/tandem mass spectrometry (LC-MS/MS) method for determination of APZ and its main metabolite, OPC-14857, in human plasma was developed and validated. Chromatographic separation was achieved isocratically on a C_{18} reversed-phase column within 7.5 min. The calibration curve, ranging from 0.1 to 100 ng/ml. The assay showed no significant interference. The LLOQ for both analytes was 0.1 ng/ml using 0.4 ml of plasma and the recovery was above 83.1%. Josefsson *et al*. (2003) report a LC-MS/MS method for the quantitation of CPZ, OLZ, CLOZ and HAL. 1.0 mL of whole blood was extracted using SPE. However, a full method validation was not performed since the objective was to evaluate the method design rather than to validate a final method set-up. Recently, Zhang *et al*. (2007b, 2007d) developed the LC-MS/MS methods for the simultaneous quantification of OLZ, CLOZ, ZIP, HAL, RISP, and its active metabolite 9-OH RISP, in rat plasma and brain tissue using midazolam as an internal standard (IS). The analytes were extracted from rat plasma or bain homogenate using LLE. The compounds were separated on a Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 μm) column using a mobile phase of acetonitrile/5 mM ammonium formate (pH 6.1 adjusted with formic acid) with gradient elution. All of the analytes were detected in positive ion mode using MRM. The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. The LLOQ was 0.1 ng/mL in plasma and 0.208 ng/g for RISP, 9-OH RISP, CLOZ and OLZ; 0.416 ng/g for HAL and ZIP in brain tissue. The relative and absolute recovery was above 77.0% for plasma and above 73.6% for brain tissue. The matrix effects were low for all the analytes except for ZIP.

This validated method has been successfully used to quantify the plasma and brain tissue concentration of the analytes after chronic treatment with antipsychotic drugs.

Ion trap mass spectrometers have the ability to perform MS^n analysis, which is a major advantage of ion trap mass spectrometers when performing structure elucidation studies. A disadvantage of ion trap mass spectrometers is that, while they are good for qualititative work, they do not work as well for quantitative studies. Calibration curves do not proportionally increase at higher concentrations, as is the case in the lower and middle portions of calibration curves (i.e. the calibration curves begin to flatten off at the high end). Newer ion trap instruments known as linear ion traps have a different design that circumvents these problems. Kollroser *et al*. (2002) described a LC-MS/MS method for the determination of OLZ, CLOZ and DMC using an ion trap mass spectrometer. The calibration curve range was from 5.0 ng/ml to 800.0 ng/ml.

Ultraviolet detection

UV detection is used most commonly with HPLCs and CEs. Compounds are detected with UV detection as absorbance of UV radiation. A wavelength of UV light at which the analyte most strongly absorbs is chosen for monitoring by the detector. Generally speaking, almost all of the antipsychotics on the market have UV absorption. HPLC-UV methods are very popular for the determination of antipsychotics in biological samples (Titer *et al*., 2002, 2003; LLerena *et al*., 2003; Dusci *et al*., 2002; Aboul-Enein *et al*., 2006; Pistos *et al*., 2003; Midha *et al*., 1981; Mosier *et al*., 2003; Garcia *et al*., 2003; Chung *et al*., 1993; Olesen *et al*., 1993; Gupta *et al*., 1995; Saracino *et al*., 2006b; Hasselstrom *et al.* 2003; Shimokawa *et al*. 2005; Sachse *et al*., 2006; Shen *et al*., 2002;

Frahnert *et al*. 2003; Zhang *et al*., 2007c; Mercolini *et al*., 2007). In addition, Lara *et al*. (2005) and Ho *et al*. (2004) described CZE-UV methods for the quantitation of antipsychotics in biological samples.

Nitrogen phosphorus detection

NPDs are commonly used with GC methods. The NPD interfaced with GC has proven to be a powerful tool in the area of underivatized drug analysis in biological extract. Since many drugs are nitrogen-containing compounds, traces of them can be selectively detected in sample screening with excellent sensitivity and minimal interference from other non-nitrogenous compounds, both endogenous and exogenous (Lora-Tamayo *et al*., 1986). Bianchetti *et al.* (1978) described a GC-NPD for the determination of HAL in 2.0 ml of plasma with the LLOQ (1.0 ng/ml). de la Torre *et al*. (2005) used a GC-NPD method for simultaneous determination of CPZ, OLZ, CLOZ and HAL in whole blood.

Fluorescence detection and electrochemical detection

FD and ED are two other detectors beside mass spectrometers for sensitive analytical methods for the determination of antipsychotics in biological samples. Suckow *et al*. (2004) described a sensitive HPLC-FD method for the quantitation of ZIP in plasma. 1.0 ml of plasma was extracted using LLE method. The recovery was above 85.0% and the LLOQ was 0.5 ng/ml. Recently, Kishikawa *et al*. (2006) report a HPLC-FD method for the determination of HAL and RHAL in serum. 0.5 ml of serum was extract with LLE followed by pre-column derivatization method with a LLOQ of 1.88 ng/ml. Higashi *et al*.

(2006) also used a HPLC-FD for the quantitation of CPHP in 0.2 ml of plasma using LLE followed by pre-column derivatization. The LLOQ was 30.0 ng/ml. In addition, LeMoing *et al*. (1993) developed a sensitive HPLC-ED method for the determination of RISP and 9-OH RISP in 1.0 ml of plasma. The recovery was above 69.0% and the LLOQ was 2.0 ng/ml. Svendesen et al. (1986) described a HPLC-ED method for the quantitation of CPZ in brain tissue. Direct protein precipitation using tetrahydrofuran was used for 1.0 ml of brain homogenate. The recovery was above 85% and the LOD was 50.0 ng/ml.

CONCLUSIONS AND PERSPECTIVES

Oral doses of antipsychotics for the treatment of chronic schizophrenia are on the order of a few milligrams per day. These antipsychotics are widely metabolized in the body. Therefore, the concentration of these drugs in plasma are quite low (pg-ng/ml levels). TDM of antipsychotics has demonstrated to be particularly useful in avoiding severe adverse effects caused by high doses of antipsychotics or due to interaction with other co-medication. In order to carry out pharmacology and toxicology studies, clinical TDM, polypharmacy treatment, and drug metabolism of antipsychotics (FGAs and SGAs), highly sensitive, selective and accurate bioanalytical methods are necessary to determine the analytes at these low concentrations. In this review, different aspects of analytical methods including separation, sample pretreatment, detectors and validation are reviewed. Many methods exist for antipsychotics and have produced valuable information. LC/MS will continue to lead in the quantitation of antipsychotics in biological samples. The increasing variety of antipsychotic drugs, the large sample numbers and

new drug candidates screening have result in the need to develop more sensitive, high throughput, automated column switching analytical methods. UPLC/MS methods, which provide a higher peak capacity, greater resolution, increased sensitivity and high speed of analysis, are attracting more attention (Li *et al*., 2007). In addition, some antisychotcs show a region-specific distribution in the brain (Kornhuber *et al*., 2006a; 2006b). Studies of this phenomena may increase our understanding of the volume changes in the basal ganglia with antipsychotics and a preferential vulnerability e.g. observed in tardive dyskinesia. Therefore, even more sensitive methods may be needed to determine the concentration of FGAs and SGAs in different regions of the brain.

LIST OF ACRONYMS USED IN THIS REVIEW

APCI	atmosphere pressure chemical ionization
APZ	aripiprazole
CE	capillary electrophoresis
CE-UV	capillary electrophoresis with ultraviolet detection
CE-MS	capillary electrophoresis interfaced with a mass spectrometer
CLOZ	clozapine
CNO	clozapine *N*-oxide
CPHP	4-(4-chlorophenyl)-4-hydroxypiperidine
CPZ	chlorpromazine
CZE	capillary zone electrophoresis
DAD	diode array detection
DMC	*N*-desmethylclozapine
DMO	desmethylolanzapine
ECL	electrochemiluminescence detection
ED	electrochemical detection
ESI	electrospray ionization
FASS-CE	field-amplified sample stacking in capillary electrophoresis
FD	fluorescence detection
FGAs	first-generation antipsychotics
GC	gas chromatography
GC-MS	gas chromatography interfaced with mass spectrometry
HAL	haloperidol
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography with ultraviolet detection
LC-UV	liquid chromatography (high performance) with ultraviolet detection
LC-MS/MS	liquid chromatography (high performance) interfaced with tandem mass spectrometry
LLE	liquid-liquid extraction
LOD	limit of detection
LLOQ	lower limit of quantitation
MRM	multiple reaction monitoring
MS	mass spectrometry
MS-MS	tandem mass spectrometry
9-OH RISP	9-hydroxylrisperidone
OLZ	olanzapine
QTP	quetiapine
RHAL	reduced haloperidol
SGAs	second-generation antipsychotics
SPE	solid-phase extraction
UPLC	ultra-high pressure liquid chromatography
ZIP	ziprasidone

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Table 2.1 Bioanalytical methods for the analysis of antipsychotics and their metabolites

Author(s)	Analyte	Matrix (volume)	Sample Preparation	Recovery	Separation Mode	Det
LeMoing *et al.* (1993)	RISP, 9-OH RISP	Plasma (1.0 ml)	SPE and LLE	Above 69.0%	HPLC	ED
Titier *et al.* (2002)	RISP, 9-OH RISP	Plasma (0.2 ml)	LLE	Above 77.0%	HPLC	UV
Llerena *et al.* (2003)	RISP, 9-OH RISP	Plasma (1.0 ml)	LLE	Above 96.8%	HPLC	UV
Raggi *et al.* (2005)	RISP, 9-OH RISP	Plasma (0.5 ml)	SPE	About 95.0%	HPLC	DA
Zhang *et al.* (2005)	RISP, 9-OH RISP	Plasma (0.5 ml)	LLE	Above 74.9%	HPLC	MS
Remmerie *et al.* (2003)	RISP, 9-OH RISP	Plasma (0.5 ml)	Mixed mode SPE	Above 90.0%	HPLC	MS
Bhatt *et al.* (2006)	RISP, 9-OH RISP	Plasma (0.1 ml)	Simple protein precipitation	Above 82.1%	HPLC	MS
Aravagiri *et al.* (2000)	RISP, 9-OH RISP	Plasma (0.5 ml)	LLE	About 84.0%	HPLC	MS
Flarakos *et al.* (2004)	RISP, 9-OH RISP	Plasma or Saliva (25 µL)	On-line extraction with column-switching after protein precipitation	Above 90.0%	HPLC	MS
Berna *et al.* (1998)	OLZ	Plasma (0.5 ml)	SPE	Above 71%	HPLC	MS
Dusci et al. (2002)	OLZ	Plasma (1.0 ml)	Back extraction LLE	N/A	HPLC	UV
Sabbioni et al. (2004)	OLZ, DMO	Plasma (0.25 ml)	SPE	Above 97.0%	HPLC	Cou det
Saracino et al. (2006a)	OLZ	Brain homogenate (0.25 ml)	HLB SPE	Above 90.0%	HPLC	Cou det
Berna *et al.* (2002)	OLZ	Blood (0.25 ml)	LLE	Above 73.6%	HPLC	MS
Bogusz *et al.* (1999)	OLZ	Serum (1.0 ml)	SPE		HPLC	MS
Catlow *et al.* (1995)	OLZ	Plasma (1.0 ml)	SPE	78.0%	HPLC	ED
Chin *et al.* (2004)	OLZ, DMO	Serum (0.2 ml)	Automated SPE	Below 70%	HPLC	MS

Table 2.1 (*continued*)

Author(s)	Analyte	Matrix (volume)	Sample Preparation	Recovery	Separation Mode	Detector	LLOQ (ng/ml or ng/g)
Gervasini *et al.* (2003)	OLZ	Plasma (0.5 ml)	LLE	Above 94%	HPLC	MS (ESI, SIM)	0.1
Nirogi *et al.* (2006)	OLZ	Plasma (0.5 ml)	LLE	About 85.5%	HPLC	MS-MS (ESI, MRM)	0.1
Bianchetti *et al.* (1978)	HAL	Plasma (2.0 ml)	Back extraction LLE	N/A	GC	NPD	1.0
Hattori *et al.* (1986)	HAL	Serum or urine (1.0 ml)	LLE	N/A	GC	MS (EI)	N/A
Arinobu *et al.* (2002)	HAL, RHAL, CPHP	Plasma or urine (1.0 ml)	Direct injection of crude biological samples without pretreatment	Above 50%	HPLC	MS (ESI, SIM)	10.0 for Hal; 15.0 for RHAL; 400.0 for CPHP
Aboul-Enein *et al.* (2006)	HAL, RHAL	Plasma (0.9ml)	Protein precipitation followed by SPE	About 76.0% for RHAL; about 85.0% for HAL	HPLC	UV	3.0 for HAL; 2.0 for RHAL
Higashi *et al.* (2006)	CPHP	Plasma (0.2 ml)	LLE followed by Pre-column derivatization	N/A	HPLC	FD	30.0
Kishikawa *et al.* (2006)	HAL, RHAL	Serum (0.5 ml)	LLE followed by Pre-column derivatization	About 99.2%	HPLC	FD	1.88
Yasui-Furukori *et al.* (2004)	HAL, RHAL	Plasma (1.0 ml)	LLE	96.2%	Automated column-switching HPLC (0.5 ml injection)	UV	1.0
McKay *et al.* (1982)	CPZ	Plasma (2.0ml)	LLE	86.0%	GC	MS (EI)	0.25
Li *et al.* (2006)	CPZ	Urine (0.2 ml)	LLE	83%	CZE	ECL	5.0
Pistos *et al.* (2003)	CPZ	Plasma (20 µL)	Direct injection of crude plasma samples without pretreatment	70.0%	HPLC	UV	100.0
Lara *et al.* (2005)	CPZ	Urine (5.0 ml)	HLB SPE	94.5%	CZE	UV	8.0
Shi et al. (2004)	CPZ	Urine (10.0ml)	Ion-pair LLE	N/A	Flow-injection analysis	Luminol chemiluminescence detection	6.0
Svendsen *et al.* (1986)	CPZ	Brain homogenate (1.0ml)	Direct protein precipitation using tetrahydrofuran	Above 85%	HPLC	ED	50.0 for LOD
Midha *et al.* (1981)	CPZ	Plasma (2.0 ml)	LLE	About 34.5%	HPLC	UV	1.0

Table 2.1 (*continued*)

Author(s)	Analyte	Matrix (volume)	Sample Preparation	Recovery	Separation Mode	Detector	LLOQ (ng/ml or ng/g)
Zhang *et al.* (2007a)	CPZ	Plasma (0.25 ml) or brain homogenate (0.2 ml)	LLE	Above 84.9%	HPLC	MS-MS (ESI, MRM)	0.2 for plasma; 0.833 for brain tissue
Niederländer *et al.* (2006)	CLOZ, DMC, CNO	Serum (0.05 ml)	On-line SPE	Above 90.0%	HPLC	MS (ESI, SIM)	50.0
Mosier *et al.* (2003)	CLOZ, DMC, CNO	Plasma (0.27 ml)	Back extraction LLE	Above 62.0%	HPLC	UV	36.0 for CLOZ; 16.0 for CNO and DMC
Garcia *et al.* (2003)	CLOZ	Plasma (1.0 ml)	LLE	N/A	HPLC	UV	15.0
Ho et al. (2004)	CLOZ, DMC, CNO	Plasma (0.2 ml)	LLE	75% for CLOZ; 60% for DMC; 25% for CNO	FASS-CE	UV	50.0 for CLOZ; 25.o for DMC; 30.0 for CNO
Raggi *et al.* (2001)	CLOZ, DMC	Plasma (0.5 ml)	HLB SPE	88.0%	CE	UV	100.0
Chung *et al.* (1993)	CLOZ, DMC	Plasma (1.0 ml)	Back extraction LLE	Above 80.0% for CLOZ; Above 55.0% for DMC	HPLC	UV	2.0
Olesen *et al.* (1993)	CLOZ, DMC	Serum (0.6 ml)	SPE	Above 80.0%	On-line automated HPLC	UV	30.0
Gupta (1995)	CLOZ, DMC	Serum (0.5 ml)	SPE	Above 90.0%	HPLC	UV	15.0
Janiszewski *et al.* (1997)	ZIP	Serum (0.3 ml)	Membrane microtiter SPE	76.0%	HPLC	MS (APCI, SIM)	1.0
Suckow *et al.* (2004)	ZIP	Plasma (1.0 ml)	LLE	Above 85.0%	HPLC	FD	0.5
Al-Dirbashi *et al.* (2006)	ZIP	Plasma (1.0 ml)	LLE	Above 84.0%	HPLC	MS-MS (ESI, MRM)	0.5
Aravagiri *et al.* (2007)	ZIP	Plasma (0.5 ml)	LLE	82.0%	HPLC	MS-MS (ESI, MRM)	0.25
Lin *et al.* (2004)	QTP	Plasma (0.1ml)	LLE	Above 62.0%	HPLC	MS-MS (ESI, MRM)	1.0
Barrett *et al.* (2007)	QTP	Plasma (0.5 ml)	HLB SPE	Above 100%	HPLC	MS-MS (ESI, MRM)	1.0
Saracino *et al.* (2006b)	QTP	Plasma (0.3 ml)	Mixed-mode cation exchange SPE	93.6%	HPLC	UV	2.5

Table 2.1 (*continued*)

Author(s)	Analyte	Matrix (volume)	Sample Preparation	Recovery	Separation Mode	Detector	LLOQ (ng/ml or ng/g)
Hasselstrom *et al.* (2003)	QTP	Serum (0.75 ml)	On-line SPE	69%	HPLC	UV	19.2
Shimokawa *et al.* (2005)	APZ	Plasma (0.5ml); brain homogenate (1.0 ml)	LLE	Above 87.2% in plasma; 41.3% in brain homogenate	HPLC	UV	10.0 for plasma; 30.0 for brain tissue
Kubo *et al.* (2005)	APZ	Plasma (0.4 ml)	LLE	Above 83.1%	HPLC	MS-MS (ESI, MRM)	0.1
de la Torre *et al.* (2005)	CPZ, OLZ, CLOZ, HAL	Blood (0.1 ml)	Bond Elut Certify SPE	56.0% for CPZ; 80.0% for OLZ; 70.0% for CLOZ; 83.0% for HAL	GC	NPD	152.0 for CPZ; 218.0 for OLZ; 122.0 for CLOZ; 515.0 for HAL
Josefsson *et al.* (2003)	CPZ, OLZ, CLOZ, HAL, ZIP, RISP	Blood (1.0 ml)	SPE	N/A	HPLC	MS-MS (ESI, MRM)	0.5 for RISP; 2.0 for HAL; 5.0 for OLZ; 1.0 for ZIP and CLOZ,
Sachse *et al.* (2006)	QTP, OLZ, CLOZ, DMC	Serum (0.1 ml)	Automated column-switching	Above 83.0%	HPLC	UV	20.0 for QTP; 105.0 for CLOZ; 10.0 for OLZ; 100.0 for DMC,
Shen *et al.* (2002)	CLOZ, DMC, CNO, RISP, 9-OH RISP	Plasma (1.0 ml)	LLE (two times)	Above 93.0%	HPLC	UV	5.0
Frahnert *et al.* (2003)	CLOZ, DMC, CNO, RISP, 9-OH RISP, QTP	Blood (1.0 ml)	SPE	Above 91.2%	HPLC	UV	5.0 for QTP, RISP and 9-OH RISP; 10.0 for CLOZ; 100.0 for DMC,
Titier *et al.* (2003)	CLOZ, HAL, OLZ, RISP, 9-OH RISP	Plasma (0.5 ml)	LLE	69.0% for OLZ; 33.5% for 9-OH RISP; 61.7% for	HPLC	DAD	10.0

Table 2.1 (*continued*)

Author(s)	Analyte	Matrix (volume)	Sample Preparation	Recovery	Separation Mode	Detector	LLOQ (ng/ml or ng/g)
Kollroser *et al.* (2002)	OLZ, CLOZ, DMC	Plasma (1.0 ml)	Direct injection of plasma samples	N/A	Column-switch HPLC	MS-MS (ESI, MRM)	5.0 for OLZ; 10.0 for CLOZ and DMC
Zhou *et al.* (2004)	CLOZ, OLZ, RISP QTP	Plasma (0.5 ml)	LLE	Above 80.7%	HPLC	MS (ESI, SIM)	20.0 for QTP and CLOZ; 1.0 for OLZ and RISP
Kirchherr *et al.* (2006)	CLOZ, RISP, 9-OH RISP, QTP, HAL, APZ, CPZ, OLZ, ZIP	Serum (0.1 ml)	Direct protein precipitation	Above 95.0%	HPLC (monolithic C18 column)	MS-MS (ESI, MRM)	1.0 for HAL, and RISP; 5.0 for 9-OH RISP; 10.0 for OLZ, APZ, QTP, ZIP, and CPZ; 100.0 for CLOZ
Li *et al.*. (2007)	QTP, APZ	*in vitro* samples (0.5 ml)	LLE	Above 73.0%	UPLC	MS-MS (ESI, MRM)	0.05
Zhang *et al.* (2007b)	CLOZ, RISP, 9-OH RISP, HAL, OLZ, ZIP	Plasma (0.25 ml)	LLE	Above 77.0%	HPLC	MS-MS (ESI, MRM)	0.1
Zhang *et al.* (2007c)	CPZ, RISP, 9-OH RISP, HAL, OLZ, ZIP	Plasma (1.0 ml)	LLE	Above 74.8%	HPLC	UV	2.0
Zhang *et al.* (2007d)	CLOZ, RISP, 9-OH RISP, HAL, OLZ, ZIP	Brain homogenate (0.20 ml)	LLE	Above 73.6%	HPLC	MS-MS (ESI, MRM)	0.208 for OLZ, RISP, 9-OH RISP and CLOZ; 0.416 for HAL and ZIP
Mercolini *et al.* (2007)	CLOZ, DMC, CNO, RISP, 9-OH RISP, HAL, OLZ, DMO, ZIP, CPZ	Plasma (0.5 ml)	SPE	Above 93%	HPLC	UV	2.6

Chapter 3

SIMULTANEOUS DETERMINATION OF FIVE ANTIPSYCHOTIC DRUGS IN RAT PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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Abstract A significant percentage of psychiatric patients who are treated with antipsychotics are treated with more than one antipsychotic drug in the clinic. Thus, it is advantageous to use a rapid and reliable assay that is suitable for determination of multiple antipsychotic drugs in plasma in a single run. A simple and sensitive HPLC-UV method was developed and validated for simultaneous quantification of olanzapine, haloperidol, chlorpromazine, ziprasidone, risperidone and its active metabolite 9-hydroxyrisperidone in rat plasma using imipramine as an internal standard (I.S.). The analytes were extracted from rat plasma using a single step liquid-liquid acid solution back extraction technique with wash procedure, which provided the very clear baseline for blank plasma extraction. The compounds were separated on an Agilent Eclipse XDB C8 (150 mm × 4.6 mm i.d., 5 μm) column using a mobile phase of acetonitrile/30 mM ammonium acetate including 0.05% triethylamine (pH 5.86 adjusted with acetic acid) with gradient elution. All of the analytes were monitored using UV detection. The method was validated and the linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries, selectivity and stability were determined. The LLOQ was 2.0 ng/ml and correlation coefficient (R^2) values for the linear range of 2.0-500.0 ng/ml were 0.998 or greater for all the analytes. The precision and accuracy for intra-day and inter-day were better than 7.44%. The recovery was above 74.8% for all of the analytes. This validated method has been successfully used to quantify the plasma concentration of the analytes for pharmacological and toxicological studies following chronic treatment with antipsychotic drugs in the rat.

Keywords: Risperidone, 9-Hydroxyrisperidone, Olanzapine, Haloperidol, Chlorpromazine, Ziprasidone

1. Introduction

Antipsychotic agents are used in psychiatric patients for the management of psychotic episodes as well as for other behavioral symptoms such as agitation. Second Generation Antipsychotics (SGAs) (such as olanzapine, ziprasidone and risperidone) and First Generation Antipsychotis (FGAs) (such as haloperidol and chlorpromazine) (Figure 1) are popular for the treatment of schizophrenia and other psychoses in the clinic [1]. It is reported that the SGAs olanzapine, risperidone, clozapine and ziprasidone are effective in the treatment of both positive and negative symptoms of schizophrenia and that they are less likely to produce extrapyramidal side effects when compared with FGAs such as haloperidol and chlorpromazine [2, 3]. However, suicide and several intoxications of antipsychotics have been published [4-6]. It has long been known that chronic exposure to FGAs such as haloperidol often result in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Furthermore, given that cognition is now recognized as a key factor that influences the long term functional outcome in schizophrenia [7-9], it is important to determine if there is a correlation between antipsychotic plasma levels (particularly in association with chronic drug exposure) and cognitive function in the rat model. Such a correlation would allow for improved clinical monitoring of these compounds. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. Therefore, the concentration of these drugs in plasma is very low. For example, the therapeutic plasma levels of olanzapine are in the range of 8-80 ng/ml [10-12]. In the case of haloperidol, levels from 5.0 to 15.0 ng/ml have also been described as the therapeutic range [13]. Plasma levels of psychoactive drugs resulting under a given dose are highly variable

between individual patients. This is primarily due to inter-individual variations in compliance and in the activities of drug metabolizing enzymes. This leads to poor predictability of drug concentrations at a given dose. Approximately 30 antipsychotic drugs are currently available in the clinic. A significant percentage of psychiatric patients who are treated with antipsychotics are treated with more than one antipsychotic drug in the clinic. Also, it is advantageous to have a method that enables determination of more than a single antipsychotic drug not only because of polypharmacy but also because of the use of so many different drugs in different patients. Laboratories therefore have to establish and validate many methods. So, for a broad and complete determination it is advantageous for the laboratory to have methods as described here that may be used for more than a single compound. At present, determination of some of these drugs has been established by high performance liquid chromatography (HPLC) with UV detection [2, 14-21], HPLC with coulometric detection [22, 23] or fluorescence detection [24, 25]. Although electrochemical detection [26-30] offers enhanced sensitivity in the low ng/ml range, electrochemical detectors require optimal working conditions, sample preparation is critical and interference from co-medications is often unavoidable. Capillary electrophoresis (CE) methods [31, 32] were reported to detect the antipsychotic drugs, but are not sensitive and robust for biological samples. Recently, several LC-MS/MS methods were reported for the quantification of the antipsychotic drugs in biological fluids [33-39]. In toxicity research and routine clinical monitoring, however, HPLC-UV may be advantageous because of lower cost and greater robustness. Few HPLC-UV methods [2, 15, 20, 21] offer the ability to measure multiple antipsychotics (i.e. both FGAs and SGAs) simultaneously in biological samples in a single run. The lower limit of

quantitation (LLOQ) for most of these methods are above 10 ng/ml. Here we describe the validation and application of a HPLC-UV method for simultaneous determination of risperidone, 9-hydroxyrisperidone, olanzapine, haloperidol, chlorpromazine and ziprasidone in rat plasma in a single run. The LLOQ of all the analytes was as low as 2 ng/ml.

2. Experimental

2.1 Chemicals and reagents

Haloperidol (HAL), risperidone (RISP) and olanzapine (OLZ) were kindly provided by Eli Lilly (Indianapolis, IN, USA). 9-Hydroxyrisperidone (9-OH RISP) was donated by the Janssen Research Foundation (Beers, Belgium). Ziprasidone was obtained from Pfizer Central Research (Groton, CT, USA). Chlorpromazine (CPZ) and imipramine (internal standard, I.S.) were from Sigma (St. Louis, MO, USA). Ethyl acetate, methyl tert-butyl ether, chloroform, hexane, diethyl ether, isopropyl ether, isoamyl alcohol, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetic acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

2.2 Instruments and chromatographic conditions

An Agilent 1100 series HPLC system, consisting of a degasser, quaternary pump, autosampler, and a variable wavelength UV detector with a thermostatted column compartment (TC-50 controller, Wisconsin, USA) was used in this study (Agilent, Palo Alto, CA, USA). The analytes were separated on an Agilent Eclipse XDB C-8 column

(150 × 4.6 mm i.d., 5 µm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted with acetic acid) and mobile phase B was acetonitrile. The flow rate was set 1.0 ml/min. An 80 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 29) (18, 60) (20, 60) (20.5, 29) (27, 29). The column temperature was maintained at 35ºC. The UV-detector program consisted of a 0-5.30 min sequence set at 277 nm for RISP and 9-OH RISP acquisition, 5.31-7.90 min sequence set at 255 nm for OLZ acquisition, 7.91-20.0 min sequence set at 245 nm for HAL, CPZ and ZIP acquisition.

2.3 Sample collection

Antipsychotic doses were based on previous rodent studies in which time dependent behavioral and neurochemical effects were detected [7, 8]. Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human [40]. Male albino Wistar rats (Harlan Inc.) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a 12-h light/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg/day), RISP (2.5 mg/kg/day), OLZ (10.0 mg/kg/day), CPZ (10.0 mg/kg/day) and ZIP (12 mg/kg/day) orally in drinking water for periods of at least 14 days to achieve a steady-state concentration of the antipsychotic drugs. Dosing antipsychotics by drinking water avoids stress of forced drug application. However, there is reduced water consumption associated with some of the antipsychotics (particularly olanzapine). In these cases (and in the study in question) we added saccharin 0.1% (w/v) to increase

water consumption to normal levels. Plasma samples were collected over the course of treatment in separate groups of rats for measurement of the antipsychotic concentrations. Rats were anesthetized with isofluorane and 3.0 ml of blood was collected via cardiac puncture to heparined tubes. The blood was centrifuged for 15 min at 2500× g at 8ºC and the resulting plasma was frozen until analysis.

2.4 Preparation of stock, working standard and quality control solutions

Individual stock solutions of OLZ, RISP, 9-OH RISP, HAL, CPZ and ZIP and I.S. (imipramine) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at -20ºC. Combined standard solutions with concentrations of 40.0, 100.0, 200.0, 400.0, 1000.0, 1500.0, 2000.0, 4000.0, and 10000.0 ng/ml were prepared by serial dilution with 0.02 M HCl water solutions. Precision and accuracy standards with concentrations of 40.0, 300.0, 3000.0, and 6000.0 ng/ml were also prepared in the same manner. A 2000.0 ng/ml of I.S. standard solution was prepared with 0.02 M HCl water solution from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at -20ºC when not use and replaced every 3 months. Fresh standard solution was prepared for each day of analysis or validation.

2.5 Preparation of calibration and QC samples

Sample for the calibration curves and QCs were prepared by adding 50.0 µl of each standard solution into 1.0 ml of blank plasma. This yields calibration standard concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, 75.0, 100.0, 200.0, and 500.0 ng/ml. The final concentrations of QCs were 2.0, 15.0, 150.0, and 300.0 ng/ml. The spiked plasma

samples (standards and quality controls) were extracted with each analytical batch along with the unknown samples.

2.6 Sample preparation

To a 1.0 ml of rat plasma sample, 50 µl of internal standard (2000.0 ng/ml, imipramine) and 0.4ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted in 8 ml of isopropyl ether: pentane (70:30) solvent for 5 min. After centrifugation at 2000× g for 10 min, the upper organic layer was removed and 100 µl of 0.05 N HCl was added to the organic layer. The mixture was shaken for 3 min and centrifuged at 2000× g for 10 min. The upper organic layer was discarded and the aqueous phase was washed by 0.3 ml of isopropyl ether and 0.1ml of pentane, respectively. Then, the upper organic layer was aspirated to waste and the aqueous phase was placed in a vacuum centrifuge under reduced pressure for 15 s to evaporate traces of the organic solvent. 80 µl of the final aqueous phase was injected into the HPLC unit for analysis.

2.7 Method validation

The method was validated for linearity, recovery, accuracy, precision and selectivity. Plasma calibration curves were constructed using the peak area ratios of OLZ, RISP, 9-OH RISP, HAL, CPZ or ZIP to that of I.S., and applying a weighted ($1/x^2$) least squares linear regression analysis, precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples (2.0, 15.0, 150.0, and 300.0 ng/ml). Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision.

Recovery was calculated as the peak area for the analytes at 2.0, 15.0, 150.0, 300.0 and 100.0 ng/ml for the I.S. in plasma spiked before extraction divided by the peak area of the pure drugs in the 0.05 M HCl water solution at the same concentration. The stability of the stock solutions were determined at their storage conditions of -20ºC for three months. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls [35]. The bench-top stability of spiked plasma samples stored at room temperature was evaluated for 2h. The freeze/thaw stability was investigated by comparing the stability samples following three freeze/thaw cycles against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 12h. The stability testing was performed at 15.0 and 300.0 ng/ml concentration levels for all of the antipsychotic drugs.

3. Results and discussion

3.1 Method development

The chromatographic conditions, especially the analytical column, the composition of mobile phase and gradient elution condition, were optimized through several trials to achieve the desired sensitivity, separation, run time, and symmetric peak shapes for the analytes and I.S. Agilent XDB C8 (150mm × 4.6 mm i.d., 5 µm) and Waters XTerra C18 (150mm × 4.6 mm i.d., 5 µm) were evaluated. As a result, Agilent XDB C8 (150mm × 4.6 mm i.d., 5 µm) was selected as it produced a satisfactory separation, peak shape and shorter analytical run time. The peaks of HAL, CPZ and ZIP showed some tailing and resulted in a longer retention time on the Waters XTerra C18 column (150mm × 4.6

mm i.d., 5 µm). Different mobile phase A (buffer), such as 30mM ammonium acetate (pH 5.80 adjusted using acetic acid), 50 mM phosphate buffer (pH 5.80), 10 mM acetic acid, or 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted using acetic acid) were attempted to improve the method for these compounds. 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted using acetic acid) resulted in the best peak shape and separation for all of the analytes. The addition of 0.05% of triethylamine in the buffer played a key role in enhancing the peak symmetry and separation capacity. In addition, the column temperature should be kept at 35ºC to obtain a baseline separation between CPZ and ZIP. Initially, we evaluated solid phase extraction (SPE) methods using different cartridges such as the Waters Oasis HLB and Varian C18 for sample preparation. However, the recovery of ZIP was very low and there were a lot of interferences from the matrix at low concentration. Finally, we evaluated a liquid-liquid acid solution back extraction method for sample preparation. We found that liquid-liquid acid solution back extraction [2, 14] produced a cleaner baseline for blank plasma when compared with direct liquid-liquid extraction when injecting the reconstitution solution after evaporating the liquid-liquid extraction organic solvent to dryness. We modified the standard liquid-liquid acid back extraction method and washed the acid back extraction solution using 0.3 ml of isopropyl ether and 0.1 ml of pentane, respectively. As a result, the baseline of blank plasma was very clean as seen in Figure 2 (C) and there was no interference for any of the analytes compared with a 2.0 ng/ml spiked sample (Figure 3 (B)). Also, the wash procedure did not significantly reduce the recovery for the analytes (data not shown). First we evaluated the direct liquid-liquid extraction method. The procedure was

as follows: to a 1.0 ml blank rat plasma sample, 100 µl of 0.02 M HCl solution and 0.4ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted in 8 ml of isopropyl ether: pentane (70:30) solvent for 5 min. After centrifugation at 2000× g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 100 µl of 0.05 M HCl was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000× g for 10 min. 80 µl of the reconstitution solution (blank plasma) was injected into HPLC-UV system. As a result, the direct liquid-liquid extraction method produced a lot of interference for the analytes as Figure 3.1 (A). The liquid-liquid acid solution back extraction method (no wash procedure) produced an interference for RISP as seen in Figure 3.1 (B). Different organic solvents, ethyl acetate, methyl tert-butyl ether, chloroform, hexane, pentane, diethyl ether, isoamyl alcohol and isopropyl ether, and their mixtures in different combinations and ratios were evaluated for extraction solvents. Finally, isopropyl ether: pentane (70:30) was found to be optimal, because it is able to produce a clean chromatogram for a blank plasma sample and yielded the highest recovery for the analytes by a liquid-liquid acid solution back extraction method. Another advantage of isopropyl ether and pentane is lower solubility in water when compared to other solvents. Low solubility and high extraction strength of the organic solvents are key factors for liquid-liquid acid back extraction methods.

3.2 Linearity and sensitivity

The calibration curves in Table 1 showed good linear response ($R^2 > 0.998$) over the range from 2.0-500.0 ng/ml for all of the analytes. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for all calibration

curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for the analytes. Table 3.1 showed the slope and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 2.0ng/ml for determination of all of the analytes in rat plasma as shown in Table 3.3. Representative chromatograms obtained from blank plasma and plasma spiked with the LLOQ standard (2.0 ng/ml) are shown in Figure 3.3. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or I.S. in blank rat plasma from six different lots. A signal-to-noise (S/N) > 10 at LLOQ (2.0 ng/ml) was observed for all of the analytes.

3.3 Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The accuracy and precision data can be seen in Table 3.2. The values for the intra-day precision and accuracy were better than 4.98% and 7.03% for all the analytes. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were better than 5.98% and 7.44% (Table 3.3).

3.4 Recovery and selectivity

The analytes and I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of the plasma sample. Hence, alkalytic modifiers were used to adjust the pH of plasma samples. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH 10.69), were evaluated. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the

highest recovery for all of the analytes. In addition, 1 M NaOH was not suitable because of the possible reduction of chlorpromazine-*N*-oxide to chlorpromazine from this solution [41]. The recoveries ranged from 74.8% to 102.3% for all of the analytes and I.S (Table 3.3).

Several drugs commonly used in psychiatric practice were tested for interference comparing their retention times with those of the analytes and the IS. Some of the main metabolites of the analytes were also checked. The results of these assays are reported in Table 3.4. The results demonstrated that there is little interfere with the determination of the analytes, granting good method selectivity.

3.5 Stability studies

Stability testing is very important for validated methods in biological samples. The stock solutions were stable at the storage conditions (-20ºC) for three months (data not shown). All the other stability studies were conducted at two concentration levels (15.0 and 300.0 ng/ml) with five determinations for each. Plasma extracts were stable in mobile phase in the HPLC autosampler for at least 12h, indicating that samples should be processed within this period of time (Table 3.5). The freeze/thaw stability tests indicate the analytes were stable in rat plasma for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for all of the analytes for at least 2h. The RE% was from 0.11% to 9.37% (< 15%) and RSD was from 0.19% to 8.62% for all the analytes (see Table 3.5).

Zhou et al. [42], reported that storage of OLZ in human plasma at room temperature for 24h produced significant degradation of OLZ. If Vitamin C was added to plasma, then OLZ was stable at room temperature for 24h. In this work, OLZ did not show significant

degradation in rat plasma kept at room temperature for up to 2h without Vitamin C addition. However, for longer storage of OLZ, freezing rat plasma is recommended [38].

3.6 Application of the method

The validated method has been successfully used to quantify antipsychotic drug concentrations in rat plasma after the chronic treatment of rats with the antipsychotic drugs in their drinking water. The steady-state concentration data for antipsychotic drugs in rat plasma are reported in Table 3.6. The representative chromatograms resulting from the analysis of real samples after chronic treatment with antipsychotic drugs is shown in Figure 3.4. There was no significant interference for any of the analytes from any other of the five analytes in the real plasma samples. In general, the concentration of HAL, RISP, OLZ and ZIP in rat plasma was relatively low, but clearly within the range that is generally considered therapeutic in humans. In addition, all the analytes were observed above the method LLOQ.

4. Conclusions

A simple, selective and sensitive HPLC-UV analytical method for the simultaneous determination of RISP, 9-OH RISP, OLZ, HAL, CPZ and ZIP in rat plasma has been developed and validated. A liquid-liquid acid solution back extraction method with wash procedure was evaluated and provided a clean baseline for blank plasma. This method provided good selectivity and a LLOQ of 2.0 ng/ml for all of the analytes. Liquid-liquid extraction sample preparation was used for 1.0 ml of rat plasma that provided high recovery for all of the analytes. The method was successfully applied to study the effect of chronic treatment of FGAs (HAL and CPZ) and SGAs (OLZ, RISP and ZIP) antipsychotic drugs on the cognitive function in rats.

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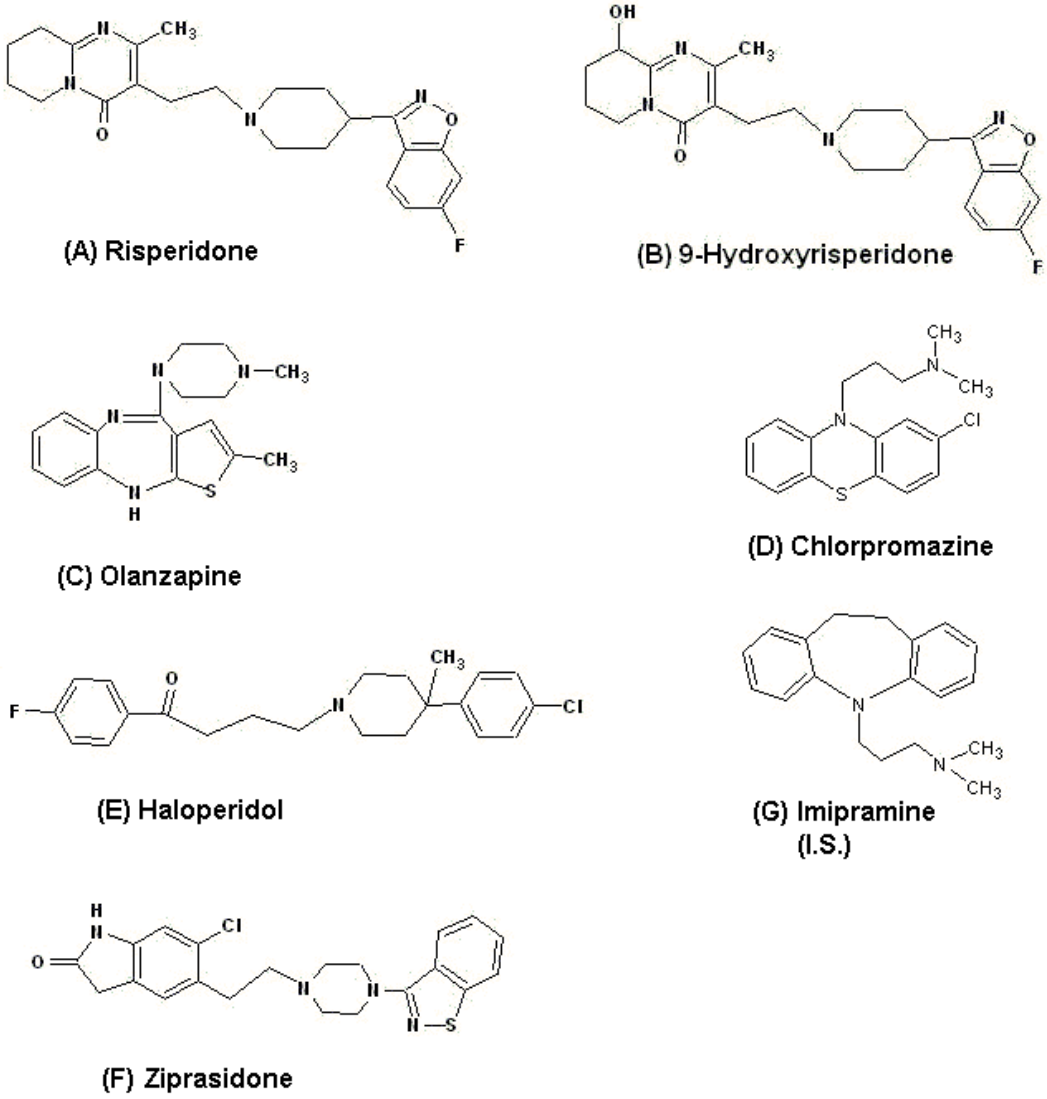

Figure 3.1. Chemical structures of RISP, 9-OH RISP, OLZ, CPZ, HAL, ZIP and I.S. (Imipramine).

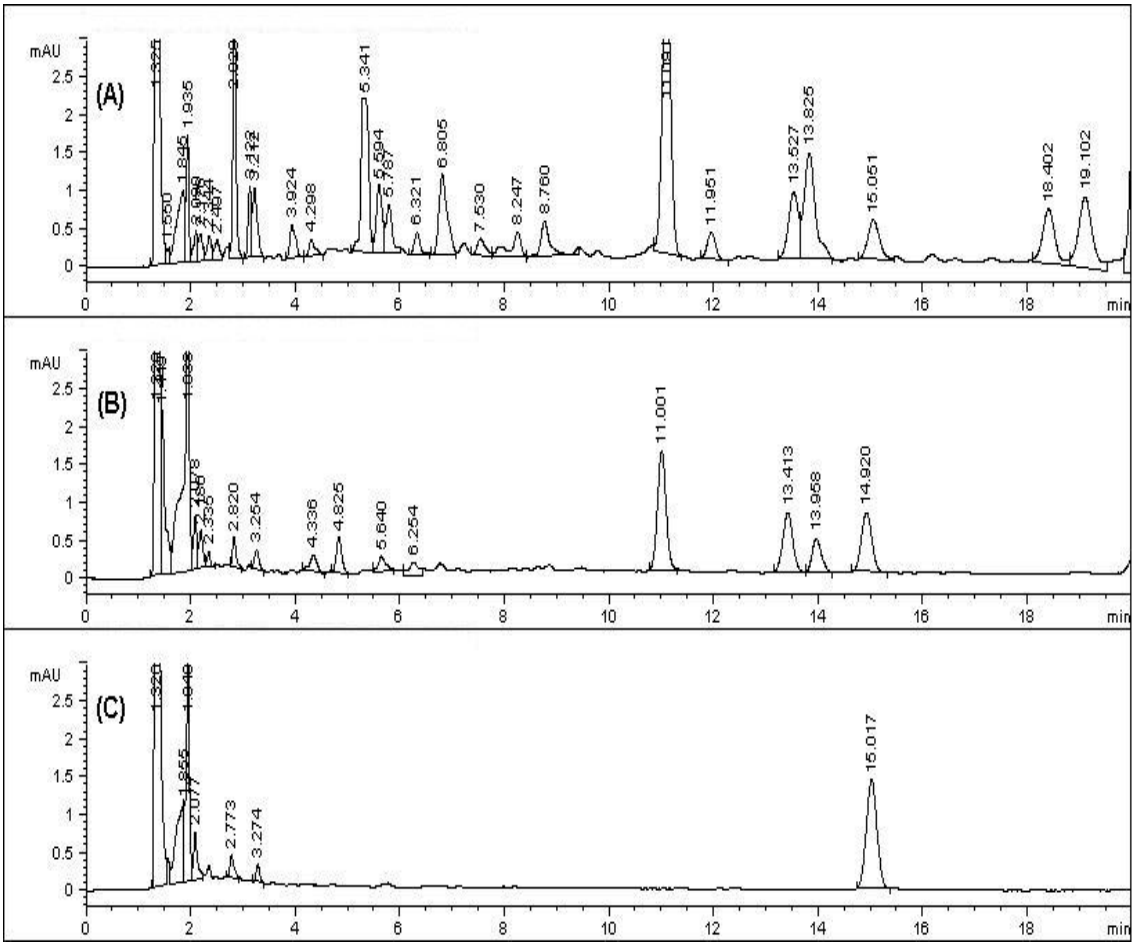

Figure 3.2. Representative baseline chromatograms of blank plasma extraction obtained from (A) direct liquid-liquid extraction method; (B) normal liquid-liquid acid solution back extraction method (no wash procedure); (C) liquid-liquid acid solution back extraction with wash procedure using 0.3 ml of isopropyl ether and 0.1 ml of pentane, respectively.

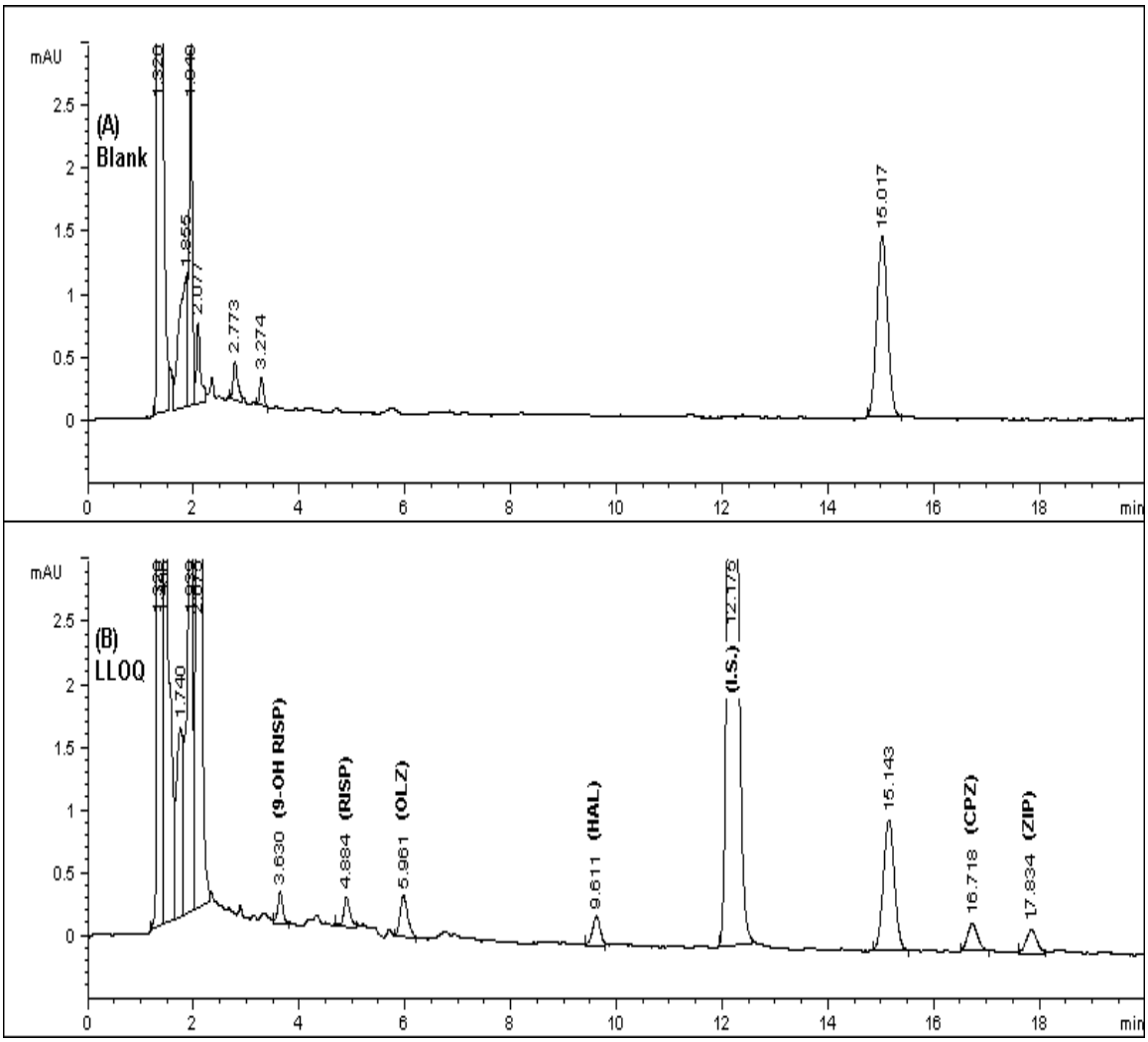

Figure 3.3. Representative chromatograms obtained from (A) blank rat plasma; (B) plasma spiked with LLOQ (2.0 ng/ml) concentration for all of the analytes and I.S. (100.0 ng/ml).

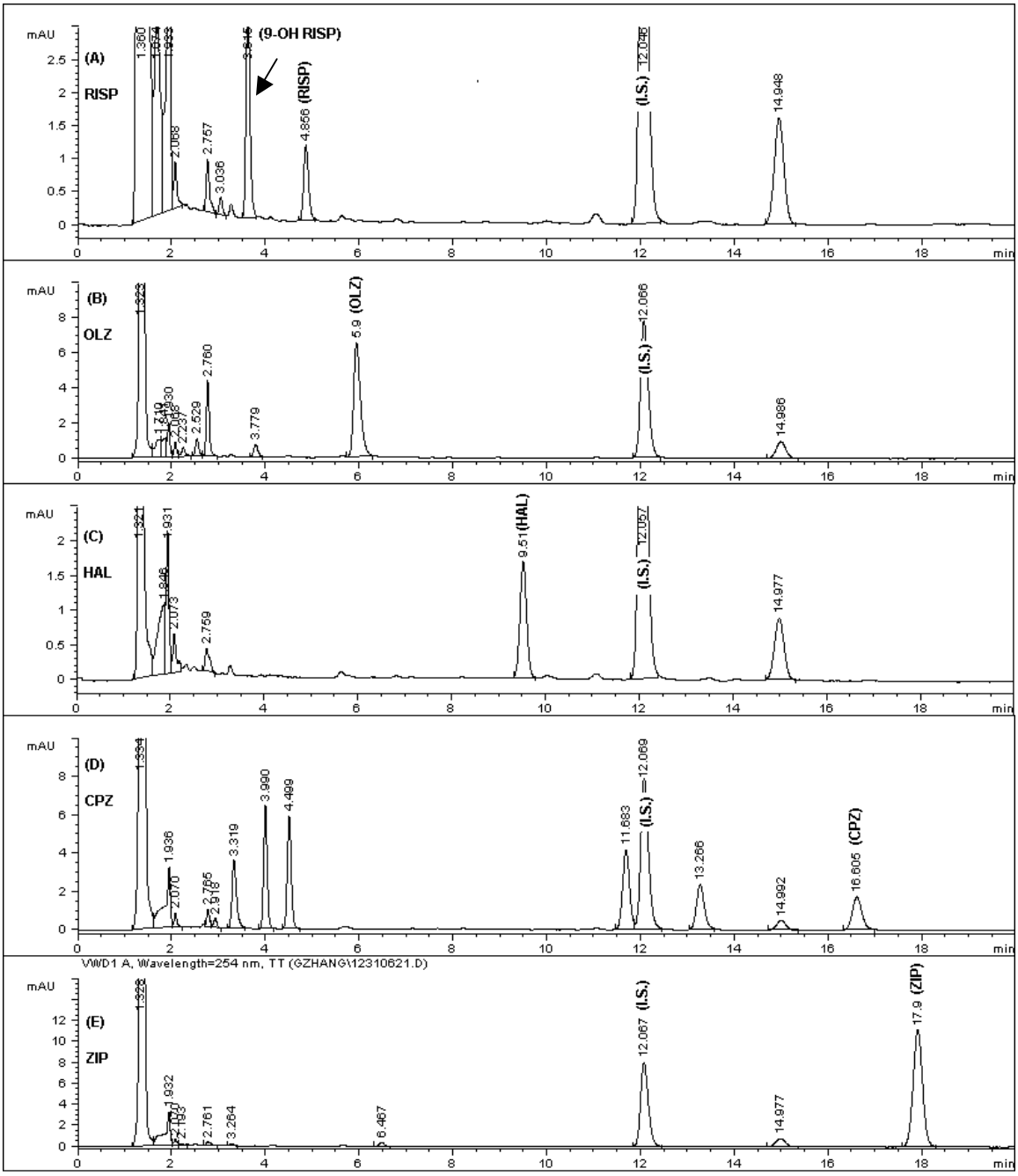

Figure 3.4. Representative chromatograms of plasma samples from chronic treatment with antipsychotic drugs: (A) a rat treated with RISP (2.5 mg/kg/day) and the concentration in plasma was 8.37 ng/ml for RISP and 20.25 ng/ml for 9-OH RISP; (B) a rat treated with OLZ (10.0 mg/kg/day) and the concentration of OLZ in plasma was 34.52 ng/ml; (C) a rat treated with HAL (2.0 mg/kg/day) and the concentration of HAL in plasma was 13.26 ng/ml; (D) a rat treated with CPZ (10.0 mg/kg/day) and the concentration of CPZ in plasma was 13.89 ng/ml.; (E) a rat treated with ZIP (12.0 mg/kg/day) and the concentration of ZIP in plasma was 140.58 ng/ml.

Table 3.1
Statistical data for linearity including standard deviation (S.D.) (linear range 2.0-500.0 ng/ml) for all of the analytes

	RISP	9-OH RISP	OLZ	HAL	CPZ	ZIP
R^2	0.9984 ± 0.0005	0.9987 ± 0.0009	0.9993 ± 0.0005	0.9995 ± 0.0002	0.9986 ± 0.0004	0.9997 ± 0.0000
Slope	0.0093 ± 0.0002	0.0089 ± 0.0003	0.0239 ± 0.0012	0.0124 ± 0.0144	0.0196 ± 0.0006	0.0125 ± 0.0002

Table 3.2
The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HPLC-UV method used to quantitate antipsychotic drugs in rat plasma

Drug		Intra-day			Inter-day		
	Concentration added (ng/ml)	Observed concentration ±S.D. (ng/ml)	R.S.D. (%)	Error (%)	Observed concentration (ng/ml)	R.S.D. (%)	Error (%)
RISP	2.0	2.05 ± 0.030	1.48	2.48	2.05 ± 0.075	3.66	3.71
	15.0	15.64 ± 0.558	3.56	4.67	15.02 ± 0.743	4.95	4.31
	150.0	154.83 ± 2.584	1.67	3.22	151.79 ± 3.997	2.63	2.23
	300.0	310.64 ± 2.381	0.77	3.55	303.16 ± 9.836	3.24	2.94
9-OH RISP	2.0	2.01 ± 0.024	1.18	0.95	1.99 ± 0.119	5.98	4.00
	15.0	16.05 ± 0.178	1.11	7.03	16.12 ± 0.178	1.11	7.44
	150.0	150.86 ± 1.678	1.11	1.07	150.10 ± 2.147	1.43	1.20
	300.0	293.24 ± 5.111	1.74	2.25	288.21 ± 6.016	2.09	3.93
OLZ	2.0	1.99 ± 0.0514	2.58	2.02	2.03 ± 0.0688	3.39	2.63
	15.0	15.25 ± 0.759	4.98	4.69	15.00 ± 0.659	4.39	4.02
	150.0	149.93 ± 2.574	1.72	1.11	150.72 ± 4.008	2.66	2.05
	300.0	303.75 ± 5.13	1.69	1.49	299.79 ± 9.338	3.12	2.51
HAL	2.0	1.97 ± 0.094	4.76	3.99	2.02 ± 0.0831	4.10	3.64
	15.0	16.00 ± 0.653	4.08	6.68	15.22 ± 0.814	5.35	4.13
	150.0	152.69 ± 2.434	1.59	1.91	151.52 ± 2.625	1.73	1.63
	300.0	304.36 ± 2.436	0.80	1.45	300.55 ± 7.998	2.66	2.11
CPZ	2.0	2.05 ± 0.0791	3.85	4.35	2.03 ± 0.0829	4.08	3.92
	15.0	15.81 ± 0.490	3.10	5.43	14.95 ± 0.824	5.51	4.35
	150.0	154.16 ± 3.972	4.26	4.35	153.10 ± 4.762	3.11	3.03
	300.0	307.56 ± 9.264	3.01	3.62	306.2 ± 13.165	4.30	3.98
ZIP	2.0	1.99 ± 0.0593	2.99	2.19	1.99 ± 0.0579	2.91	2.26
	15.0	15.51 ± 0.428	2.76	3.75	15.09 ± 0.525	3.48	2.88
	150.0	148.58 ± 2.864	1.93	1.41	149.35 ± 2.984	2.00	1.47
	300.0	298.01 ± 3.434	1.15	0.92	296.24 ± 5.861	1.98	1.70

Table 3.3
Recovery (%, mean ± S.D.) of analytes in rat plasma (n=5); recovery of I.S. was 96.3 ± 3.9 (n=5) at 100.0 ng/ml in rat plasma.

Concentration (ng/ml)	RISP	9-OH RISP	OLZ	HAL	CPZ	ZIP
2.0	87.1 ± 2.0	84.0 ± 1.6	93.8 ± 3.3	102.3 ± 3.5	95.9 ± 3.5	100.3 ± 2.1
15.0	88.4 ± 1.2	82.5 ± 1.0	90.4 ± 3.3	97.4 ± 2.3	92.4 ± 3.0	95.3 ± 1.0
150.0	89.6 ± 1.5	74.8 ± 0.5	94.9 ± 1.9	97.4 ± 1.9	89.5 ± 2.3	94.2 ± 2.0
300.0	88.7 ± 0.7	75.0 ± 1.8	94.7 ± 1.5	95.1 ± 1.3	87.5 ± 2.6	91.6 ± 1.4

Table 3.4
Retention times of some commonly used drugs and their metabolites

Drugs	Retention times(min)
Caffeine	1.84
9-Hydroxyrisperidone	**3.62**
Risperidone	**4.83**
Proprapanolol	5.24
Olanzapine	**5.90**
Lidocaine	6.33
Normethylclozapine	6.55
Oxazepam	9.09
Haloperidol	**9.52**
Desipramine	9.94
Clozapine *N*-oxide	10.42
Promazine	10.65
Clonazepam	10.96
Imipramine (I.S.)	**12.05**
Clozapine	13.37
Verapermil	13.49
Amitriptyline	13.88
Trimipramine	15.16
Chlorpromazine	**16.55**
Midazolam	16.88
Ziprasidone	**17.71**
Diazepam	18.50
Morphine	N.D.
Codeine	N.D.
Loxapine	N.D.

N.D.: not detected within a 20.0-min run.

Table 3.5
Stability testing of antipsychotic drugs used in this study (n=5)

Drugs	Stability	Spiked conc. (ng/ml)	Observed conc. ± S.D. (ng/ml)	R.S.D. (%)	Relative error (%)
RISP	Three freeze-thaw cycles	15.0	15.31 ± 0.562	3.67	2.05
		300.0	306.79 ± 8.012	2.62	2.26
	Bench top (2h)	15.0	15.47 ± 0.406	2.62	3.15
		300.0	313.77 ± 13.421	4.28	4.59
	Autosampler stability (12h)	15.0	14.26 ± 0.500	3.50	-4.91
		300.0	300.32 ± 7.126	2.37	0.11
9-OH RISP	Three freeze-thaw cycles	15.0	15.97 ± 0.139	0.87	6.47
		300.0	297.88 ± 1.372	0.46	-0.71
	Bench top (2h)	15.0	16.41 ± 0.169	1.03	9.37
		300.0	297.81 ± 6.588	2.21	-0.73
	Autosampler stability (12h)	15.0	15.98 ± 0.218	1.37	6.54
		300.0	292.24 ± 2.635	0.90	-2.59
OLZ	Three freeze-thaw cycles	15.0	15.56 ± 0.505	3.25	3.71
		300.0	298.68 ± 11.505	3.85	-0.44
	Bench top (2h)	15.0	15.53 ± 0.246	1.59	3.54
		300.0	305.37 ± 10.811	3.54	1.79
	Autosampler stability (12h)	15.0	15.53 ± 0.525	3.38	3.54
		300.0	302.19 ± 3.502	1.16	0.73
HAL	Three freeze-thaw cycles	15.0	15.23 ± 0.261	1.71	1.53
		300.0	298.29 ± 2.906	0.97	-0.57
	Bench top (2h)	15.0	14.97 ± 0.2494	1.67	-0.24
		300.0	304.93 ± 3.779	1.24	1.64
	Autosampler stability (12h)	15.0	15.18 ± 0.702	4.62	1.19
		300.0	300.86 ± 0.564	0.19	0.29
CPZ	Three freeze-thaw cycles	15.0	15.37 ± 1.325	8.62	2.47
		300.0	303.49 ± 4.587	1.51	1.16
	Bench top (2h)	15.0	15.96 ± 0.968	6.07	6.42
		300.0	301.52 ± 4.107	1.36	0.51
	Autosampler stability (12h)	15.0	14.85 ± 0.714	4.81	-1.01
		300.0	295.74 ± 5.948	2.01	-1.42
ZIP	Three freeze-thaw cycles	15.0	15.58 ± 0.231	1.48	3.89
		300.0	311.02 ± 3.484	1.12	3.67
	Bench top (2h)	15.0	14.69 ± 0.591	4.02	-2.06
		300.0	308.31 ± 9.837	3.19	2.77
	Autosampler stability (12h)	15.0	15.22 ± 0.411	2.70	1.44
		300.0	309.25 ± 2.077	0.67	3.08

Table 3.6
Steady-state plasma concentrations of the analytes after the chronic treatment with antipsychotic drugs for rats (n=3)

Drugs administered	Dose (mg/kg/day)	Concentrations (ng/ml ± S.D.)
RISP	2.5	RISP: 12.91 ± 4.608
		9-OH RISP: 23.05 ± 2.456
HAL	2.0	11.15 ± 2.200
OLZ	10.0	22.24 ± 11.749
CPZ	10.0	11.85 ± 2.421
ZIP	12.0	111.09 ± 86.563

Chapter 4

LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY METHOD FOR SIMULTANEOUS DETERMINATION OF OLANZAPINE, RISPERIDONE, 9-HYDROXYRISPERIDONE, CLOZAPINE, RISPERIDONE, AND ZIPRASIDOEN IN RAT PLASMA

Zhang G, Terry AV Jr and Bartlett, MG. *Rapid Communications in Mass Spectrometry* 2007; **21:** 920-928. Reprinted here with the permission of publisher.

Abstract

A simple, sensitive and rapid liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method was developed and validated for simultaneous quantification of olanzapine, clozapine, ziprasidone, haloperidol, risperidone, and its active metabolite 9-hydroxyrisperidone in rat plasma using midazolam as internal standard (I.S.). The analytes were extracted from rat plasma using a single step liquid-liquid extraction technique. The compounds were separated on a Waters Atlantis^{TM} dC-18 (2.1 × 30 mm, 3 µm) column using a mobile phase of acetonitrile/5 mM ammonium formate (pH 6.1 adjusted with formic acid) with gradient elution. All the analytes were detected in positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. LLOQ was 0.1 ng/ml and correlation coefficient (R^2) values for the linear range of 0.1-100 ng/ml were 0.997 or greater for all the analytes. The precision and accuracy for intra-day and inter-day were better than 8.05%. The relative and absolute recovery was above 77% and matrix effects were low for all the analytes except for ziprasidone. This validated method has been successfully used to quantify the plasma concentration of the analytes after chronic treatment with antipsychotic drugs.

The use of drugs with well-demonstrated efficacy in psychiatric disorders has become common since the 1950s. Currently, about 20% of prescriptions written in the United States are for medications which modify mental processes.[1] Second Generation Antipsychotics (SGAs) (such as olanzapine, clozapine, ziprasidone, risperidone and its active metabolite, 9-hydroxyrisperidone) and First Generation Antipsychotis (FGAs) (such as haloperidol) (Figure 1) make up a large market share for the treatment of schizophrenia and other psychoses.[2] It is reported that the SGAs olanzapine, risperidone, clozapine and ziprasidone are effective in the treatment of both positive and negative symptoms of schizophrenia and that they are less likely to produce extrapyramidal side effects when compared with classical antipsychotics such as haloperidol and chlorpromazine.[3,4] However, suicide and suicide attempts are very frequent in populations using antipsychotics.[5] In addition, several intoxications of antipschotics have been published.[6,7] It has long been known that chronic exposure to FGAs such as haloperidol often results in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Furthermore, given that cognition is now recognized as a key factor that influences long term functional outcome in schizophrenia,[8-10] it is important to determine if there is a correlation between antipsychotic plasma levels (particularly in association with chronic drug exposure) and cognitive function. Such a correlation would allow for improved clinical monitoring of these compounds. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. In addition, they are widely metabolized in the body. Therefore, the concentration of these drugs in plasma is very low (pg-ng/ml levels). For example, the therapeutic plasma levels of olanzapine are in the range of 8-80 ng/ml.[11-13]

In the case of haloperidol levels as low as 2.0 ng/ml have also been described as within the therapeutic range,[14] though most references indicate that 5.0 ng/ml represents the lower end of the therapeutic range. The upper range of haloperidol is 20 ng/ml.[15] 9-Hydroxyrisperidone, the main metabolite of risperidone, had the same antipsychotic activity profile as that of the parent drug .[16] For adequate support of clinical and toxicity studies with risperidone, an analytical method is required for the determination of plasma levels of both risperidone and its active metabolite 9-hydroxyrisperidone. Usually, oral doses of risperidone in the treatment of chronic schizophrenia are 2-6 mg per day and the typical plasma range are 1-10 ng/ml for risperidone.[17] The combination concentration of risperidone and the active 9-hydroxyrisperidone metabolite should also be provided since this combination is considered the most clinically relevant measurement.[18] The range for the combination has been estimated at 20-60 ng/ml.[15,17] From above mentioned information, to quantify relevant plasma levels of these antipsychotic drugs, an analytical method with high sensitivity is required. At present, determination of some of these drugs has been established by high performance liquid chromatography (HPLC) with UV detection,[19-26] HPLC with coulmetric detection[27,28] or fluorescence detection[29,30]. Although electrochemical detection[31-35] offers enhanced sensitivity in the low ng/ml range, electrochemical detectors require optimal working conditions and sample preparation is critical and interference from co-medications is often unavoidable. Capillary electrophoresis (CE) methods[36,37] were reported to detect the antipsychotic drugs, but it is not sensitive and robust for biological samples. An additional draw back of most of these methods was that at least 1 ml of plasma was needed to obtain the reported detection limit. So, most of these methods were not

suitable for the study of pharmacokinetics and metabolism for antipsychotic drugs. The usefulness of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields[38-40]. LC/MS methods offer several significant advantages as compared with other previous methods, the small sample volume required, minimization of mobile phase requirement, rapid analytical run time and improved sensitivity, selectivity and specificity[41]. Several LC-MS/MS methods were reported for the quantification of the antipsychotic drugs in biological fluids[42-47]. However, only a few of the published LC-MS/MS methods have reported improved sensitivity and specificity[44-48] and few LC/MS methods[47,49] offer the ability to measure multiple antipsychotics (i.e. both FGAs and SGAs) simultaneously in the single run. The purpose of this investigation was to develop and validate a highly selective, sensitive and robust LC-MS/MS method for simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat plasma in a single run. The lower limit of quantitation (LLOQ) of all the analytes was 100 pg/ml.

EXPERIMENTAL

Chemicals and reagents

Haloperidol (HAL), risperidone (RISP), clozapine (CLOZ) and olanzapine (OLZ) were kindly provided by Eli Lilly (Indianapolis, IN, USA). 9-Hydroxyrisperidone (9-OH RISP) was donated by the Janssen Research Foundation (Beers, Belgium). Ziprasidone was obtained from Pfizer Central Research (Groton, CT, USA). Midazolam (internal standard, I.S.) was from Sigma (St. Louis, MO, USA). Ethyl acetate, butyl ter-methyl ether, chloroform, hexane, diethylether, isopropylether, HPLC-grade acetonitrile and

methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). The formic acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate, ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler, and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA).

Liquid chromatographic and mass spectrometric conditions

The analytes were separated on a Waters Atlantis^{TM} dC-18 (2.1 × 30 mm; 3 µm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 5 mM ammonium formate in water with pH adjusted to 6.1 using formic acid and mobile phase B was acetonitrile. The flow rate was set 0.3 ml/min. A 15 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 15) (1, 15) (5, 50) (10, 72) (10.5, 80) (13.5, 80) (14.0, 15) (20.0, 15). The column temperature was maintained at 25ºC. The LC flow was introduced directly to the mass spectrometer from 5.0-12.0 min and diverted to waste at other times using a six-port switching valve. The autosampler injection needle was washed with methanol to reduce carry-over after each injection. The mass

spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350ºC. The cone gas flow was set to 50 L/h. Argon was the collision gas and the collision cell pressure was 2.4 × 10^{-3} mbar. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes are presented in Table 1. The source temperature and capillary voltage were set at 130ºC and 3.5 kV, respectively.

Sample collection

Antipsychotic doses were based on previous rodent studies in which time dependent behavioral and neurochemical effects were detected.[8,9] Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human.[50] Male albino Wistar rats (Harlan Inc.) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a 12-h ligh/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg/day), RISP (2.5mg/kg/day), OLZ (10.0mg/kg/day), ZIP (12 mg/kg/day) and CLOZ (20 mg/kg/day) orally in drinking water for periods of 90 or 180 days, plasma samples were collected at days 15, 45, 90 and 180 days of treatment in a separate groups of rats (N=6) for measurements of antipsychotic concentrations. Rats were anesthetized with isofluorane and 3.0 ml of blood was collected via cardiac puncture to heparined tubes. The blood was centrifuged for 15 min at 2500× g at 8ºC and the resulting plasma was frozen until analysis.

Preparation of stock, working standard and quality control solutions

Individual stock solutions of OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP and I.S. (midazolam) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at -20ºC. Combined standard solutions with concentrations of 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0, 400.0 and 1000.0 ng/ml were prepared by serial dilution with 5 mM ammonium formate – acetonitrile solutions (30:70). Precision and accuracy standards with concentrations of 1.0, 3.0, 30.0, and 800.0 ng/ml were also prepared in the same manner. A 40.0 ng/ml I.S. standard solution was prepared with 5 mM ammonium formate – acetonitrile solutions (30:70) from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at -20ºC when not use and replaced every 3 months. Fresh standard solution was prepared for each day of analysis or validation.

Preparation of calibration and QC samples

Sample for the calibration curves and QCs were prepared by adding 25.0 µl of each standard into 250 µl blank plasma. This yields calibration standard concentrations of 0.1, 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0 and 100.0 ng/ml. The final concentrations of QCs were 0.1, 0.3, 3.0, 80.0 ng/ml. The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

Sample preparation

To a 250 µl rat plasma sample, 25 µl of internal standard (40.0 ng/ml, midazolam), 0.2ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted in 3 ml isopropyl ether for 5 min. After centrifugation at 2000× g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in

a vacuum centrifuge. To the residue, 100 µl of methanol : 20 mM ammonium formate (pH 3.86, adjusted by formic acid) (70:30) was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000× g prior to LC MS/MS analysis.

Method validation

The method was validated for linearity, recovery, matrix effect, accuracy and precision. Plasma calibration curves were constructed using the peak area ratios of OLZ, RISP, 9-OH RISP, CLOZ, HAL or ZIP to that of I.S., and applying a weight ($1/x^2$) least squares linear regression analysis, precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples (0.1, 0.3, 3.0, and 80.0 ng/ml). Five replicates of each QC points were analyzed to determine the inta-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Absolute and relative recoveries, and matrix effects were calculated for spiked samples at 0.1, 0.3, 3.0, 80.0 ng/ml and 4.0 ng/ml I.S. samples (n=5) in rat plasma. Absolute recovery was calculated as the peak area for the analytes in plasma spiked before extraction divided by the peak area of the pure drugs in the methanol : 20 mM ammonium formate (pH 3.86, adjusted with formic acid) (70:30) solution at the same concentration. Relative recovery was calculated by dividing the peak area for analytes spiked before extraction by peak area for an equal concentration of the antipsychotic drugs sample in the same matrix spiked after extraction. The matrix effects were calculated by dividing the response for the analytes in biological matrix spiked after extraction by an equal concentration of the analytes in the methanol : 20 mM ammonium formate (pH 3.86, adjusted with formic acid) (70:30) solution.[51] The stability

of the stock solutions was determined at their storage conditions of -20ºC for three months. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls.[44] The bench-top stability of spiked plasma samples stored at room temperature was evaluated for 8h except for OLZ which was evaluated for 2h. The freeze/thaw stability was investigated by comparing the stability samples following three freeze/thaw cycles against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 24h. The stability testing was performed at 0.3 and 3.0 ng/ml concentration levels for all of the antipsychotic drugs.

RESULTS AND DISCUSSION

Method development

In order to develop and validate a highly sensitive and selective method with the designed LLOQ (100 pg/ml), during method development different options were evaluated to optimize the detection (MRM) parameters, chromatography and sample preparation methodology. MS-MS detection provided improved sensitivity for trace mixture analysis.[52] ESI(+) MS-MS product ion spectra were produced by CAD of the protonated molecule ion $(M+H)^+$. The most favorable transitions were selected and the instrument parameter settings were optimized individually for each pure compound by constant infusion at 10 µl/min of a 2 µg/ml solution. The major MS-MS transitions utilized for LC-MS/MS analysis were m/z 316→256 for OLZ, m/z 411→191 for RISP, m/z 427→207 for 9-OH RISP, m/z 327→270 for CLOZ, m/z 413→194 for ZIP, m/z

376→165 for HAL and m/z 326→291 for I.S. (midazolam) (shown in Figure 4.2). The relevant optimized MRM parameters are summarized in Table 4.1.

The chromatographic conditions, especially the analytical column and the composition of mobile phase, were optimized through several trials to achieve the desired sensitivity, separation, run time, and symmetric peak shapes for the analytes and I.S. Agilent XDB C8 (150 × 2.1 mm, 5 µm), Waters XTerra C18 (50 × 2.1 mm, 5 µm) and Waters Atlantis^{TM} dC-18 (2.1 × 30 mm; 3 µm) were evaluated. As a result, Waters Atlantis^{TM} dC-18 (2.1 × 30 mm; 3 µm) was selected as it produced the satisfactory separation, peak shape and shorter analytical time. Different mobile phase A (buffer), 5 mM ammonium formate (pH 6.1 or 4.6 adjusted by formic acid), 5 mM ammonium acetate, 10 mM formic acid, or 20 mM ammonium formate were evaluated. Finally, 5 mM ammonium formate (pH 6.1) buffer combination with acetonitrile resulted in the highest signals with optimized gradient elution for all of the analytes. Initially, we evaluated a one-step protein precipitation method using methanol or acetonitrile for sample preparation. However, the LLOQ was poorer because of dilution of the sample. Also, the recovery was very low for ZIP and HAL. Next, we evaluated solid phase extraction (SPE) methods using different cartridges such as Waters Oasis HLB and Varian C18. However, the recovery of ZIP was still very low. Finally, we evaluated liquid-liquid extraction (LLE) for the sample preparation. LLE can be helpful in producing a clean sample and avoiding the introduction of non-volatile materials on the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effects in LC-MS/MS analysis. Different organic solvents, ethyl acetate, butyl tert-methyl ether, chloroform, hexane, diethyl ether and isopropylether, and their mixtures in different

combinations and ratios were evaluated. Finally, isopropylether was found to optimal, because it is able to produce a clean chromatogram for a blank plasma sample and yielded the highest recovery for the analytes by a single-step liquid-liquid extraction.

Specificity

Representative chromatograms obtained from blank plasma and plasma spiked with the LLOQ standard (0.1 ng/ml) are shown in Figure 4.3. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or I.S. in blank rat plasma from six different lots. Utilization of the major precursor-to-product transition for each compound enhanced the mass spectrometric selectivity. The analytes and I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of the plasma sample. Hence, alkalytic modifiers were used to adjust the pH of plasma samples. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH 10.69), were evaluated. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for all of the analytes.

Linearity and sensitivity

Table 4.2 shows the calibration curves for each day of validation. The curves showed good linear response ($R^2 > 0.997$) over the range from 0.1-100 ng/ml. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for the analytes. Table 4.2 showed the slope and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was

0.1ng/ml for determination of all of the analytes in rat plasma as shown in Table 4.3. A signal-to-noise (S/N) > 10 at LLOQ (0.1 ng/ml) was observed for all of the analytes. To our knowledge, this was the first time to report that LLOQ for CLOZ, HAL and ZIP in validated methods for biological samples was as low as 0.1 ng/ml.

Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The accuracy and precision data can be seen in Table 4.3. The values for the intra-day precision and accuracy were better than 5.92% and 8.05% for all the analytes. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were better than 4.20% and 5.75% (Table 4.3).

Recovery and matrix effect

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 4.4. Absolute recoveries range from 77.1% to 96.8% and relative recoveries from 81.7% to 98.8% for all of the analytes and I.S. Matrix effects are an important issue in ESI. Matrix effects are the results of co-eluting components, generally from the matrix, that cause variable suppression or enhancement of analyte response.[53] In the positive ion mode, if the ionized analyte is transferred to the gas phase, gas phase proton transfer reactions may cause neutralization if another neutral species is present with a higher proton affinity. In addition, other ionic species, such as salts, in biological samples with high ionization efficiency or surface activity may compete with the analytes during ion evaporation. LLE used in this study can be helpful in producing clean samples, which are essential to reduce matrix effect. The matrix effect of plasma for all of the analytes

in this work was lower than 16.57% suppression or enhancement (Table 4.4). This demonstrated that the sample preparation procedure of LLE method provided a clean extraction solution for LC-MS/MS system.

Stability studies

Stability testing is very important for validated method in biological sample. The stock solutions were stable at the storage conditions (-20ºC) for three months (Data not shown). All the other stability studies were conducted at two concentration levels (0.3 and 3.0 ng/ml) with five determinations for each. Plasma extracts were stable in mobile phase in the HPLC autosampler for at least 24h, indicating that samples should be processed within this period of time (Table 4.5). The freeze/thaw stability tests indicate the analytes were stable in rat plasma for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for all of the analytes for 8h except OLZ which was only stable for 2h. The RE% was from 0.64% to 8.07% (< 15%) and RSD was from 0.45% to 6.92% for all the analytes (see Table 4.5).

Zhou et al.,[49] reported that storage of OLZ in human plasma at room temperature for 24h produced significant degradation of OLZ. If Vitamin C was added to plasma, then OLZ was stable at room temperature for 24h. In this work, OLZ did not show significant degradation in rat plasma kept at room temperature for up to 2h without Vitamin C addition. However, for longer storage of OLZ, freezing rat plasma is recommended.[47]

Application of the method

The validated method has been successfully used to quantify antipsychotic drug concentrations in rat plasma after the chronic treatment of rats with the antipsychotic drugs in their drinking water. The concentration data for antipsychotic drugs in rat

plasma are reported in Table 4.6. The representative of MRM chromatograms resulting from the analysis of real samples after the chronic treatment of antipsychotic drugs is shown in Figure 4. In general, the concentration of HAL, RISP, OLZ and ZIP in rat plasma was relatively low. However, all the analytes were observed above the LLOQ. The concentration of active metabolite 9-OH RISP was higher than that of its parent drug RISP in plasma. Balant-Gorgia et al. reported the same result.[17]

CONCLUSIONS

A simple, specific, rapid and sensitive LC-MS/MS analytical method for the simultaneous determination of OLZ, RISP, 9-OH RISP, HAL, CLOZ and ZIP in rat plasma has been developed and validated. This method provided excellent specificity, wide linear range and a LLOQ of 0.1ng/ml for all of the analytes. A one-step liquid-liquid extraction sample preparation was used for 0.25 ml rat plasma that provided low matrix effects and high recovery for all the analytes. The method was successfully applied to study the effect of chronic treatment of FGAs (HAL) and SGAs (OLZ, RISP and ZIP) antipsychotic drugs on the cognitive function in rats. This method also meets the requirements for pharmacokinetic and metabolic studies of these antipsychotic drugs.

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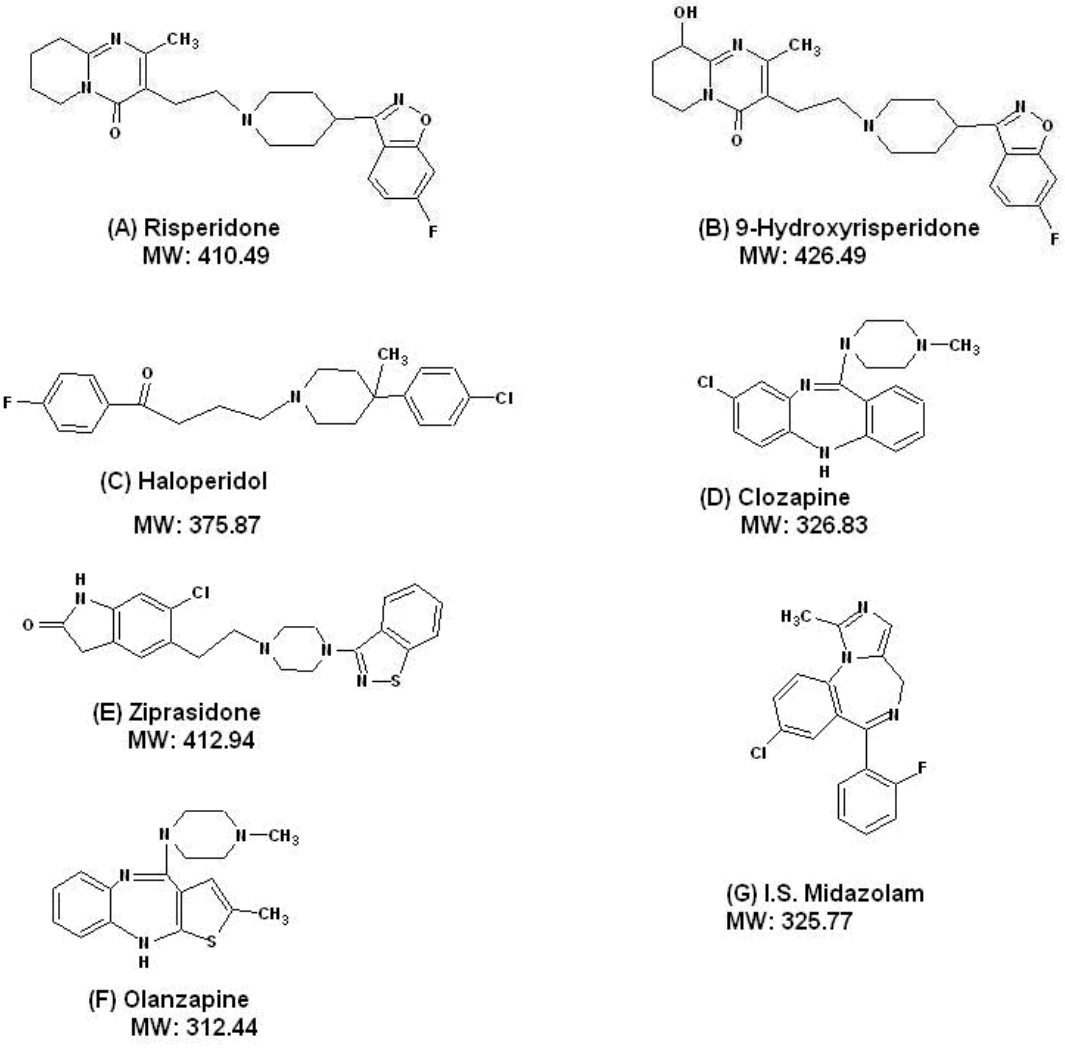

Figure 4.1. Chemical structures of OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP and I.S. (midazolam).

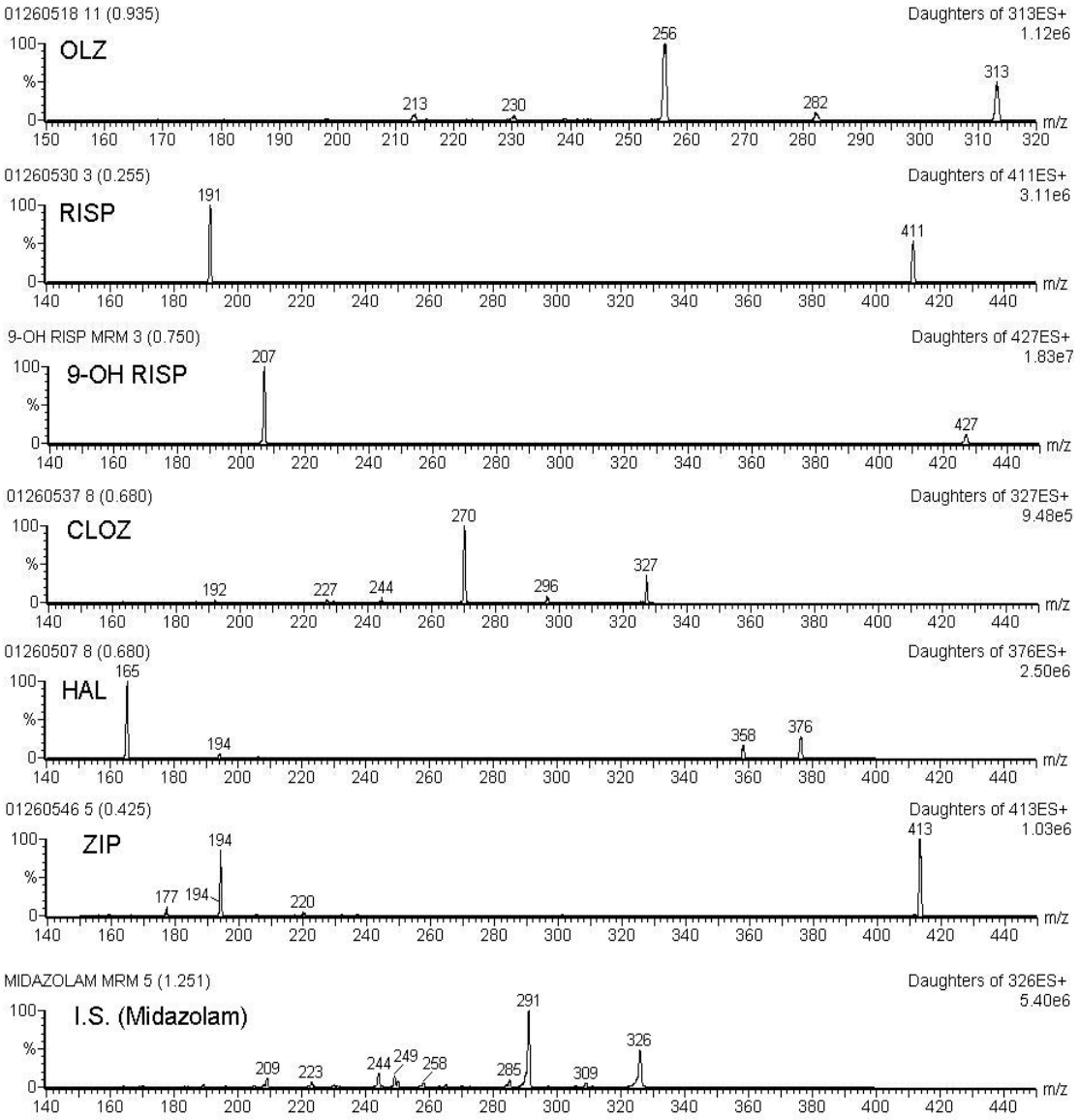

Figure 4.2. MS/MS spectra of OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP and I.S.

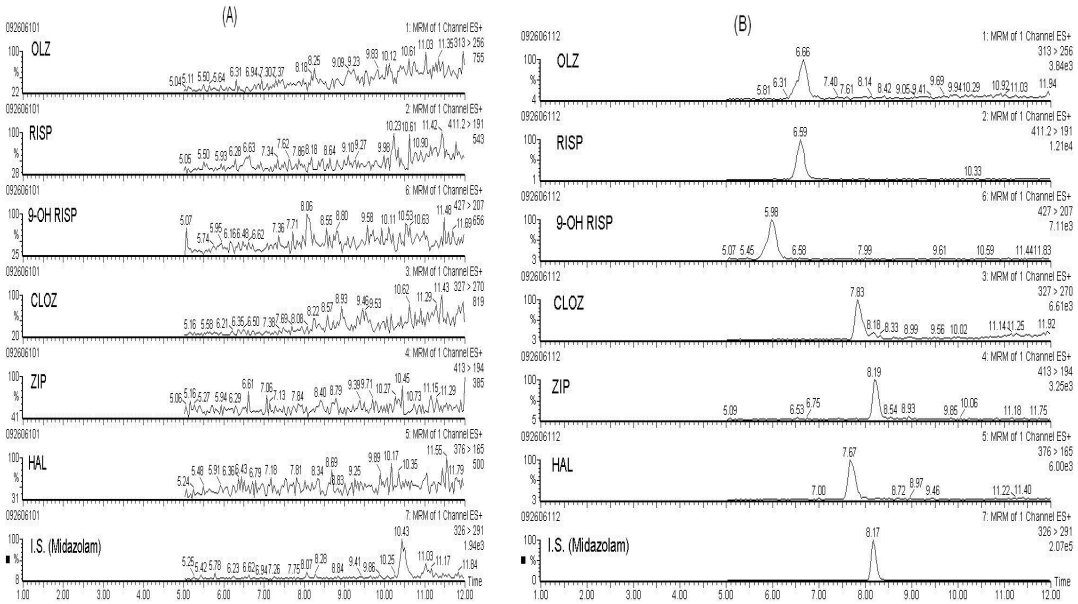

Figure 4.3. Representative chromatograms obtained from (A) blank rat plasma; (B) plasma spiked with LLOQ (0.1 ng/ml) concentration for all of the analytes and I.S. (4 ng/ml).

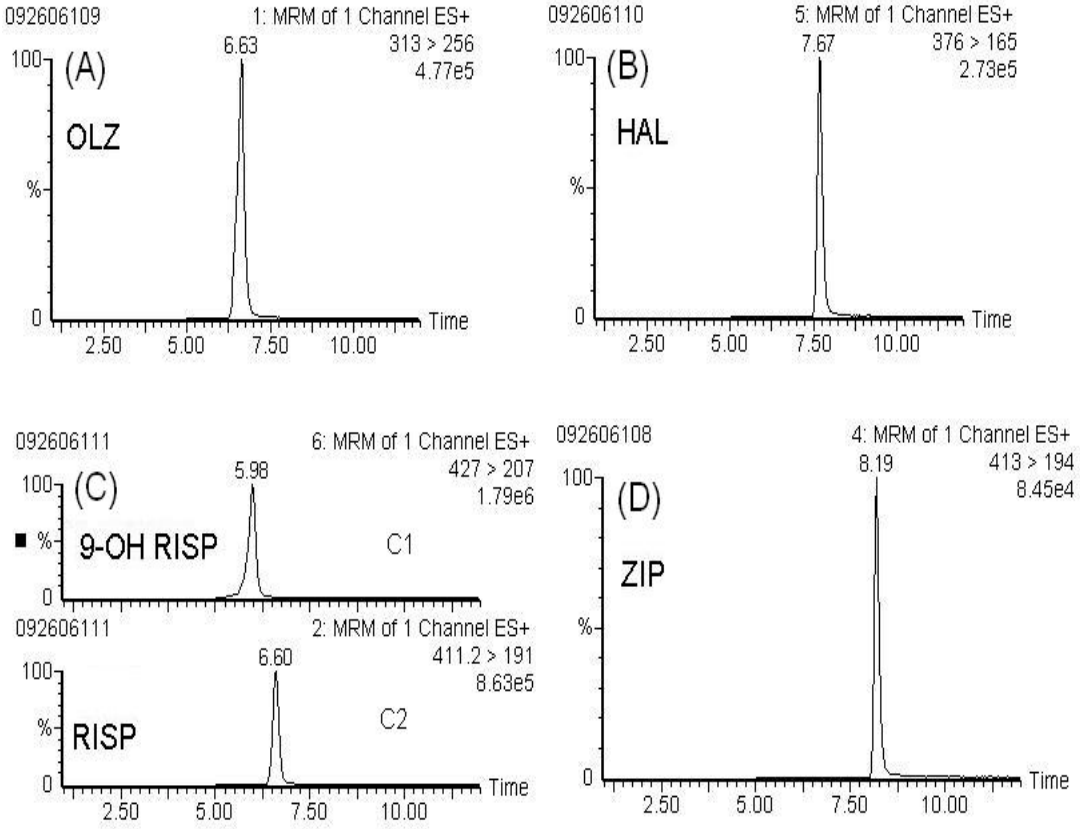

Figure 4.4. Representative chromatograms of real plasma samples from chronic treatment of psychotic drugs for 45 days: (A) a rat treated with OLZ (10.0 mg/kg/day) and the concentration of OLZ in plasma was 15.41 ng/ml; (B) a rat treated with HAL (2.0 mg/kg/day) and the concentration of HAL in plasma was 4.94 ng/ml; (C) a rat treated with RISP (2.5 mg/kg/day) and the concentration of RISP and its active metabolite (9-OH RISP) in plasma was 24.54 ng/ml (C1) and 7.16 ng/ml (C2), respectively; (D) a rat treated with ZIP (12.0 mg/kg/day) and the concentration of ZIP in plasma was 3.20 ng/ml.

Table 4.1. Optimal positive ion ESI mass spectrometric conditions for multiple reaction monitoring (MRM)

Drugs	Ion Transition	Cone Voltage (V)	Coll Energy (eV)
OLZ	316→256	32	23
RISP	411→191	35	24
9-OH RISP	427→207	35	27
CLOZ	327→270	35	22
ZIP	413→194	37	26
HAL	376→165	30	21
Midazolam (I.S.)	326→291	32	30

Table 4.2. Statistical data for linearity including standard deviation (S.D.) (linear range 0.1-100ng/ml) for all of the analytes

	OLZ	RISP	9-OH RISP	CLOZ	HAL	ZIP
R^2	0.9982± 0.0006	0.9994± 0.0011	0.9994± 0.0004	0.9993± 0.0001	0.9994± 0.0004	0.9976± 0.0008
Slope	0.3144± 0.0165	0.9327± 0.0414	0.6036± 0.0144	0.5117± 0.0160	0.4583± 0.0187	0.2690± 0.0116

Table 4.3. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the LC-MS/MS method used to quantitate antipsychotic drugs in rat plasma

Drug		Intra-day			Inter-day		
	Concentration added (ng/ml)	Observed concentration ±S.D. (ng/ml)	R.S.D. (%)	Error (%)	Observed concentration (ng/ml)	R.S.D. (%)	Error (%)
OLZ	0.1	0.0966± 0.0057	5.92	4.15	0.0995 ± 0.0042	4.20	2.78
	0.3	0.283 ± 0.0083	2.94	5.74	0.286 ± 0.0096	3.36	4.79
	3.0	2.956 ± 0.0358	1.21	1.51	2.942 ± 0.0743	2.52	2.64
	80.0	79.753 ± 1.2113	1.52	1.15	80.827 ± 1.5631	1.93	1.66
RISP	0.1	0.0983 ± 0.0033	3.37	2.03	0.0993 ± 0.0033	3.33	2.39
	0.3	0.289 ± 0.0056	1.95	3.63	0.294 ± 0.0069	2.36	2.59
	3.0	2.964 ± 0.0601	2.03	1.81	2.973 ± 0.0504	1.70	1.58
	80.0	77.560 ± 1.573	2.03	3.05	77.896 ± 1.221	1.57	2.65
9-OH RISP	0.1	0.0974 ± 0.0019	1.97	2.74	0.0987 ± 0.0024	2.45	1.87
	0.3	0.207 ± 0.0072	2.44	1.71	0.290 ± 0.0091	3.13	3.55
	3.0	3.087 ± 0.0044	1.44	2.89	2.992 ± 0.1097	3.67	2.90
	80.0	78.335 ± 1.819	2.32	2.76	80.347 ± 2.7258	3.39	2.94
CLOZ	0.1	0.0984 ± 0.0022	2.25	2.17	0.0994 ± 0.0031	3.13	2.46
	0.3	0.298 ± 0.0069	2.32	2.01	0.297 ± 0.0047	1.57	1.56
	3.0	2.891 ± 0.0422	1.46	3.63	2.967 ± 0.0713	2.40	1.97
	80.0	78.86 ± 1.4296	1.81	1.79	78.004 ± 1.4112	1.81	2.63
ZIP	0.1	0.0982 ± 0.0037	3.74	3.48	0.0980 ± 0.0026	2.61	2.70
	0.3	0.293 ± 0.0147	5.02	4.92	0.302 ± 0.0114	3.77	3.28
	3.0	3.100 ± 0.0956	3.08	3.35	3.096 ± 0.0693	2.24	3.20
	80.0	73.557 ± 2.171	2.95	8.05	75.401 ± 2.703	3.58	5.75
HAL	0.1	0.0980 ± 0.0029	2.96	2.09	0.0985±0.0030	3.07	2.39
	0.3	0.291 ± 0.0046	1.59	2.84	0.296 ± 0.0069	2.34	2.05
	3.0	2.904 ± 0.0228	0.79	3.20	2.967 ± 0.0616	2.08	1.77
	80.0	78.083 ± 1.567	2.01	2.43	77.235 ± 1.764	2.28	3.47

Table 4.4. Absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of analytes and I.S. in rat plasma (n=5)

Drugs	Concentration (ng/ml)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
OLZ	0.1	95.7 ± 1.71	92.8 ± 1.66	103.15	3.15% enhancement
	0.3	96.8 ± 0.69	93.4 ± 0.65	103.61	3.61% enhancement
	3.0	93.6 ± 2.25	89.1 ± 2.13	105.0	5.0% enhancement
	80.0	92.0 ± 3.18	92.8 ± 3.22	99.12	0.88% suppression
RISP	0.1	94.7 ± 1.43	91.5 ± 1.38	103.47	3.47% enhancement
	0.3	91.1 ± 0.69	89.9 ± 0.66	101.30	1.30% enhancement
	3.0	86.4 ± 1.51	86.5 ± 1.54	99.81	0.19% suppression
	80.0	87.1 ± 0.87	90.5 ± 0.91	96.19	3.81% suppression
9-OH RISP	0.1	88.2 ± 1.64	90.6 ± 1.68	97.26	2.74% suppression
	0.3	88.1 ± 0.97	85.4 ± 0.94	103.16	1.36% enhancement
	3.0	80.9 ± 2.17	81.7 ± 2.19	99.06	0.94% suppression
	80.0	81.7 ± 1.11	84.1 ± 1.15	97.15	2.85% suppression
CLOZ	0.1	91.9 ± 1.71	96.4 ± 1.79	95.36	4.65% suppression
	0.3	86.0 ± 1.61	89.7 ± 1.67	95.85	4.15% suppression
	3.0	88.2 ± 1.62	91.2 ± 1.67	96.75	3.25% suppression
	80.0	87.7 ± 1.79	91.9 ± 1.88	95.46	4.54% suppression
HAL	0.1	93.1 ± 1.36	96.9 ± 1.42	96.05	3.95% suppression
	0.3	93.7 ± 0.94	91.7 ± 0.89	102.16	1.02% enhancement
	3.0	89.5 ± 2.36	92.6 ± 1.47	96.62	3.38% suppression
	80.0	89.7 ± 1.71	94.7 ± 1.85	94.77	5.23% suppression
ZIP	0.1	80.0 ± 1.88	95.9 ± 2.36	83.43	16.57% suppression
	0.3	80.8 ± 1.04	96.8 ± 1.26	83.51	16.49% suppression
	3.0	77.1 ± 1.28	86.9 ± 1.46	88.66	11.34% suppression
	80.0	77.2 ± 2.83	91.9 ± 3.37	83.89	16.11% suppression
I.S.	4.0	94.9 ± 1.55	98.8 ± 1.62	96.05	3.95% suppression

Table 4.5. Stability testing of antipsychotic drugs used in this study (n=5)

Drugs	Stability	Spiked conc. (ng/ml)	Observed conc. ± S.D. (ng/ml)	R.S.D. (%)	Relative error (%)
OLZ	Three freeze-thaw cycle	0.3	0.287 ± 0.0169	5.90	-4.49
		3.0	3.068 ± 0.0440	1.43	2.27
	Bench top (2h)	0.3	0.284 ± 0.0178	6.28	-5.49
		3.0	2.818 ± 0.122	4.31	-6.06
	Autosampler stability (24h)	0.3	0.315 ± 0.0108	3.44	5.12
		3.0	3.095 ± 0.214	6.92	3.15
RISP	Three freeze-thaw cycle	0.3	0.291 ± 0.0108	3.70	-2.90
		3.0	2.859 ± 0.0919	3.22	-4.69
	Bench top (8h)	0.3	0.285 ± 0.0157	5.51	-4.93
		3.0	3.090 ± 0.1328	4.30	3.00
	Autosampler stability (24h)	0.3	0.306 ± 0.0032	1.03	2.13
		3.0	3.035 ± 0.0543	1.79	1.16
9-OH RISP	Three freeze-thaw cycle	0.3	0.302 ± 0.0188	6.23	0.64
		3.0	3.07 ± 0.0356	1.16	2.48
	Bench top (8h)	0.3	0.291 ± 0.0094	3.24	-2.96
		3.0	3.07 ± 0.0508	1.65	2.44
	Autosampler stability (24h)	0.3	0.324 ± 0.0128	3.94	8.11
		3.0	3.145 ± 0.0768	2.44	4.82
CLOZ	Three freeze-thaw cycle	0.3	0.289 ± 0.0028	0.98	-3.52
		3.0	2.758 ± 0.181	6.57	-8.07
	Bench top (8h)	0.3	0.291 ± 0.0059	2.04	-3.07
		3.0	2.907 ± 0.0541	1.86	-3.09
	Autosampler stability (24h)	0.3	0.296 ± 0.0050	1.69	-1.31
		3.0	3.07 ± 0.121	3.96	2.26
HAL	Three freeze-thaw cycle	0.3	0.291 ± 0.0067	2.29	-3.05
		3.0	2.917 ± 0.412	1.41	-2.75
	Bench top (8h)	0.3	0.286 ± 0.0013	0.45	-4.55
		3.0	3.050 ± 0.1482	4.86	1.67
	Autosampler stability (24h)	0.3	0.312 ± 0.0062	1.99	3.84
		3.0	3.110 ± 0.0212	0.68	3.65
ZIP	Three freeze-thaw cycle	0.3	0.292 ± 0.0069	2.37	-2.82
		3.0	2.91 ± 0.0294	2.04	-3.02
	Bench top (8h)	0.3	0.289 ± 0.0099	3.44	-3.60
		3.0	2.946 ± 0.1050	3.57	-1.81
	Autosampler stability (24h)	0.3	0.291 ± 0.0018	0.62	-2.95
		3.0	3.02 ± 0.0094	1.16	0.66

Table 4.6. Plasma concentrations of the analytes after the chronic treatment of antipsychotic drugs for rats (n=6)

Drugs administered	Dose (mg/kg/day)	Time of treatment (days)	Concentrations (ng/ml ± S.D.)
RISP	2.5	15	RISP: 4.61 ± 3.11
			9-OH RISP: 22.50 ± 5.58
	2.5	45	RISP: 2.92 ± 2.61
			9-OH RISP: 10.51 ± 8.86
	2.5	90	RISP: 1.33 ± 0.75
			9-OH RISP: 9.91 ± 5.29
	2.5	180	RISP: 4.30 ± 3.64
			9-OH RISP: 22.72 ± 16.35
HAL	2.0	15	6.38 ± 2.26
	2.0	45	2.68 ± 3.63
	2.0	90	3.81 ± 1.83
	2.0	180	12.04 ± 6.68
OLZ	10.0	45	23.74 ± 17.86
	10.0	90	23.21 ± 24.36
	10.0	180	80.28 ± 91.83
ZIP	12.0	45	7.57 ± 5.64

Chapter 5

SENSITIVE LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY METHOD FOR THE SIMULTANEOUS DETERMINATION OF OLANZAPINE, RISPERIDONE, 9-HYDROXYRISPERIDONE, CLOZAPINE, RISPERIDONE AND ZIPRASIDONE IN RAT BRAIN TISSUE

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Abstract One prerequisite for therapeutic effects of psychotic drugs is its ability to pass the blood brain barrier. Hence, it is very important to know the concentration of antipsychotic drugs in brain tissue. A simple, sensitive and rapid liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method was developed and validated for simultaneous quantification of olanzapine, clozapine, ziprasidone, haloperidol, risperidone, and its active metabolite 9-hydroxyrisperidone in rat brain tissue. The analytes were extracted from rat brain homogenate using liquid-liquid extraction technique. The compounds were separated on a Waters Atlantis^{TM} dC-18 (2.1 × 30 mm, 3 µm) column using a mobile phase of acetonitrile/5 mM ammonium formate (pH 6.1 adjusted with formic acid) with gradient elution. All the analytes were detected in positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. LLOQ was 0.208 ng/g for olanzapine, risperidone, 9-hydroxyrisperidone and clozapine and 0.416 ng/g for haloperidol and ziprasidone. The method was linear in the concentration range from 0.208-416.0 ng/g for risperidone, 9-hydroxyrisperidone and clozapine and 0.416-416.0 ng/g for risperidone and ziprasidoen. The correlation coefficient (R^2) values were 0.997 or greater for all the analytes. The precision and accuracy for intra-day and inter-day were better than 7.67%. The relative and absolute recovery was above 73.6% and matrix effects were low for all the analytes except for ziprasidone. This validated method has been successfully used to quantify the rat brain tissue concentration of the analytes after chronic treatment with antipsychotic drugs.
Keywords: Brain; Risperidone; Olanzapine; Clozapine; Ziprasidone; Haloperidol

1. Introduction

Atypical antipsychotics (olanzapine, clozapine, ziprasidone, risperidone and its active metabolite, 9-hydroxyrisperidone) and haloperidol (Figure 1) make up a large market share for the treatment of schizophrenia and other psychoses [1]. It is reported that the atypical antipsychotic drugs olanzapine, risperidone, clozapine and ziprasidone are effective in the treatment of both positive and negative symptoms of schizophrenia and that they are less likely to produce extrapyramidal side effects when compared with classical antipsychotics such as haloperidol and chlorpromazine [2, 3]. However, several intoxications of antipschotics have already been published [4, 5]. It has long been known that chronic exposure to typical neuroleptics such as haloperidol often results in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Thus the chronic effects of typical and atypical antipsychotics on cognitive function are attracting more and more attention [6-8]. One prerequisite for the therapeutic effects of antipsychotic drugs is its ability to pass the blood brain barrier. Therefore, it is important to know the concentrations of antipsychotic drugs in brain, the target compartment for therapeutic actions and determine the extent to which there is a correlation between the concentration of antipsychotic drugs in the brain tissue and the loss of cognitive function in the brain region when antipsychotics doses are given at chronic low dose levels. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. 9-Hydroxyrisperidone, the main metabolite of risperidone, had the same antipsychotic activity profile as that of the parent drug [9]. Usually, oral doses of risperidone in the treatment of chronic schizophrenia are 2-6 mg per day and the typical plasma range are 1-10 ng/ml for risperidone and 10-70 ng/ml for

9-hydroxyrisperidone [10]. However, the concentrations of risperidone and its active metabolite, 9-hydroxyrisperidone, are very low in brain tissue. The concentration ratios of brain/plasma were 0.22 for risperidone and 0.04 for 9-hydroxyrisperidone [11]. In order to quantify relevant brain tissue levels of risperidone and 9-hydroxyrisperidone, an analytical method with high sensitivity is required. At present, determination of some of these drugs in plasma has been established by high performance liquid chromatography (HPLC) with UV detection [12-19], coulmetric detection [20, 21], fluorescence detection [22, 23] or electrochemical detection [24-25]. But, to date, very few papers reported the determination of antipsychotic drugs in brain tissue [11, 26-30]. Most of these methods were not validated. The usefulness of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields [31, 32]. LC/MS methods offer several significant advantages as compared with other previous methods, the small sample volume required, minimization of mobile phase requirement, rapid analytical run time and improved sensitivity, selectivity and specificity [33]. Several LC-MS/MS methods were reported for the quantification of the antipsychotic drugs in biological fluids [34-36]. However, no study has been conducted to determine the olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, ziprasidone and haloperidol simultaneously in brain tissue by LC-MS/MS method. The purpose of this investigation was to develop and validate a highly selective, sensitive and robust LC-MS/MS method for simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat brain tissue in a single

run. To our knowledge, our paper for the first time reported the validated method for determination of ziprasidone in brain tissue.

2. Experimental

2.1 Chemicals and reagents

Haloperidol (HAL), risperidone (RISP), clozapine (CLOZ) and olanzapine (OLZ) were kindly provided by Eli Lilly (Indianapolis, IN, USA). 9-Hydroxyrisperidone (9-OH RISP) was donated by the Janssen Foundation (Beers, Belgium). Ziprasidone was obtained from Pfizer Central Research (Groton, CT, USA). Midazolam (internal standard, I.S.) was from Sigma (St. Louis, MO, USA). Ethyl acetate, butyl ter-methyl ether, chloroform, hexane, diethylether, isopropylether, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). The formic acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate, ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

2.2 Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler, and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA).

2.3 Liquid chromatographic and mass spectrometric conditions

The analytes were separated on a Waters Atlantis^{TM} dC-18 (2.1 × 30 mm; 3 µm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 5 mM ammonium formate in water with pH adjusted to 6.1 using formic acid and mobile phase B was acetonitrile. The flow rate was set 0.3 ml/min. A 15 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 15) (1, 15) (5, 50) (10, 72) (10.5, 80) (13.5, 80) (14.0, 15) (20.0, 15). The column temperature was maintained at 25ºC. The LC flow was introduced directly to the mass spectrometer from 5.0-12.0 min and diverted to waste at other times using a six-port switching valve. The autosampler injection needle was washed with methanol to reduce carry-over after each injection. The mass spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350ºC. The cone gas flow was set to 50 L/h. Argon was the collision gas and the collision cell pressure was 2.4 × 10^{-3} mbar. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes are presented in Table 5.1. The source temperature and capillary voltage were set at 130ºC and 3.5 kV, respectively.

2.4 Sample collection

Antipsychotic doses were based on previous rodent studies in which time dependent behavioral and neurochemical effects were detected [6, 7]. Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human [37]. Male albino Wistar rats (Harlan, Inc.) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a

12-h ligh/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg/day), RISP (2.5mg/kg/day), OLZ (10.0mg/kg/day), ZIP (12 mg/kg/day) and CLOZ (20 mg/kg/day) orally in drinking water for periods of 90 or 180 days. The whole brains of sacrificed animals at days 90 or 180 days were removed and kept frozen at -70 ºC until analysis.

2.5 Preparation of stock, working standard and quality control solutions

Individual stock solutions of OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP and I.S. (midazolam) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at -20ºC. Combined standard solutions with concentrations of 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0, 400.0 and 1000.0 ng/ml were prepared by serial dilution with 5 mM ammonium formate – acetonitrile solutions (30:70). Precision and accuracy standards with concentrations of 0.5, 3.0, 30.0, and 300.0 ng/ml for OLZ, RISP, 9-OH RISP and CLOZ and 1.0, 3.0, 30.0, and 300.0 ng/ml for HAL and ZIP were also prepared in the same manner. A 40.0 ng/ml I.S. standard solution was prepared with 5 mM ammonium formate – acetonitrile solutions (30:70) from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at -20ºC when not use and replaced every 3 months. Fresh standard solution was prepared for each day of analysis or validation.

2.6 Preparation of calibration and QC samples

The brains were minced and homogenized in a volume of deionized water (in ml) equal to twice the weight (in g) of the tissue using a Brinkmann (Westburg, NY, USA) Polytron TP 1020 homogenizer. Sample for the calibration curves and QCs were prepared by

adding 25.0 µl of each standard into 200 µl blank brain homogenate. This yields calibration standard concentrations of 0.208, 0.416, 0.832, 1.664, 4.16, 8.32, 16.64, 41.6, 83.2, 166.4 and 416.0 ng/g. The final concentrations of QCs were 0.208, 1.25, 12.5, 125.0 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416, 1.25, 12.5, 125.0 ng/g for HAL and ZIP. The spiked brain homogenate samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

2.7 Sample preparation

To a 200 µl rat brain homogenate, 25 µl of internal standard (40.0 ng/ml, midazolam), 0.4ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted in 3 ml isopropyl ether for twice. After centrifugation at 2000× g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 100 µl of methanol : 20 mM ammonium formate (pH 3.86, adjusted by formic acid) (70:30) was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000× g prior to LC MS/MS analysis.

2.8 Method validation

The method was validated for linearity, recovery, matrix effect, accuracy and precision. Brain homogenate calibration curves were constructed using the peak area ratios of OLZ, RISP, 9-OH RISP, CLOZ, HAL or ZIP to that of I.S., and applying a weight ($1/x^2$) least squares linear regression analysis, precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples. Five replicates of each QC points were analyzed to determine the inta-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Absolute and relative

recoveries, and matrix effects were calculated for spiked samples at 0.208, 1.25, 12.5, 125.0 ng/g for OLZ, RISP, 9-OH RISP and CLOZ, 0.416, 1.25, 12.5, 125.0 ng/g for HAL and ZIP and 16.64 ng/g I.S. samples (n=5) in rat brain homogenate. Absolute recovery was calculated as the peak area for the analytes in plasma spiked before extraction divided by the peak area of the pure drugs in the methanol : 20 mM ammonium formate (pH 3.86, adjusted by formic acid) (70:30) solution at the same concentration. Relative recovery was calculated by dividing the peak area for analytes spiked before extraction by peak area for an equal concentration of the antipsychotic drugs sample in the same matrix spiked after extraction. The matrix effects were determined using the method of Maluszewski et al. [38]. The stability of the stock solutions was determined at their storage conditions of -20ºC for three months. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls [34]. The bench-top stability of spiked plasma samples stored at room temperature was evaluated for 2h. The freeze/thaw stability was investigated by comparing the stability samples following three freeze/thaw cycles, against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted brain homogenate samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 24h. The stability testing was performed at 1.25 and 12.5 ng/g concentration levels for all of the antipsychotic drugs.

3. Results and discussion

3.1 Method development

In order to develop and validate a highly sensitive and selective method with the designed LLOQ (0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP), during method development different options were evaluated to optimize the detection (MRM) parameters, chromatography and sample preparation methodology. MS-MS detection provided improved sensitivity for trace mixture analysis . ESI(+) MS-MS product ion spectra were produced by CAD of the protonated molecule ion $(M+H)^+$. The most favorable transitions were selected and the instrument parameter settings were optimized individually for each pure compound by constant infusion at 10 µl/min of a 2 µg/ml solution. The major MS-MS transitions utilized for LC-MS/MS analysis were m/z 316→256 for OLZ, m/z 411→191 for RISP, m/z 427→207 for 9-OH RISP, m/z 327→270 for CLOZ, m/z 413→194 for ZIP, m/z 376→165 for HAL and m/z 326→291 for I.S. (midazolam). The relevant optimized MRM parameters are summarized in Table 5.1.

The chromatographic conditions were optimized and Waters Atlantis^{TM} dC-18 (2.1 × 30 mm; 3 µm) was selected as it produced the satisfactory separation, peak shape and shorter analytical time. 5 mM ammonium formate (pH 6.1) buffer combination with acetonitrile resulted in the sensitive signals with optimized gradient elution for all of the analytes. Initially, we evaluated a one-step protein precipitation method using methanol or acetonitrile for sample preparation. However, the LLOQ was poorer because of dilution of the sample. Also, the recovery was very low for ZIP, HAL and I.S. Next, we evaluated solid phase extraction (SPE) methods using different cartridges such as Waters Oasis HLB and Varian C18. However, the recovery of ZIP, HAL and I.S. was still very low. Some of ZIP was deadly absorbed on the SPE cartridge. Finally, we evaluated

liquid-liquid extraction (LLE) for the sample preparation. LLE can be helpful in producing a clean sample and avoiding the introduction of non-volatile materials on the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effects in LC-MS/MS analysis [36]. Different organic solvents, ethyl acetate, butyl tert-methyl ether, chloroform, hexane, diethyl ether and isopropylether, and their mixtures in different combinations and ratios were evaluated. Finally, isopropylether was found to optimal, because it was able to produce a clean chromatogram for a blank brain homogenate sample and yield the highest recovery for the analytes by liquid-liquid extraction.

3.2 Specificity

Representative chromatograms obtained from blank brain homogenate and brain homogenate spiked with the LLOQ standard (0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP) are shown in Figure 5.2. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or I.S. in blank brain homogenate. Utilization of the major precursor-to-product transition for each compound enhanced the mass spectrometric selectivity. The analytes and I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of brain homogenate sample. Hence, alkalytic modifiers were used to adjust the pH of brain homogenate. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH 10.69), were evaluated. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for all of the analytes.

3.3 Linearity and sensitivity

Table 5.2 shows the calibration curves for each day of validation. The curves showed good linear response (R^2 > 0.997) over the range of 0.208-416.0 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416-416.0 ng/g for HAL and ZIP. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for the analytes. Table 5.2 showed the slope and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP in rat brain homogenate as shown in Table 5.3. A signal-to-noise (S/N) > 10 at LLOQ was observed for all of the analytes.

3.4 Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The accuracy and precision data can be seen in Table 5.3. The values for the intra-day precision and accuracy were better than 4.43% and 7.67% for all the analytes. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were better than 7.22% and 6.02% (Table 5.3).

3.5 Recovery and matrix effect

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 5.4. Absolute recoveries range from 73.6% to 95.0% and relative recoveries from 83.7% to 94.2% for all of the analytes and I.S. Matrix effects are an important issue in ESI. Matrix effects are the results of co-eluting components, generally from the matrix, that

cause variable suppression or enhancement of analyte response [39]. In the positive ion mode, if the ionized analyte is transferred to the gas phase, gas phase proton transfer reactions may cause neutralization if another neutral species is present with a higher proton affinity. In addition, other ionic species, such as salts, in biological samples with high ionization efficiency or surface activity may compete with the analytes during ion evaporation. LLE used in this study can be helpful in producing clean samples, which are essential to reduce matrix effect. The matrix effect of brain homogenate for all of the analytes in this work was lower than 16.19% suppression or enhancement (Table 5.4). This demonstrated that the sample preparation procedure of LLE method provided a clean extraction solution for LC-MS/MS system.

3.6 Stability studies

Stability testing is very important for validated method in biological sample. The stock solutions were stable at the storage conditions (-20ºC) for three months (Data not shown). All the other stability studies were conducted at two concentration levels (1.25 and 12.5 ng/g) with five determinations for each. Brain homogenate extracts were stable in mobile phase in the HPLC autosampler for at least 24h, indicating that samples should be processed within this period of time (Table 5.5). The freeze/thaw stability tests indicate the analytes were stable in rat brain for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for all of the analytes for 2h. The RE% was from 1.01% to 6.59% (< 15%) and RSD was from 0.75% to 5.64% for all the analytes (see Table 5.5). We found the storage of OLZ in rat brain homogenate at room temperature for 12h produced 18% significant degradation of OLZ. Saracino et al. [29] reported that Vitamin C was added to the brain homogenate to

prevent OLZ degradation. In this work, OLZ did not show significant degradation in rat brain homogenate kept at room temperature for up to 2h without Vitamin C addition. However, for longer storage of OLZ, freezing rat brain homogenate is recommended. In addition, the storage of brain homogenate at room temperature over 8h produced increased viscosity of brain homogenate. The increased viscosity in brain homogenate resulted in the reduced recovery of HAL, ZIP and I.S. So the freshly brain homogenate should be analyzed during 2h or kept frozen immediately.

3.7 Application of the method

The validated method has been successfully used to quantify antipsychotic drug concentrations in rat brain tissue after the chronic treatment of rats with the antipsychotic drugs in their drinking water. The concentration data for antipsychotic drugs in rat brain tissue are reported in Table 6. The representative of MRM chromatograms resulting from the analysis of real brain tissue samples after the chronic treatment of antipsychotic drugs is shown in Figure 5.3. In general, the concentration of RISP and 9-OH RISP in rat brain homogenate was relative low. However, all the analytes were observed above the LLOQ.

4. Conclusions

A simple, specific, rapid and sensitive LC-MS/MS analytical method for the simultaneous determination of OLZ, RISP, 9-OH RISP, HAL, CLOZ and ZIP in rat brain tissue has been developed and validated. This method provided excellent specificity, wide linear range and a LLOQ of 0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP. Liquid-liquid extraction sample preparation was used for 0.20 ml rat brain homogenate that provided low matrix effects and high recovery for all

the analytes. The method was successfully applied to study the effect of chronic treatment of typical (HAL) and atypical (OLZ, RISP and ZIP) antipsychotic drugs on the cognitive function in rats. Another advantage of this method was that only 0.2 ml brain homogenate is necessary for sample preparation. This method will also be used to determine the concentration of antipsychotic drugs in different areas in brain region in the future.

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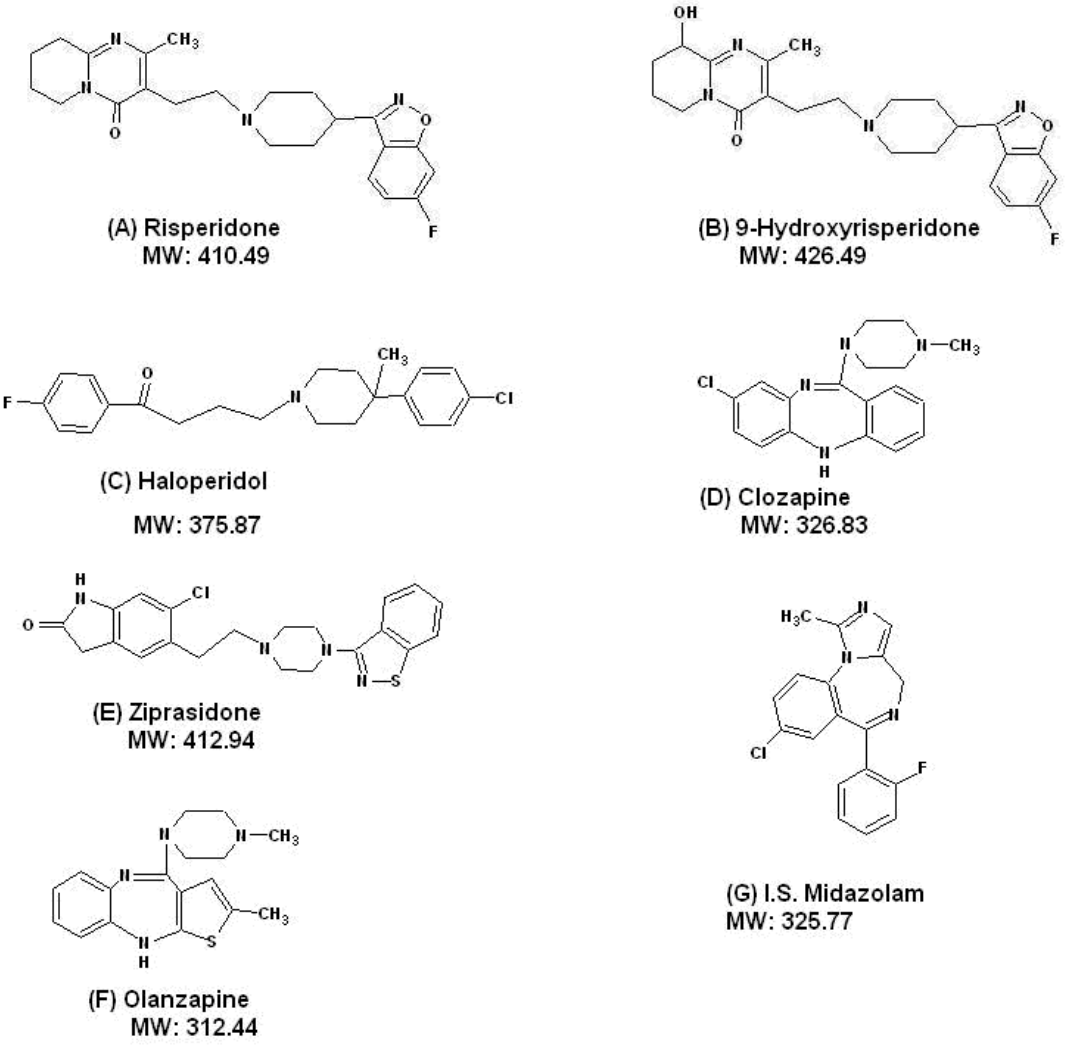

Figure 5.1. Chemical structures of OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP and I.S. (midazolam).

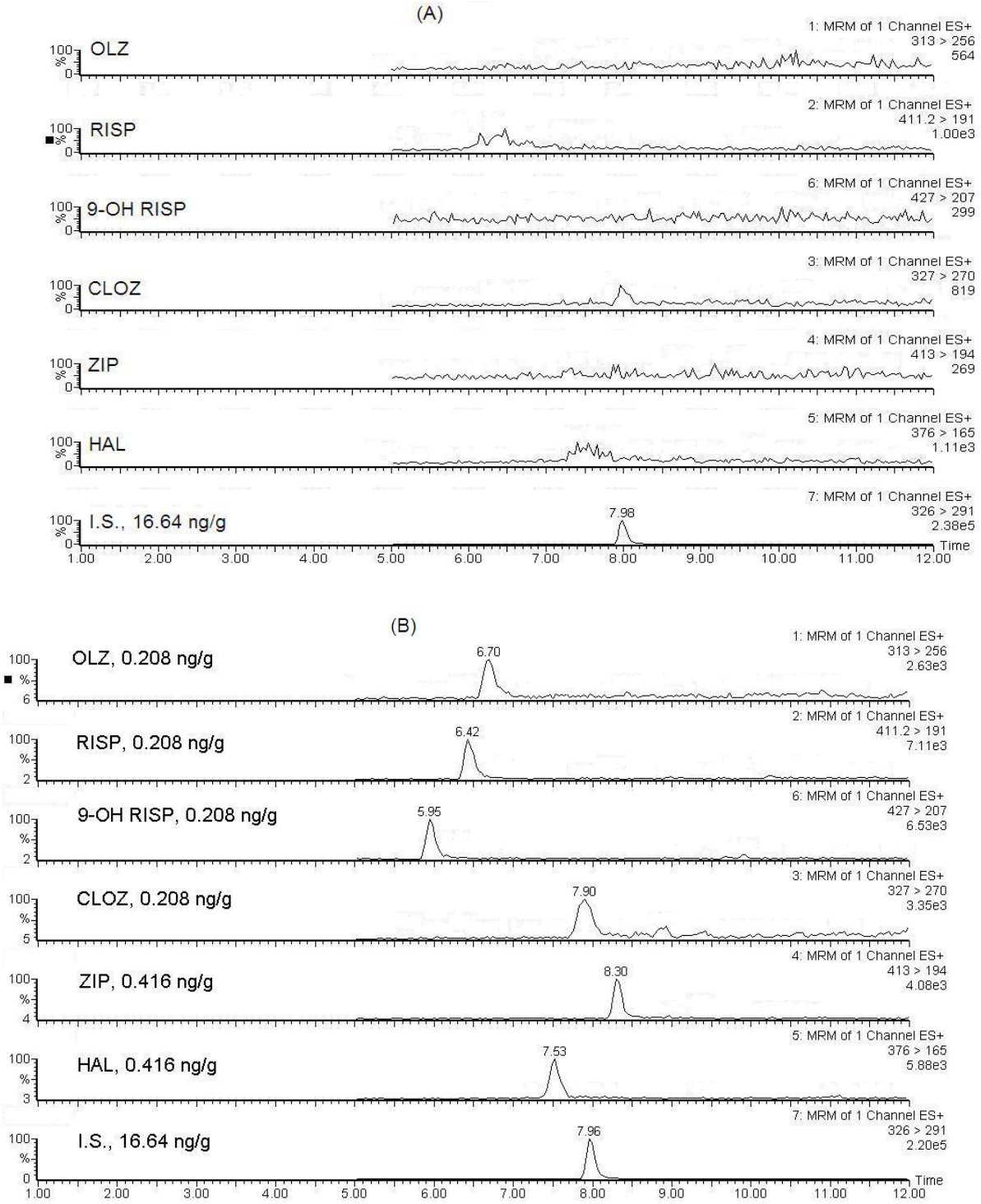

Figure 5.2. Representative chromatograms obtained from (A) blank rat brain homogenate; (B) plasma spiked with LLOQ (0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP) concentration for all of the analytes and I.S. (16.64 ng/g).

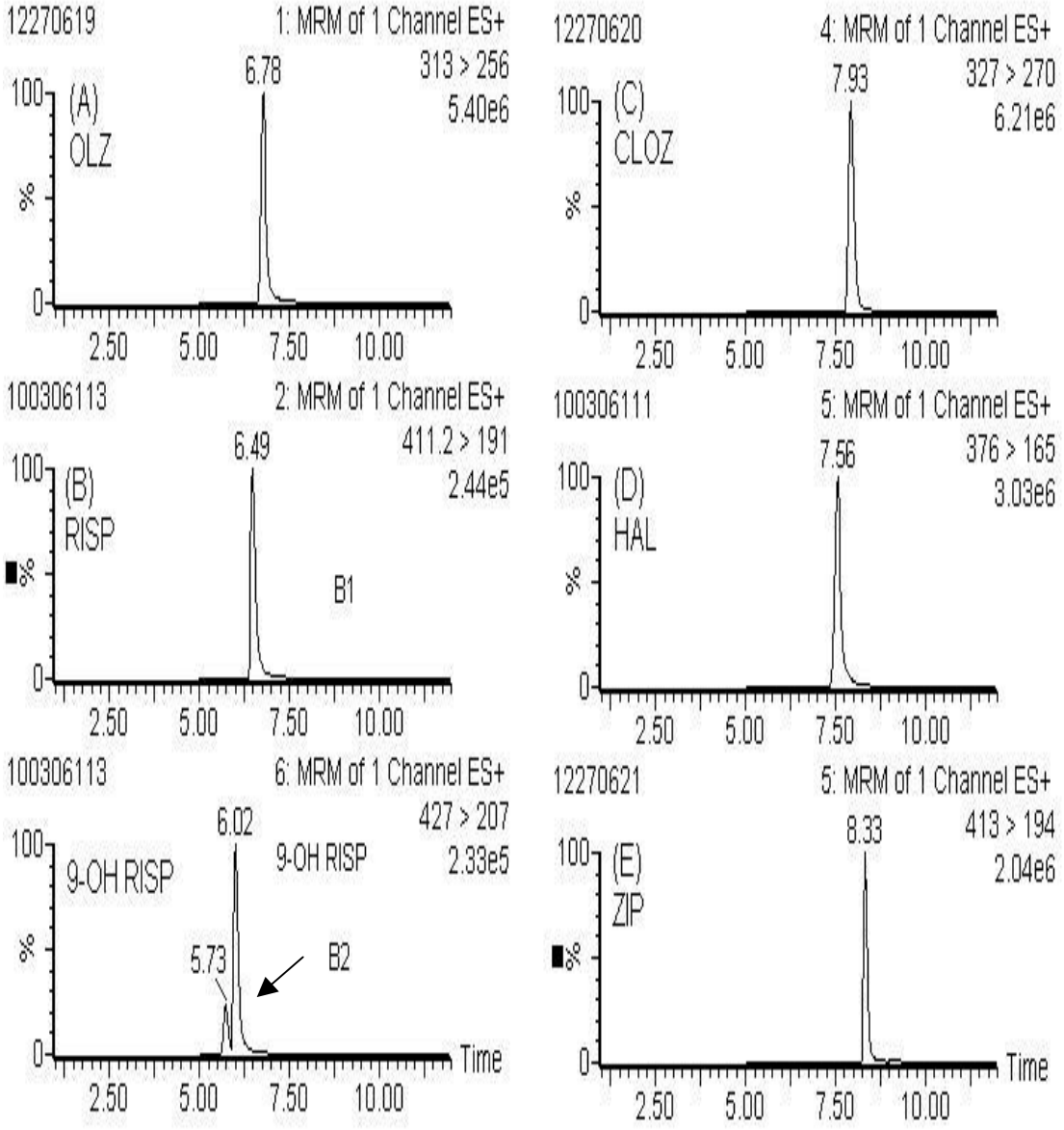

Figure 5.3. Representative chromatograms of brain tissue samples from chronic treatment of antipsychotic drugs: (A) a rat treated with OLZ (10.0 mg/kg/day) the concentration of OLZ in brain tissue was 392.93 ng/g; (B) a rat treated with RISP (2.5 mg/kg/day) the concentration of RISP and its active metabolite (9-OH RISP) in brain tissue was 6.5 ng/g (B1) and 7.0 ng/g (B2), respectively; (C) a rat treated with CLOZ (20.0 mg/kg/day) the concentration of CLOZ in brain tissue was 341.56 ng/g; (D) a rat treated with HAL (2.0 mg/kg/day) the concentration of HAL in brain tissue was 300.0 ng/g; (E) a rat treated with ZIP (12.0 mg/kg/day) the concentration of ZIP in brain tissue was 206.25 ng/g.

Table 5.1
Optimal positive ion ESI mass spectrometric conditions for multiple reaction monitoring (MRM)

Drugs	Ion Transition	Cone Voltage (V)	Coll Energy (eV)
OLZ	316→256	32	23
RISP	411→191	35	24
9-OH RISP	427→207	35	27
CLOZ	327→270	35	22
ZIP	413→194	37	26
HAL	376→165	30	21
Midazolam (I.S.)	326→291	32	30

Table 5.2
Statistical data for linearity including standard deviation (S.D.) (linear range 0.208-416.0 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416-416.0 ng/g for HAL and ZIP).

	OLZ	RISP	9-OH RISP	CLOZ	HAL	ZIP
R^2	0.9989± 0.0008	0.9995± 0.0004	0.9990± 0.0004	0.9991± 0.0011	0.9995± 0.0003	0.9973± 0.0008
Slope	0.1047± 0.0055	0.2281± 0.0073	0.1782± 0.0114	0.1211± 0.0071	0.0791± 0.0055	0.0369± 0.0037

Table 5.3
The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the LC-MS/MS method used to quantitate antipsychotic drugs in rat brain tissue.

Drug		Intra-day			Inter-day		
	Concentration added (ng/g)	Observed concentration ±S.D. (ng/g)	R.S.D. (%)	Error (%)	Observed concentration (ng/g)	R.S.D. (%)	Error (%)
OLZ	0.208	0.209 ± 0.0071	3.40	2.66	0.211 ± 0.0060	2.84	2.65
	1.25	1.210±0.0340	2.81	3.23	1.206±0.0550	4.57	4.28
	12.5	12.30±0.1112	0.90	1.43	12.168±0.935	1.59	2.52
	125.0	129.219±0.827	0.64	3.54	127.217±2.123	1.67	2.09
RISP	0.208	0.205±0.0063	3.08	2.85	0.209±0.0082	3.93	2.56
	1.25	1.225±0.0217	1.77	2.05	1.236±0.0284	2.30	2.11
	12.5	11.821±0.1585	1.34	5.28	12.146±0.2735	2.25	2.68
	125.0	125.093±1.068	0.85	0.75	125.503±1.664	1.33	1.16
9-OH RISP	0.208	0.203 ± 0.0090	4.43	2.93	0.206 ± 0.0077	3.72	2.51
	1.25	1.28 ± 0.0391	3.05	2.70	1.21 ± 0.0565	4.66	4.52
	12.5	12.81 ± 0.4906	3.83	4.21	12.70 ± 0.3533	2.78	2.61
	125.0	123.5 ± 3.0007	2.43	2.18	121.3 ± 2.7345	2.25	3.22
CLOZ	0.208	0.208±0.0091	4.38	3.21	0.209±0.0108	5.18	3.97
	1.25	1.249±0.0428	3.43	2.46	1.262±0.0466	3.69	2.74
	12.5	12.402±0.0662	0.53	0.70	12.579±0.3572	2.84	1.79
	125.0	126.328±1.816	1.44	1.22	122.817±5.946	4.84	3.46
ZIP	0.416	0.408±0.01626	3.99	3.35	0.432±0.0312	7.22	5.92
	1.25	1.152±0.0374	3.25	7.67	1.180±0.0497	4.21	6.02
	12.5	11.901±0.1560	1.31	4.64	12.285±0.6129	4.99	4.55
	125.0	131.395±2.795	2.13	5.28	124.076±6.411	5.17	4.24
HAL	0.416	0.407±0.0133	3.26	2.46	0.419±0.0161	3.85	2.72
	1.25	1.229±0.0425	3.46	2.87	1.225±0.0340	2.78	2.65
	12.5	12.591±0.2177	1.73	1.47	12.454±0.3028	2.43	2.08
	125.0	127.880±3.260	2.55	2.47	124.178±3.712	2.99	2.18

Table 5.4
Absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of analytes and I.S. in rat brain tissue (n=5)

Drugs	Concentration (ng/ml)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
OLZ	0.208	94.6 ± 5.30	86.67 ± 4.85	109.14	9.14% enhancement
	1.25	94.1 ± 1.31	92.3 ± 1.28	101.93	1.93% enhancement
	12.5	94.2 ± 2.57	92.4 ± 2.52	102.00	2.00% enhancement
	125.0	92.4 ± 1.80	88.1 ± 1.70	104.92	4.92% enhancement
RISP	0.208	92.0 ± 3.38	86.2 ± 3.16	106.82	6.82% enhancement
	1.25	95.0 ± 1.11	93.2 ± 1.09	101.98	1.98% enhancement
	12.5	91.7 ± 2.71	92.8 ± 2.74	98.72	1.28% suppression
	125.0	92.8 ± 1.81	90.1 ± 1.75	103.00	3.00% enhancement
9-OH RISP	0.208	90.4 ± 3.30	83.7 ± 3.05	107.97	7.97% enhancement
	1.25	93.9 ± 2.35	92.5 ± 2.31	101.53	1.53% enhancement
	12.5	94.2 ± 2.54	92.3 ± 2.48	102.06	2.06% enhancement
	125.0	91.4 ± 2.13	88.5 ± 2.05	103.29	3.29% enhancement
CLOZ	0.208	79.3 ± 2.59	86.2 ± 2.81	92.06	7.94% suppression
	1.25	86.3 ± 2.11	94.2 ± 2.30	91.66	8.34% suppression
	12.5	84.0 ± 2.55	93.1 ± 2.83	90.16	9.84% suppression
	125.0	83.7 ± 1.97	84.6 ± 1.99	98.99	1.01% suppression
HAL	0.416	80.5 ± 1.95	89.1 ± 2.16	90.32	9.68% suppression
	1.25	82.4 ± 4.01	85.1 ± 4.14	96.85	3.15% suppression
	12.5	81.0 ± 2.05	89.8 ± 2.28	90.19	9.81% suppression
	125.0	80.1 ± 2.03	87.1 ± 2.21	92.00	8.00% suppression
ZIP	0.416	76.6 ± 1.21	90.0 ± 1.43	85.11	14.89% suppression
	1.25	74.8 ± 2.79	87.9 ± 3.28	85.05	14.95% suppression
	12.5	73.6 ± 3.49	87.8 ± 4.16	83.81	16.19% suppression
	125.0	76.4 ± 1.95	84.8 ± 2.17	90.14	9.86% suppression
I.S.	16.64	85.7 ± 2.32	94.0 ± 2.54	91.11	8.89% suppression

Table 5.5
Stability testing of antipsychotic drugs in brain homogenate (n=5).

Drugs	Stability	Spiked conc. (ng/g)	Observed conc. ± S.D. (ng/g)	R.S.D. (%)	Relative error (%)
OLZ	Three freeze-thaw cycle	1.25	0.287 ± 0.0169	5.90	-4.49
		3.0	3.068 ± 0.0440	1.43	2.27
	Bench top (2h)	0.3	0.284 ± 0.0178	6.28	-5.49
		3.0	2.818 ± 0.122	4.31	-6.06
	Autosampler stability (24h)	0.3	0.315 ± 0.0108	3.44	5.12
		3.0	3.095 ± 0.214	6.92	3.15
RISP	Three freeze-thaw cycle	0.3	0.291 ± 0.0108	3.70	-2.90
		3.0	2.859 ± 0.0919	3.22	-4.69
	Bench top (2h)	0.3	0.285 ± 0.0157	5.51	-4.93
		3.0	3.090 ± 0.1328	4.30	3.00
	Autosampler stability (24h)	0.3	0.306 ± 0.0032	1.03	2.13
		3.0	3.035 ± 0.0543	1.79	1.16
9-OH RISP	Three freeze-thaw cycle	0.3	0.302 ± 0.0188	6.23	0.64
		3.0	3.07 ± 0.0356	1.16	2.48
	Bench top (2h)	0.3	0.291 ± 0.0094	3.24	-2.96
		3.0	3.07 ± 0.0508	1.65	2.44
	Autosampler stability (24h)	0.3	0.324 ± 0.0128	3.94	8.11
		3.0	3.145 ± 0.0768	2.44	4.82
CLOZ	Three freeze-thaw cycle	0.3	0.289 ± 0.0028	0.98	-3.52
		3.0	2.758 ± 0.181	6.57	-8.07
	Bench top (2h)	0.3	0.291 ± 0.0059	2.04	-3.07
		3.0	2.907 ± 0.0541	1.86	-3.09
	Autosampler stability (24h)	0.3	0.296 ± 0.0050	1.69	-1.31
		3.0	3.07 ± 0.121	3.96	2.26
HAL	Three freeze-thaw cycle	0.3	0.291 ± 0.0067	2.29	-3.05
		3.0	2.917 ± 0.412	1.41	-2.75
	Bench top (2h)	0.3	0.286 ± 0.0013	0.45	-4.55
		3.0	3.050 ± 0.1482	4.86	1.67
	Autosampler stability (24h)	0.3	0.312 ± 0.0062	1.99	3.84
		3.0	3.110 ± 0.0212	0.68	3.65
ZIP	Three freeze-thaw cycle	0.3	0.292 ± 0.0069	2.37	-2.82
		3.0	2.91 ± 0.0294	2.04	-3.02
	Bench top (2h)	0.3	0.289 ± 0.0099	3.44	-3.60
		3.0	2.946 ± 0.1050	3.57	-1.81
	Autosampler stability (24h)	0.3	0.291 ± 0.0018	0.62	-2.95
		3.0	3.02 ± 0.0094	1.16	0.66

Table 5.6. Steady-state brain tissue concentrations of the analytes after the chronic treatment of antipsychotic drugs for rats and the comparison of brain to plasma ratio (N=6).

Drugs administered	Concentrations in plasma (ng/ml ± S.D.)[a]	Concentrations in brain (ng/g ± S.D.)	Brain/plasma ratio	Published brain/plasma ratio[b]
RISP	RISP: 10.88 ± 13.77	5.17 ± 4.28	0.48	0.22
	9-OH RISP: 30.96 ± 24.87	4.85 ± 2.29	0.16	0.04
HAL	18.43 ± 4.88	334.66 ± 63.01	18.16	22
OLZ	175.74 ± 21.91	348.11 ± 44.06[c]	1.98	5.4
CLOZ	28.05 ± 7.60	286.57 ± 64.33[c]	10.22	24
ZIP	103.61 ± 70.79	246.22 ± 73.02[c]	2.38	--

a. The concentration of all of the analytes in rat plasma were determined using the reported LC-MS/MS method developed by Zhang et al.[40]

b. See references: Aravagiri et al.[11] for RISP and 9-OH RISP; Sunderland and Cohen[41] for HAL; Aravagiri et al.[42] for OLZ; Weigmann et al.[43] for CLOZ.

c. n=4.

Chapter 6

SENSITIVE LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF THE LIPOPHILIC ANTIPSYCHOTIC DRUG CHLORPROMAZINE IN RAT PLASMA AND BRAIN TISSUE

Zhang G, Terry AV Jr and Bartlett, MG. *Journal of Chromatograpgy B* 2007; **854:** 68-76.

Abstract

A simple, sensitive and robust liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method was developed and validated for quantification of chlorpromazine in rat plasma and brain tissue. Chlorpromazine was extracted from rat plasma and brain homogenate using liquid-liquid extraction. The compounds were separated on a Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 µm) column using a mobile phase of acetonitrile/20 mM ammonium formate (pH 4.25 adjusted with formic acid) with gradient elution. Chlorpromazine was detected in positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. The LLOQ was 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. The method was linear over the concentration range from 0.2-200.0 ng/ml for plasma and 0.833-833.3 ng/g for brain tissue. The correlation coefficient (R^2) values were more than 0.998 for both plasma and brain homogenate. The precision and accuracy for intra-day and inter-day were better than 7.54%. The relative and absolute recovery was above 84.9% and matrix effects were lower than 5.6%. This validated method has been successfully used to quantify the rat plasma and brain tissue concentration of chlorpromazine after chronic treatment.

Keywords: Chlorpromazine; Plasma; Brain Tissue; LC-MS/MS

1. Introduction

Chlorpromazine (CPZ, Figure 6.1), one of the First Generation Antipsychotics (FGAs), was discovered in the early 1950s. The advent of powerful phenothiazine psychopharmacological agents represents a landmark achievement in the history of medical and psychiatric sciences [1]. CPZ is the most important compound in the large group of phenothiazine derivatives. It is widely used as a therapeutic agent for controlling excitement, agitation and other psychomotor disturbances in schizophrenic patients and reduces the manic phase of manic-depressive conditions. It is used to treat hyperkinetic states and aggression and is sometimes given in other psychiatric conditions for the control of anxiety and tension. The antipsychotic effect of CPZ is believed to be closely related to its dopamine receptor blocking activity [2]. However, CPZ often produces extrapyramidal side effects. Recently, the chronic effects of FGAs and Second Generation Antipsychotics (SGAs) on cognitive function are attracting more attention [3-5]. One prerequisite for the therapeutic effects of CPZ is its ability to pass the blood brain barrier. Given that cognition is now recognized as a key factor that influences long term functional outcome in schizophrenia, it is important to determine the concentration of CPZ in both plasma and brain (the target compartment for therapeutic action). Knowing these concentrations would allow the determination of extent to which there is a correlation between the concentration of CPZ in the plasma or brain tissue and alterations in cognitive function when CPZ is given chronically in rat model. Such a correlation would allow for improved clinical monitoring of CPZ. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. In addition, CPZ is widely metabolized in the body. Therefore, the concentration of CPZ

in plasma is low. From a bioanalytical and clinical point of view, sensitive and accurate methods are needed to determine CPZ in biological fluids for obtaining optimum therapeutic concentrations and controlling its side effects. At present, determination of CPZ in plasma has been accomplished by GC [6, 7], high performance liquid chromatography (HPLC) with UV detection [8-12] and HPLC with fluorescence detection [13]. Radioimmunoassay [14, 15] provides the sensitive assay, but little was known about the metabolites due to assay selectivity. Although electrochemical detection [16] offers enhanced sensitivity in the low ng/ml range, electrochemical detectors require optimal working conditions and sample preparation is critical. Interference from co-medications is often unavoidable. Another apparent limitation of electrochemical detection is the presence of wide solvent fronts, which may be due to the response of endogenous materials coextracted from plasma. This limitation prevents operation of the detector at its lowest limits of detection [16]. An additional draw back of most of these methods was that at least 1 ml of plasma is needed to obtain the reported detection limit. So, most of these methods were not suitable for the study of the pharmacokinetics and metabolism of CPZ. The usefulness of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields [17, 18]. LC-MS methods offer several significant advantages when compared with the previous methods, such as the small sample volume required, minimization of mobile phase, rapid analytical run time and improved sensitivity, selectivity and specificity [19]. To date, very few LC-MS/MS methods have been reported for the quantitation of CPZ in plasma [20]. In addition, a few papers have reported the determination of CPZ in brain

tissue using HPLC with electrochemical detection [21-23] or UV detection [24]. However, most of these methods were not validated. Also, due to the lipophilic nature of CPZ, it is readily absorbed by myelin and other lipid constituents of brain homogenates. Hence, the recovery of CPZ in brain tissue was very low for most methods. It was a challenge to improve the recovery of the hydrophobic CPZ from lipophilic brain tissue. The purpose of this investigation was to develop and validate a highly selective, sensitive and robust LC-MS/MS method for the determination of the lipophilic drug CPZ in rat plasma and brain tissue. To our knowledge, our paper is the first to report a validated LC-MS/MS method for determination of CPZ in brain tissue.

2. Experimental

2.1 Chemicals and reagents

Chlorpromazine and midazolam (internal standard, I.S.) were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, tert-butyl methyl ether, chloroform, hexane, diethyl ether, isopropyl ether, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). The formic acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate, ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

2.2 Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler, and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro

triple-quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA).

2.3 Liquid chromatographic and mass spectrometric conditions

The analytes were separated on a Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 µm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 20 mM ammonium formate in water with pH adjusted to 4.25 using formic acid and mobile phase B was acetonitrile. The flow rate was set to 0.3 ml/min. A 10 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B, flow rate (ml/min)) (0, 15, 0.3) (5, 70, 0.3) (7.5, 77, 0.3) (7.6, 90, 0.6) (8.8, 90, 0.6) (8.9, 15, 0.3) (12.5, 15, 0.3). From 7.6 to 8.8 min, it was very important to use a high concentration of acetonitrile (90%) in the mobile phase to wash the column at a flow rate of 0.6 ml/min. This procedure can remove the remaining brain extract residue from the column following each injection. Otherwise there would be decreased response of CPZ in next injection because of ion suppression coming from the brain extract residue of the previous injection. The column temperature was maintained at 25ºC. The LC flow was introduced directly to the mass spectrometer from 5.5-8.5 min and diverted to waste at other times using a six-port switching valve. The autosampler injection needle was washed with methanol to reduce carry-over after each injection. The mass spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350ºC. The cone gas flow was set to 50 L/h. Argon was the collision

gas and the collision cell pressure was 2.4×10^{-3} mbar. For quantitation, an MRM transition from m/z 319→86 was performed for CPZ with a cone voltage of 32 V and a collision energy of 20 eV. An MRM transition from m/z 326→291 was performed for the I.S. (midazolam) with a cone voltage of 32 V and a collision energy of 30 eV. The source temperature and capillary voltage were set at 130ºC and 3.5 kV, respectively.

2.4 Sample collection

CPZ chronic dosing was derived from previously published data [4, 5]. Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human [25]. Male albino Wistar rats (Harlan, Inc.) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a 12-h ligh/dark cycle with free access to food. Rats were thus treated with CPZ (10.0 mg/kg/day) orally in drinking water for periods of up to 180 days. Plasma samples were collected at various days between 15 and 180 days of treatment in a separate groups of rats (N=6). (Steady-state concentrations of CPZ were obtained after two weeks of chronic dosing). Rats were anesthetized with isofluorane and 3.0 ml of blood was collected via cardiac puncture to heparined tubes. The blood was centrifuged for 15 min at 2500× g at 8ºC and the resulting plasma was frozen until analysis. The whole brains of sacrificed animals were removed and kept frozen at -70 ºC until analysis.

2.5 Preparation of stock, working standard and quality control solutions

Individual stock solutions of CPZ and I.S. (midazolam) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at -20ºC. Standard solutions with concentrations of 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0, 400.0, 1000.0 and 2000.0

ng/ml were prepared by serial dilution with acetonitrile – methanol solutions (60:40). Precision and accuracy standards with concentrations of 2.0, 6.0, 150.0, and 1500.0 ng/ml were also prepared in the same manner. An 80.0 ng/ml I.S. standard solution was prepared with acetonitrile – methanol solutions (60:40) from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at -20ºC when not in use and replaced every 3 months. A fresh standard solution was prepared for each day of analysis or validation.

2.6 Preparation of calibration and QC samples

The brains were minced and homogenized in a volume of deionized water (in ml) equal to twice the weight (in g) of the tissue using a Brinkmann (Westburg, NY, USA) Polytron TP 1020 homogenizer. Samples for the calibration curves and QCs were prepared by adding 25.0 µl of each standard into 250 µl blank rat plasma or 200 µl blank brain homogenate. This yields calibration standard concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0 and 200.0 ng/ml for plasma and 0.833, 1.666, 4.165, 8.33, 16.66, 41.65, 83.33, 166.6, 416.5 and 833.3 ng/g for brain tissue. The final concentrations of QCs were 0.2, 0.6, 15.0, 150.0 ng/ml for plasma and 0.833, 2.5, 62.5, 625.0 ng/g for brain tissue. The spiked plasma and brain homogenate samples (standards and quality controls) were extracted with each analytical batch along with the unknown samples.

2.7 Sample preparation

To a 250 µl rat plasma or 200 µl brain homogenate sample, 25 µl of internal standard (80.0 ng/ml, midazolam), 0.2ml of 0.5 M Na_2HPO_4 (pH 10.69) for plasma and 0.4ml of 0.5 M Na_2HPO_4 (pH 10.69) for brain homogenate were added. The samples were briefly

mixed and extracted in 3 ml isopropyl ether once for plasma and twice for brain homogenate. After centrifugation at 2000× g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 200 µl of acetonitrile – methanol solutions (60:40) solution was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000× g prior to LC- MS/MS analysis.

2.8 Method validation

The method was validated for linearity, recovery, matrix effect, accuracy and precision. Brain homogenate calibration curves were constructed using the peak area ratios of CPZ to that of I.S., and applying a weighted ($1/x^2$) least squares linear regression analysis. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples. Five replicates of each QC points were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Absolute and relative recoveries, and matrix effects were calculated for spiked samples at 0.2, 0.6, 15.0, 150.0 ng/ml for plasma and 0.833, 2.5, 62.5, 625.0 ng/g for brain tissue and 8.0 ng/ml I.S. in plasma samples and 16.64 ng/g I.S. in brain tissue (n=5). Absolute recovery was calculated as the peak area for CPZ and I.S. in plasma or brain homogenate spiked before extraction divided by the peak area of the pure drugs in the acetonitrile – methanol solutions (60:40) solution at the same concentration. Relative recovery was calculated by dividing the peak area for CPZ and I.S. spiked before extraction by the peak area for an equal concentration of the sample in the same matrix spiked after extraction. The matrix effects were calculated by

dividing the response for a CPZ sample in biological matrix spiked after extraction by an equal concentration of CPZ in an acetonitrile – methanol (60:40) solution [26]. The stability of the stock solutions was determined at its storage conditions of -20ºC for three months. CPZ was considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls [27]. The bench-top stability of spiked plasma samples stored at room temperature was evaluated for 2h. The freeze/thaw stability was investigated by comparing the stability samples following three freeze/thaw cycles, against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted brain homogenate samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 24h. The stability testing was performed at 0.6 and 150.0 ng/ml for plasma and 2.5 and 625.0 ng/g for brain tissue concentration levels.

3. Results and discussion

3.1 Method development

In order to develop and validate a highly sensitive and selective method with the designed LLOQ (0.2 ng/ml for plasma and 0.833 ng/g for brain tissue), during method development different options were evaluated to optimize the detection (MRM) parameters, chromatography and sample preparation methodology. MS/MS detection provided improved sensitivity for trace mixture analysis. ESI(+) MS/MS product ion spectra were produced by CAD of the protonated molecular ion $(M+H)^+$. The most favorable transition was selected and the instrument parameter settings were optimized individually for CPZ and I.S. by constant infusion at 10 µl/min of a 2 µg/ml solution. The

major MS/MS transitions utilized for LC-MS/MS quantitate analysis were m/z 319→86 for CPZ and m/z 326→291 for I.S. (midazolam).

The chromatographic conditions, especially the analytical column and the composition of mobile phase, were optimized through several trials to achieve the desired sensitivity, separation, run time, and symmetric peak shapes for CPZ and I.S. Agilent XDB C8 (150 mm × 2.1 mm i.d., 5 µm), Waters XTerra C18 (50 mm × 2.1 mm i.d., 5 µm) and Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 µm) columns were evaluated. The peak shape of CPZ on the XTerra C18 (50 mm × 2.1 mm i.d., 5 µm) column showed tailing. As a result, Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d.; 3 µm) was selected as it produced the satisfactory separation, peak shape and a shorter analytical run time than the other columns. 20 mM ammonium formate (pH 4.25 using formic acid) buffer combination with acetonitrile resulted in the sensitive signals with optimized gradient elution. CPZ is a lipophilic drug and produces low concentrations in biological samples. We initially pursued isocratic elution to obtain shorter run times but this was not suitable for brain tissue samples. Brain samples contained substantial amount of lipid residue after evaporation of the extraction solvent to dryness. Next, we pursued gradient elution with a low concentration of acetonitrile in the mobile phase at the start of the run to improve the peak shape and thus the assay sensitivity. Another reason we used gradient elution was that the separation capacity between CPZ and lipid residue could be improved which reduced ion suppression from brain tissue samples. When using an isocratic method, the peak shape of CPZ was broad which decreased sensitivity. We have optimized the total method run time to 12.5 min. From 7.6 to 8.8 min, it was very important to use a high concentration of acetonitrile (90%) in the mobile phase to wash

the column with a flow rate of 0.6 ml/min. This procedure can remove the brain extract residue from the column following each run. Failure to remove the residue matrix would decrease the response of CPZ for subsequent injections because of ion suppression coming from the brain extract residue from the previous injection on the column. It is necessary to wash out the brain extract residue from the column during each running. Initially, we evaluated a one-step protein precipitation method using methanol or acetonitrile for sample preparation. However, the LLOQ was poorer because of dilution of the sample. Other authors [28] found recoveries of 96% for CPZ using protein precipitation for plasma. However, we found the recovery using protein precipitation was very low for CPZ and the I.S in brain tissue. Next, we evaluated solid phase extraction (SPE) methods using different cartridges such as the Waters Oasis HLB and Varian C18. Although Ohkubo et al [29] reported about 80 to 88% recovery of CPZ was obtained in serum and human breast milk, we found that the recovery of CPZ and I.S. was still very low using SPE from brain tissue. Finally, we evaluated liquid-liquid extraction (LLE) for the sample preparation. LLE can be helpful in producing a clean sample and avoiding the introduction of non-volatile materials on the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effects in LC-MS/MS analysis [28]. Different organic solvents, ethyl acetate, tert-butyl methyl ether, chloroform, hexane, diethyl ether and isopropyl ether, and their mixtures in different combinations and ratios were evaluated. Finally, isopropyl ether was found to optimal, because it was able to produce a clean chromatogram for a blank brain homogenate sample and yielded the highest recovery for the analytes by liquid-liquid extraction.

3.2 Linearity and sensitivity

Table 6.1 shows the calibration curves for each day of validation. The curves showed good linear response (R^2 > 0.9984) over the range of 0.2-200.0 ng/g for plasma and 0.833-833.3 ng/g for brain tissue. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for the calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for CPZ. Table 6.1 showed the slope, intercept and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.2 ng/g for plasma and 0.833 ng/g for brain tissue as shown in Table 6.2. A signal-to-noise (S/N) > 10 at LLOQ was observed for CPZ in biological matrix.

3.3 Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The accuracy and precision data can be seen in Table 6.2. The values for the intra-day precision and accuracy were better than 4.58% and 7.54%. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were better than 4.78% and 5.95% (Table 6.2).

3.4 Recovery and matrix effect

CPZ and the I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of brain homogenate samples. Hence, alkalytic modifiers were used to adjust the pH of brain homogenate. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH 10.69), were evaluated.

1.0 M NaOH is not suitable as a pH modifier because of the possible reduction from chlorpromazine-N-oxide (one metabolite of CPZ) to CPZ from this solution [30]. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for CPZ and the I.S.

Due to non-specific binding of lipophilic CPZ to brain constituents [31], it is a challenge to improve the recovery of CPZ in brain homogenate. We found that reconstitution solution plays a key role for improved recovery and reduced matrix effects in brain homogenate residue after liquid-liquid extraction. Methanol: 20 mM ammonium formate (pH 4.25) (80:20), pure methanol, acetonitrile: 20 mM ammonium formate (pH 4.25) (80:20), and acetonitrile: methanol (60:40) were evaluated as reconstitution solutions, respectively. Table 3 showed the effect of different reconstitution solutions on absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of CPZ in rat plasma (15.0 ng/ml) and brain tissue (62.5 ng/g) (n=3). The peak shape of CPZ in different reconstitution solution was acceptable in plasma or brain tissue extracts. For plasma, the effect of different reconstitution solution on recovery and matrix effects was not significantly different. However, for brain tissue, the effect of different reconstitution solution on recovery and matrix effects was highly variable. Methanol: 20 mM ammonium formate (pH 4.25) (80:20) as reconstitution solution produced the lowest absolute recovery (66.7%) and the highest ion suppression (19.6%). Finally, acetonitrile: methanol (60:40) solution was chosen because it produced the highest absolute recovery (90.8%) and the lowest matrix effects (2.1 % ion suppression) for brain tissue. In addition, absolute recovery, relative recovery, and matrix effect data of CPZ in different concentration in plasma or brain tissue using acetonitrile: methanol (60:40)

solution as reconstitution solution are summarized in Table 6.4. Absolute recoveries range from 89.6% to 93.2% and relative recoveries from 84.9% to 98.2% for CPZ and the I.S. in plasma or brain homogenate. Matrix effects are an important issue in ESI. Matrix effects are the results of co-eluting components, generally from the matrix, that cause variable suppression or enhancement of analyte response [32]. In the positive ion mode, if the ionized analyte is transferred to the gas phase, gas phase proton transfer reactions may cause neutralization if another neutral species is present with a higher proton affinity. In addition, other ionic species, such as salts, in biological samples with high ionization efficiency or surface activity may compete with the analytes during ion evaporation. LLE used in this study can be helpful in producing clean samples, which are essential to reduce matrix effect. The matrix effect of plasma for all of the analytes in this work was lower than 5.6% suppression or enhancement (Table 6.4).

3.5 Specificity

Representative chromatograms obtained from blank biological matrix and spiked with LLOQ standard (0.2 ng/ml for plasma and 0.833 ng/g for brain homogenate) are shown in Figure 6.2 and Figure 6.3. No interfering peaks from endogenous compounds were observed at the retention times of CPZ or I.S. in blank plasma or brain homogenate. Three MRM transitions have been used: one transition (319→86) for quantification and two transitions (319→58 and 319→246) for identification. Table 6.5 contains the data for the first identification MRM transition (319→58), the peak area ratio (319→86 / 319→58) in plasma or brain tissue is from 4.60 to 4.79 over the calibration curve range. The peak area ratio (319→86 / 319→58) from real samples was 4.71 for plasma and 4.78 for brain tissue, respectively. Also, for the second identification MRM transition

(319→246), the peak area ratio (319→86 / 319→246) in plasma or brain tissue is from 20.89 to 21.77 over the calibration curve range. The peak area ratio (319→86 / 319→246) from real samples was 21.12 for plasma and 21.08 for brain tissue, respectively. These results showed that no significant interference existed for CPZ in plasma or brain tissue. The addition of the two MRM transitions (319→58 and 319→246) from CPZ for identification improved the specificity of the validated method.

3.6 Stability studies

Stability testing is very important for validated methods in biological samples. The stock solution was stable at the storage conditions (-20ºC) for three months (Data not shown). All the other stability studies were conducted at two concentration levels (0.6 and 150.0 ng/ml for plasma or 2.5 and 625.0 ng/g for brain tissue) with five determinations for each. Plasma and brain homogenate extracts were stable in the mobile phase in the HPLC autosampler for at least 24h, indicating that samples could be processed within this period of time (Table 6.6). The freeze/thaw stability tests indicate CPZ was stable in rat plasma and brain homogenate for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for 2h. The %RE was from 0.39% to 2.59% for plasma and from 1.80% to 3.19% for brain homogenate, and RSD was from 1.20% to 1.98% for plasma and from 0.13% to 2.34% for brain homogenate (see Table 6.6). Finally, the storage of brain homogenate at room temperature over 8h caused an increase in the viscosity of the brain homogenate samples. This increased viscosity of the brain homogenate resulted in reduced recovery of CPZ and the I.S. Therefore, fresh brain homogenate should be analyzed within 2h or frozen immediately for later analysis.

3.7 Application of the method

The validated method has been successfully used to quantify CPZ concentrations in rat plasma and brain tissue after the chronic treatment of rats with CPZ in their drinking water. The concentration data for CPZ in rat plasma and brain tissue are reported in Table 6.7. The representative of MRM chromatograms resulting from the analysis of real plasma and brain tissue samples after the chronic treatment of antipsychotic drugs is shown in Figure 6.4. The concentration of CPZ in brain tissue is much higher than in plasma. Titier et al. reported the same result [24].

4. Conclusions

A simple, specific and sensitive LC-MS/MS analytical method for the determination of CPZ in rat plasma and brain tissue has been developed and validated. This method provided excellent specificity, wide linear range and a LLOQ of 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. Liquid-liquid extraction sample preparation was used for 0.25 ml plasma and 0.20 ml rat brain homogenate that provided low matrix effects and high recovery for CPZ. The method is currently being used in a study in which the effects of chronic CPZ treatment on cognitive function in rats is being assessed. Another advantage of this method was that only 0.25ml for plasma and 0.2 ml for brain homogenate (small sample volume) are necessary for sample preparation. This method will also be used to determine the concentration of CPZ in different brain regions in the future.

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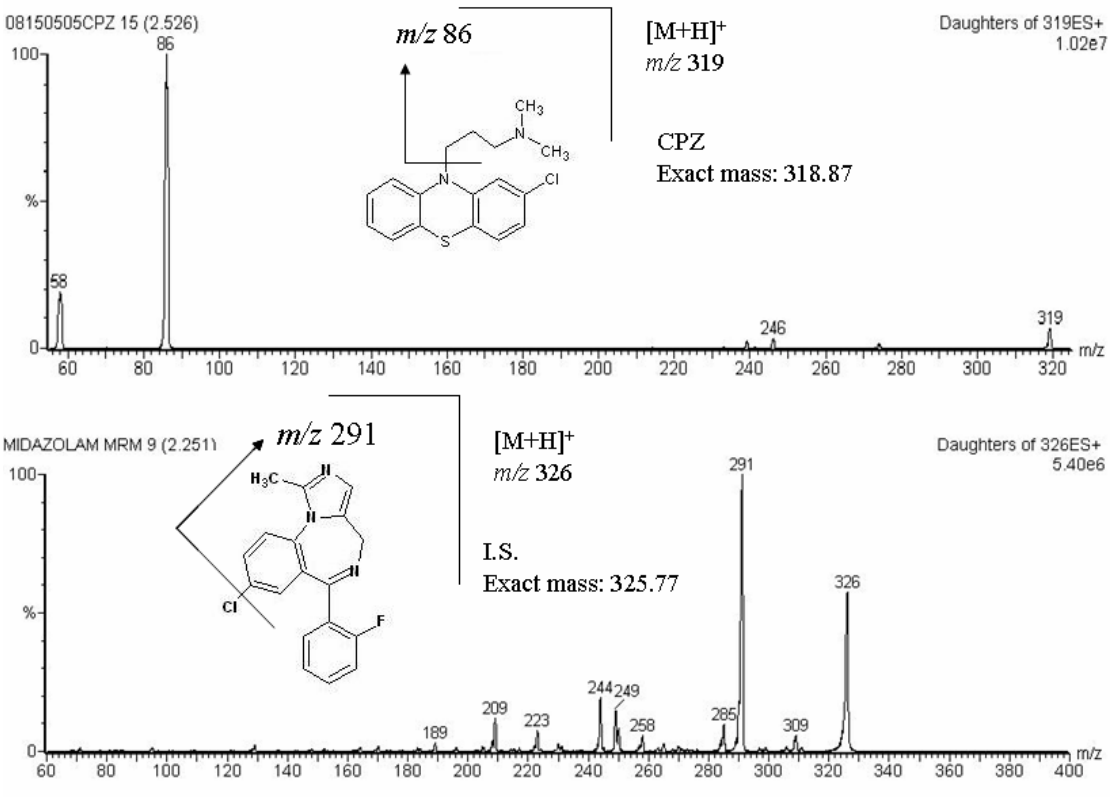

Figure 6.1. Product ion mass spectra of (A) CPZ, (B) I.S. and respective chemical structures.

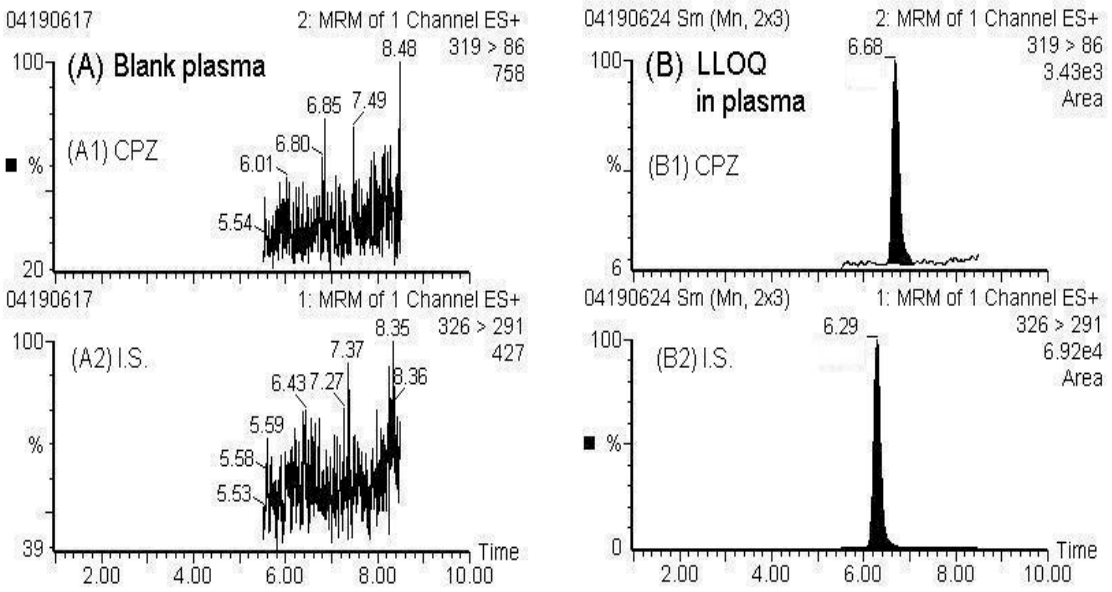

Figure 6.2. Representative chromatograms obtained from rat plasma, (A) blank rat plasma; (B) plasma spiked with LLOQ for CPZ at 0.2 ng/ml (B1) and I.S. at 8.0 ng/ml (B2).

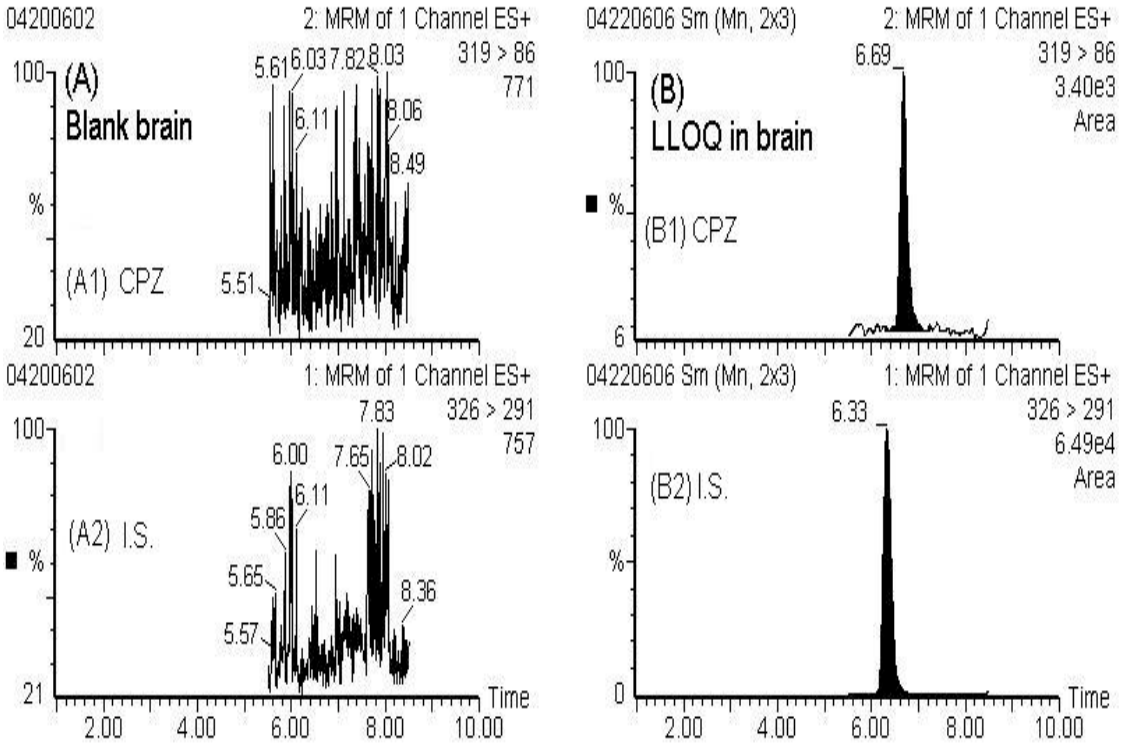

Figure 6.3. Representative chromatograms obtained from rat brain tissue, (A) blank rat tissue; (B) brain tissue homogenate spiked with LLOQ for CPZ at 0.833 ng/g (B1) and I.S. at 33.33 ng/g (B2).

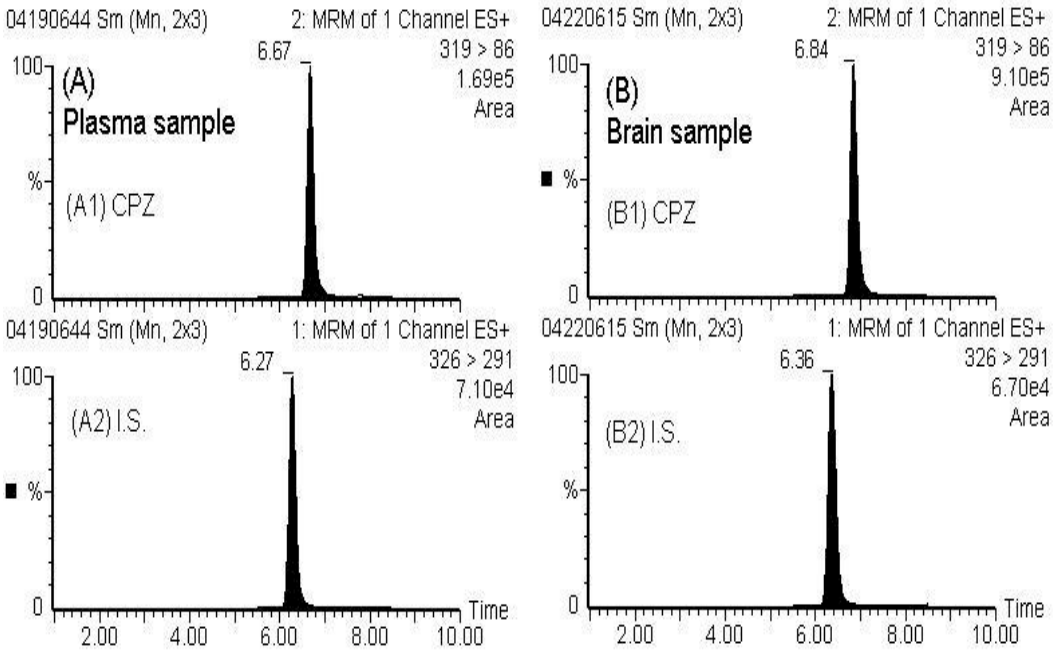

Figure 6.4. Representative chromatograms of plasma and brain tissue samples from rats following chronic treatment with CPZ for 90 days at 10.0 mg/kg/day: (A) the concentration of CPZ in the plasma sample was 9.98 ng/ml (A1); (B) the concentration of CPZ in brain tissue sample was 254.73 ng/g (B1).

Table 6.1
Statistical data for linearity including standard deviation (S.D.) (linear range 0.2-200.0 ng/ml for plasma and 0.833-833.3 ng/g for brain)

Biological matrix	R^2	Slope	Intercept
Plasma	0.9984 ± 0.00035	0.22176 ± 0.00748	0.003593 ± 0.002256
Brain tissue	0.9985 ± 0.00101	0.04916 ± 0.00273	0.00264 ± 0.001146

Table 6.2
The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the LC-MS/MS method used to quantitate antipsychotic drugs in rat plasma and brain tissue

Matrix		Intra-day			Inter-day		
	Concentration added (ng/ml or ng/g)	Observed concentration (ng/ml or ng/g)	R.S.D (%)	Error (%)	Observed concentration (ng/ml or ng/g)	R.S.D. (%)	Error (%)
Plasma	0.2	0.196 ± 0.0071	3.63	3.41	0.197 ± 0.0057	2.88	2.57
	0.6	0.645 ± 0.0087	1.35	7.54	0.626 ± 0.02154	3.44	4.67
	15.0	15.88 ± 0.1534	0.97	5.87	14.963 ± 0.7026	4.70	4.16
	150.0	156.06 ± 0.991	0.64	4.04	153.75 ± 7.3508	4.78	4.68
Brain	0.833	0.800 ± 0.0367	4.58	4.96	0.824 ± 0.0331	4.01	3.29
	2.5	2.636 ± 0.0521	1.98	5.42	2.649 ± 0.0482	1.82	5.95
	62.5	61.935 ± 1.5940	2.57	2.08	62.933 ± 2.0714	3.29	2.71
	625.0	599.98 ± 9.1217	1.52	4.00	609.54 ± 18.384	3.02	3.45

Table 6.3
Effect of different reconstitution solutions on absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of CPZ in rat plasma (15.0 ng/ml) and brain tissue (62.5 ng/g) (n=3).

Matrix	Reconstitution solution* (v: v)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
Plasma	ACN: MeOH = 60: 40	93.3 ± 1.47	90.8 ± 1.75	102.7 ± 0.37	2.7% enhancement
	ACN: Buffer = 80: 20	92.4 ± 1.03	91.2 ± 0.95	101.2 ± 0.37	1.2% enhancement
	MeOH: Buffer = 80: 20	88.9 ± 0.56	90.1 ± 0.83	98.7 ± 0.95	1.3% suppression
	MeOH	95.4 ± 1.48	97.7 ± 2.15	97.1 ± 0.94	2.9% suppression
Brain	ACN: MeOH = 60: 40	90.8 ± 0.84	92.3 ± 0.86	97.9 ± 0.22	2.1% suppression
	ACN: Buffer = 80: 20	70.5 ± 1.55	81.4 ± 0.71	86.7 ± 1.16	13.3% suppression
	MeOH: Buffer = 80: 20	66.7 ± 2.69	82.8 ± 3.98	80.4 ± 0.64	19.6% suppression
	MeOH	76.1 ± 1.17	87.4 ± 2.31	87.1 ± 1.55	12.9% suppression

* ACN: acetonitrile; MeOH: methanol; Buffer: 20 mM ammonium formate (pH 4.25 adjusted using formic acid).

Table 6.4
Absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of analytes and I.S. in rat plasma and brain tissue at different concentrations (n = 5) using acetonitrile : methanol (60 : 40) as the reconstitution solution.

Matrix	Concentration (ng/ml or ng/g)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
Plasma	0.2	93.2 ± 1.37	84.9 ± 1.25	104.5 ± 4.57	4.5% enhancement
	0.6	91.0 ± 1.59	89.3 ± 1.56	101.7 ± 0.39	1.7% enhancement
	15.0	91.5 ± 0.85	89.1 ± 0.83	102.0 ± 0.84	2.0% enhancement
	150.0	90.2 ± 1.18	89.1 ± 1.16	101.8 ± 1.33	1.8% enhancement
I.S. (Plasma)	8.0	89.8 ± 1.08	91.1 ± 1.10	97.8 ± 0.67	2.2% suppression
Brain	0.833	90.0 ± 2.02	91.9 ± 2.06	96.8 ± 4.18	3.2% suppression
	2.5	90.6 ± 1.33	98.2 ± 1.44	94.4 ± 2.80	5.6% suppression
	62.5	91.2 ± 3.40	94.8 ± 3.54	95.6 ± 0.56	4.4% suppression
	625.0	89.6 ± 1.51	93.1 ± 1.57	96.5 ± 1.82	3.5% suppression
I.S. (Brain)	33.33	95.1 ± 1.23	97.3 ± 1.26	97.7 ± 0.58	2.3% suppression

Table 6.5
Enhanced specificity of CPZ by multiple MRM transitions (319→86 for quantification; 319→58 and 319→246 for identification for CPZ in plasma and brain tissue).

Concentration (ng/ml in plasma / ng/g in brain tissue	0.2/0.833	0.4/1.666	1.0/4.165	4.0/16.66	40.0/166.6	200.0/833.3	Real samples plasma / brain
Peak area ratio in plasma (319→86 / 319→58)	n.d.	4.68	4.73	4.60	4.71	4.71	4.71
Peak area ratio in brain (319→86 / 319→58)	n.d.	4.70	4.71	4.79	4.74	4.72	4.78
Peak area ratio in plasma (319→86 / 319→246)	n.d.	n.d.	n.d.	21.72	21.05	20.89	21.12
Peak area ratio in brain (319→86 / 319→246)	n.d.	n.d.	n.d.	21.77	21.11	21.53	21.08

n.d.: not detected with 319→58 or 319→246 MRM transition

Table 6.6
Stability testing of CPZ in rat plasma and brain homogenate (n=5).

Matrix	Stability	Spiked conc. (ng/ml or ng/g)	Observed conc. ± S.D. (ng/g)	R.S.D. (%)	Relative error (%)
Plasma	Three freeze-thaw cycle	0.6	0.584 ± 0.0115	1.97	-2.59
		150.0	150.58 ± 1.8110	1.20	0.39
	Bench top (2h)	0.6	0.589 ± 0.0117	1.98	-1.76
		150.0	152.099 ± 2.596	1.71	1.40
	Autosampler stability (24h)	0.6	0.594 ± 0.0090	1.51	-0.95
		150.0	151.70 ± 0.3392	0.22	1.14
Brain	Three freeze-thaw cycle	2.5	2.427 ± 0.0567	2.34	-2.93
		625.0	613.08 ± 9.788	1.60	-1.91
	Bench top (2h)	2.5	2.45 ± 0.0550	2.25	-1.99
		625.0	610.11 ± 1.727	0.28	-2.38
	Autosampler stability (24h)	2.5	2.54 ± 0.0964	3.79	1.80
		625.0	605.09 ± 0.8125	0.13	-3.19

Table 6.7
Steady-state plasma and brain tissue concentrations of CPZ after the chronic treatment of CPZ in rats.

Matrix	Dose (mg/kg/day)	Steady-state concentrations (ng/ml or ng/g ± S.D.)	Concentration range (ng/ml or ng/g)
Plasma (n=18)	10.0	11.78 ± 7.06	2.98-26.58
Brain tissue (n=6)	10.0	251.02 ± 66.14	125.46-312.60

Chapter 7

DETERMINATION OF THE LIPOPHILIC ANTIPSYCHOTIC DRUG ZIPRASIDONE IN RAT PLASMA AND BRAIN TISSUE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Zhang G, Terry AV Jr and Bartlett, MG. To be submitted to ***Biomedical Chromatography***.

Abstract

A simple, sensitive and robust liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method with low matrix effects was developed and validated for the quantification of the lipophilic antipsychotic ziprasidone from rat plasma and brain tissue. Ziprasidone was extracted from rat plasma and brain homogenate using a single step liquid-liquid extraction. Ziprasidone was separated on an Agilent Eclipse XDB C-8 column (150 mm × 2.1 mm i.d., 5 µm) column using a mobile phase of acetonitrile/0.02% ammonia in water (pH 7.20 adjusted with formic acid) using gradient elution. Ziprasidone was detected in the positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recovery, matrix effects and stability were determined. The LLOQ was 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. The method was linear over the concentration range from 0.2-200.0 ng/ml for plasma and 0.833-833.3 ng/g for brain tissue. The correlation coefficient (R^2) values were more than 0.996 for both plasma and brain homogenate. The precision and accuracy for intra-day and inter-day were better than 8.13%. The relative and absolute recovery was above 81.0% and matrix effects were lower than 5.2%. This validated method has been successfully used to quantify the rat plasma and brain tissue concentration of ziprasidone after chronic treatment.

KEYWORDS: ziprasidone; plasma; brain tissue; LC-MS/MS; matrix effect

INTRODUCTION

Ziprasidone (ZIP, Figure 1) was one of the more recent generation antipsychotics (SGAs) to gain FDA approval (February 2001). It is chemically different from the phenothiazine and butyrophenone type antipsychotic drugs. Oral ZIP (ziprasidone hydrochloride) is approved by the U.S. FDA for the treatment of schizophrenia, and acute manic or mixed episodes associated with bipolar disorder (with or without psychotic features). ZIP intramuscular (ziprasidone mesylate) is FDA-approved for acute agitation in patients with schizophrenia. Oral ZIP appears efficacious, and has been shown to have some limited clinical advantages over chlorpromazine and haloperidol in ameliorating the negative symptoms of schizophrenia (Greenberg and Citrome; 2007). ZIP exhibits a potent and highly selective antagonistic activity on the D_2 and 5-HT_{2A} receptors. It also has a high affinity for the 5-HT_{1A}, 5-HT_{1D}, and 5-HT_{2C} receptor subtypes that could contribute to the overall therapeutic effect (Seeger *et al*, 1995; Schmidt *et al*., 2001). ZIP is metabolized extensively resulting in several metabolites which are far less pharmacologically active. Ziprasidone sulfoxide and ziprasidone sulfone are the major circulating metabolites in humans and are predominantly produced by the cytochrome P450 (CYP) 3A4 isozyme. Other metabolites produced include N-dealkylation, benzisothiazole cleavage and hydration of the C–N bond followed by oxidation and dealkylation (Prakash *et al*., 1997, 2000; Wilner *et al*., 2000).

One prerequisite for the therapeutic effects of ZIP is its ability to pass the blood brain barrier. Given that cognition is now recognized as a key factor that influences long

term functional outcome in schizophrenia, it is important to determine the concentration of ZIP in both plasma and the brain (the target organ for therapeutic action). Knowing these concentrations would allow the determination of the extent to which there is a correlation between the concentration of ZIP in the plasma or brain tissue and alterations in cognitive function when ZIP is given chronically. Such a correlation would allow for improved clinical monitoring of ZIP. Since ZIP is widely metabolized in the body, its concentration in plasma is low. From a bioanalytical and clinical point of view, sensitive and accurate methods are needed to determine ZIP in biological fluids for obtaining optimum therapeutic concentrations and controlling its side effects. At present, the determination of ZIP in plasma has been accomplished by high performance liquid chromatography (HPLC) with UV detection (Janiszewski *et al.*, 1995) and HPLC with fluorescence detection (Suckow *et al*., 2004). The usefulness of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields. LC-MS methods offer several significant advantages when compared with previous methods, such as the small sample volume required, minimization of mobile phase, rapid analytical run time and improved sensitivity, selectivity and specificity (Srinivas, 2006). To date, only a few LC-MS/MS methods have been reported for the quantitation of ZIP in plasma (Janiszewski *et al*., 1997; Al-Dirbashi *et al*., 2006; Aravagiri *et al*., 2007). However, no papers report the matrix effects for ZIP from plasma using LC-MS methods. Recently, our lab (Zhang *et al*., 2007a and 2007b) described comprehensive LC-

MS/MS methods for determination of several antipsychotics including ZIP from plasma and brain tissue. However, the matix effects were high for ZIP (ion suppression: 17.1% for plasma and 16.2% for brain tissue). Matrix effects are an important issue in ESI resulting from co-eluting components, generally from the matrix, that cause variable suppression or enhancement of analyte response (King *et al*., 2000). In addition, liquid-liquid extraction was repeated twice to extract ZIP from brain tissue which was time consuming. Due to the lipophilic nature of ZIP, it was readily absorbed by myelin and other lipid constituents of brain homogenates. We found that ZIP was, to some extent, irreversibly bound to the inner surface of plastic or untreated glass tubes. Hence, the recovery of ZIP from brain tissue in particular was low. It was a challenge to improve the recovery of the hydrophobic ZIP from lipophilic brain tissue.

The purpose of this investigation was to develop and validate a simple, rapid, highly selective, sensitive and robust LC-MS/MS method for the determination of the lipophilic drug ZIP from rat plasma and brain tissue with low matrix effects.

EXPERIMENTAL

Chemicals and reagents

Ziprasidone was obtained from Pfizer Central Research (Groton, CT, USA). Midazolam (internal standard, I.S.) and hexamethyldisilazane were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, tert-butyl methyl ether, chloroform, hexane, methyl chloride, diethyl ether, isopropyl ether, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). The formic

acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate, ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler, and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA).

Liquid chromatographic and mass spectrometric conditions

The analytes were separated on an Agilent Eclipse XDB C-8 column (150 mm × 2.1 mm i.d., 5 µm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 0.02% ammonia in water (adjusted to pH 7.20 using formic acid) and mobile phase B was acetonitrile. A 10 µl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B, flow rate (ml/min)) (0, 60, 0.3) (2.5, 60, 0.3) (2.6, 90, 0.6) (5.5, 90, 0.6) (5.6, 60, 0.3) (12.6, 60, 0.3). From 2.6 to 5.5 min, it was very important to use a high concentration of acetonitrile (90%) in the mobile phase to wash the column at a flow rate of 0.6 ml/min. This procedure removed the remaining

brain extract residue from the column following each injection. Otherwise there was decreased response for ZIP in the next injection due to ion suppression resulting from brain extract residue from the previous injection. The column temperature was maintained at 25ºC. The LC flow was introduced directly to the mass spectrometer from 1.7-3.5 min and diverted to waste at other times using a six-port column switching valve. The autosampler injection needle was washed with methanol to reduce carry-over after each injection. The mass spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 600 L/h with a temperature of 380ºC. Argon was the collision gas and the collision cell pressure was 2.4×10^{-3} mbar. For quantitation, an MRM transition from m/z 413→194 was performed and 413→177 and 413→220 (Figure 1.) was monitored for identification with a cone voltage of 37 V and a collision energy of 26 eV. An MRM transition from m/z 326→291 was performed for the I.S. (midazolam) with a cone voltage of 32 V and a collision energy of 30 eV. The source temperature and capillary voltage were set at 120ºC and 3.20 kV, respectively.

Sample collection

ZIP chronic dosing was derived from previously published data (Terry *et al*., 2005 and 2006). Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human. Male albino Wistar rats (Harlan, Inc.) 2-3 months old were housed individually in a temperature-controlled room (25ºC), maintained on a 12-h ligh/dark cycle with free

access to food. Rats were treated with ZIP (12.0 mg/kg/day) orally in drinking water for periods of at least 14 days to achieve a steady-state concentration of ZIP. Plasma samples were collected at steady-state concentrations of ZIP. Rats were anesthetized with isofluorane and 3.0 ml of blood was collected via cardiac puncture to heparined tubes. The blood was centrifuged for 15 min at 2500× g at 8ºC and the resulting plasma was frozen until analysis. The whole brains of sacrificed animals were removed and kept frozen at -70 ºC until analysis.

Preparation of stock, working standard and quality control solutions

Individual stock solutions of ZIP and I.S. (midazolam) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at -20ºC. Standard solutions with concentrations of 5.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0, 2500.0 and 5000.0 ng/ml were prepared by serial dilution with acetonitrile. Precision and accuracy standards with concentrations of 5.0, 15.0, 375.0, and 3750.0 ng/ml were also prepared in the same manner. An 100.0 ng/ml I.S. standard solution was prepared with acetonitrile from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at -20ºC when not in use and replaced every 3 months. Fresh standard solutions were prepared for each day of analysis or validation.

Preparation of calibration and QC samples

The brains were minced and homogenized in a volume of deionized water (in ml) equal to twice the weight (in g) of the tissue using a Brinkmann (Westburg, NY, USA) Polytron TP 1020 homogenizer. Samples for the calibration curves and QCs were

prepared by adding 10.0 µl of each standard into 250 µl blank rat plasma or 200 µl blank brain homogenate. This yielded calibration standard concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0 and 200.0 ng/ml for plasma and 0.833, 1.667, 4.165, 8.33, 16.67, 41.67, 83.33, 166.7, 416.7 and 833.3 ng/g for brain tissue. The final concentrations of QCs were 0.2, 0.6, 15.0, 150.0 ng/ml for plasma and 0.833, 2.5, 62.5, 625.0 ng/g for brain tissue. The spiked plasma and brain homogenate samples (standards and quality controls) were extracted with each analytical batch along with the unknown samples.

Sample preparation

Prior to use, all of glass extraction tubes were silanised by rinsing with 10% (v/v) hexamethyldisilazane in ethyl acetate and heated 1 hr at 150ºC. To a 250 µl rat plasma or 200 µl brain homogenate sample, 10.0 µl of internal standard (100.0 ng/ml, midazolam) and 0.1 ml of 0.5 M Na_2HPO_4 (pH 10.69) for plasma or for brain homogenate were added. The samples were briefly mixed and extracted with 3 ml of isopropyl ether: methyl chloride (80: 20). After centrifugation at 2000× g for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 200 µl of acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) solutions were added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000× g prior to LC-MS/MS analysis.

Method validation

The method was validated for linearity, recovery, matrix effect, accuracy and precision. Brain homogenate calibration curves were constructed using the peak area ratios of ZIP to that of I.S., and applying a weighted ($1/x^2$) least squares linear regression analysis. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples. Five replicates of each QC points were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Absolute and relative recoveries, and matrix effects were calculated for spiked samples at 0.2, 0.6, 15.0, 150.0 ng/ml for plasma and 0.833, 2.5, 62.5, 625.0 ng/g for brain tissue and 4.0 ng/ml I.S. in plasma samples and 16.67 ng/g I.S. in brain tissue (n=5). Absolute recovery was calculated as the peak area for ZIP and the I.S. in plasma or brain homogenate spiked before extraction divided by the peak area of the pure drugs in the acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) solution at the same concentration. Relative recovery was calculated by dividing the peak area for ZIP and the I.S. spiked before extraction by the peak area for an equal concentration of the sample in the same matrix spiked after extraction. The matrix effects were calculated by dividing the response for a ZIP sample in biological matrix spiked after extraction by an equal concentration of ZIP in an acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) solutions (Matuszewski *et al.*, 2003). The stability of the stock solution was determined at its storage conditions of -20ºC for three months. ZIP was

considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls. The bench-top stability of spiked samples stored at room temperature was evaluated for 2h. The freeze/thaw stability was investigated by comparing the stability of samples following three freeze/thaw cycles, against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 24h. The stability testing was performed at 0.6 and 150.0 ng/ml for plasma and 2.5 and 625.0 ng/g for brain tissue concentration levels.

RESULTS AND DISCUSSION

Method development

In order to develop and validate a highly sensitive and selective method with the designed LLOQ (0.2 ng/ml for plasma and 0.833 ng/g for brain tissue), during method development different options were evaluated to optimize the detection (MRM) parameters, chromatography and sample preparation methodology. MS/MS detection provided improved sensitivity for trace mixture analysis. ESI(+) MS/MS product ion spectra were produced by CAD of the protonated molecular ion $(M+H)^+$. The most favorable transition was selected and the instrument parameter settings were optimized individually for ZIP and the I.S. by constant infusion at 10 µl/min of a 2 µg/ml solution. The major MS/MS transitions utilized for LC-MS/MS quantitative analysis were m/z 413→194 for ZIP and m/z 326→291 for I.S. (midazolam).

ZIP is a high hydrophobic drug and was found to easily irreversibly absorb on to the surface of plastic and untreated glass tubes. Silanised glass tubes reduced this absorption greatly. Brain samples contained substantial amount of lipid residue after evaporation of the extraction solvent to dryness. We evaluated solid phase extraction (SPE) methods using different cartridges such as the Waters Oasis HLB and Varian C18. We found that the recovery of ZIP and the I.S. was still very low using SPE from brain tissue because of the irreversible absorption of ZIP to the SPE cartridges. So liquid-liquid extraction was optimized using the extraction solvent isopropyl ether: methyl chloride (80: 20), which produced the least residue and high recovery using only a single liquid-liquid extraction. We next optimized the gradient elution conditions. From 2.6 to 5.5 min, it was important to use a high concentration of acetonitrile (90%) in the mobile phase and to wash the column at a flow rate of 0.6 ml/min. This procedure removed the residual brain extract from the column following each run. Failure to remove this residue decreased the response of ZIP for subsequent injections due to ion suppression from the brain extract residue from previous injections on the column.

Linearity and sensitivity

Table 1 shows the calibration curves for each day of validation. The curves showed good linear response (R^2 > 0.9968) over the range of 0.2-200.0 ng/g for plasma and 0.833-833.3 ng/g for brain tissue. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for the calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for ZIP.

Table 7.1 showed the slope, intercept and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with accuracy within 20% and a precision < 20%, was 0.2 ng/g for plasma and 0.833 ng/g for brain tissue as shown in Table 2. A signal-to-noise (S/N) > 10 at the LLOQ was observed for ZIP in biological matrices.

Precision and accuracy

Precision and accuracy measurements were acquired for the QC points. The accuracy and precision data can be seen in Table 2. The values for the intra-day precision and accuracy were better than 4.68% and 6.19%. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were better than 7.17% and 8.13% (Table 7.2).

Recovery and matrix effect

ZIP and the I.S. are basic compounds. Therefore, extraction was influenced by the pH of the brain homogenate samples. 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for ZIP and the I.S.

Due to non-specific binding of lipophilic ZIP to brain constituents, it was a challenge to improve the recovery of ZIP from brain homogenate. We found that the reconstitution solution played a key role in the recovery and reduced the matrix effects from the brain homogenate residue after liquid-liquid extraction. Our lab (Zhang *et al*., 2007a and 2007b) previously described LC-MS/MS methods for the determination of several antipsychotics including ZIP from plasma and brain tissue.

However, the matix effects were high (ion suppression: 17.1% for plasma and 16.2% for brain tissue) for ZIP using methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (70: 30). Finally, we found 0.2 ml of acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) as reconstitution solution and produced higher recovery (above 81.0%) and the lowest matrix effects (5.2% ion suppression). The absolute recovery, relative recovery, and matrix effect data for ZIP at different concentrations in plasma or brain tissue using acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) as the reconstitution solution are summarized in Table 7.3. The absolute recoveries ranged from 81.0% to 103.4% and the relative recoveries from 83.7% to 102.4% for ZIP and the I.S. from plasma or brain homogenate.

Specificity

Representative chromatograms obtained from blank biological matrices and samples spiked with the LLOQ standard (0.2 ng/ml for plasma and 0.833 ng/g for brain homogenate) are shown in Figures 7.2 and 7.3. No interfering peaks from endogenous compounds were observed at the retention times of ZIP or I.S. in blank plasma or brain homogenate. Three MRM transitions have been used: one transition (413→194) for quantification and two transitions (413→177 and 413→220) for identification. Table 7.4 contains the data for the first identification MRM transition (413→177), the peak area ratio (413→194/ 413→177) in plasma or brain tissue is from 7.16 to 7.29 over the calibration curve range. The peak area

ratio (413→194 / 413→177) from real samples was 7.18 for plasma and 7.25 for brain tissue, respectively. Also, for the second identification MRM transition (413→220), the peak area ratio (413→194 / 413→220) in plasma or brain tissue is from 23.90 to 25.03 over the calibration curve range. The peak area ratio (413→194 / 413→220) from real samples was 24.26 for plasma and 24.56 for brain tissue, respectively. These results showed that no significant interference existed for ZIP from plasma or brain tissue. The addition of the two MRM transitions (413→177 and 413→220) from ZIP for identification improved the specificity of the validated method.

Stability studies

Stability testing is very important for validated methods in biological samples. The stock solution was stable at the storage conditions (-20ºC) for three months (Data not shown). All the other stability studies were conducted at two concentration levels (0.6 and 150.0 ng/ml for plasma or 2.5 and 625.0 ng/g for brain tissue) with five determinations for each. Plasma and brain homogenate extracts were stable in the mobile phase in the HPLC autosampler for at least 24h, indicating that samples should be processed within this period of time (Table 7.5). The freeze/thaw stability tests indicated ZIP was stable in rat plasma and brain homogenate for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for 2h. The %RE was from 2.38% to 4.26% for plasma and from 2.32% to 6.76% for brain homogenate, and RSD was from 0.45% to 8.95% for plasma and from 1.93% to 5.61% for brain homogenate (see Table 7.5). Finally, the storage of

brain homogenate at room temperature over 8h resulted in an increase in the viscosity of the brain homogenate samples. This increased viscosity of the brain homogenate resulted in reduced recovery of ZIP and the I.S. Therefore, fresh brain homogenate should be analyzed within 2h or frozen immediately for later analysis.

Application of the method

The validated method has been successfully used to quantify ZIP concentrations in rat plasma and brain tissue following the chronic treatment of rats with ZIP in their drinking water. The concentration data for ZIP in rat plasma and brain tissue are reported in Table 7.6. The representative of MRM chromatograms resulting from the analysis of real plasma and brain tissue samples after the chronic treatment of antipsychotic drugs are shown in Figure 7.4. The concentration of ZIP in brain tissue was higher than in plasma. The result was the same as olanzapine (Aravagiri et al., 1999).

CONCLUSIONS

A simple, specific and sensitive LC-MS/MS method with low matrix effects for the determination of ZIP from rat plasma and brain tissue has been developed and validated. This method provided excellent specificity, wide linear range and a LLOQ of 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. A single step liquid-liquid extraction for sample preparation was used for 0.25 ml plasma and 0.20 ml rat brain homogenate that provided low matrix effects and high recovery for ZIP. Another advantage of this method was that only 0.25ml for plasma and 0.2 ml for brain homogenate (small sample volume) were necessary for sample preparation. This

method will also be used to determine the concentration of ZIP in different brain regions in the future.

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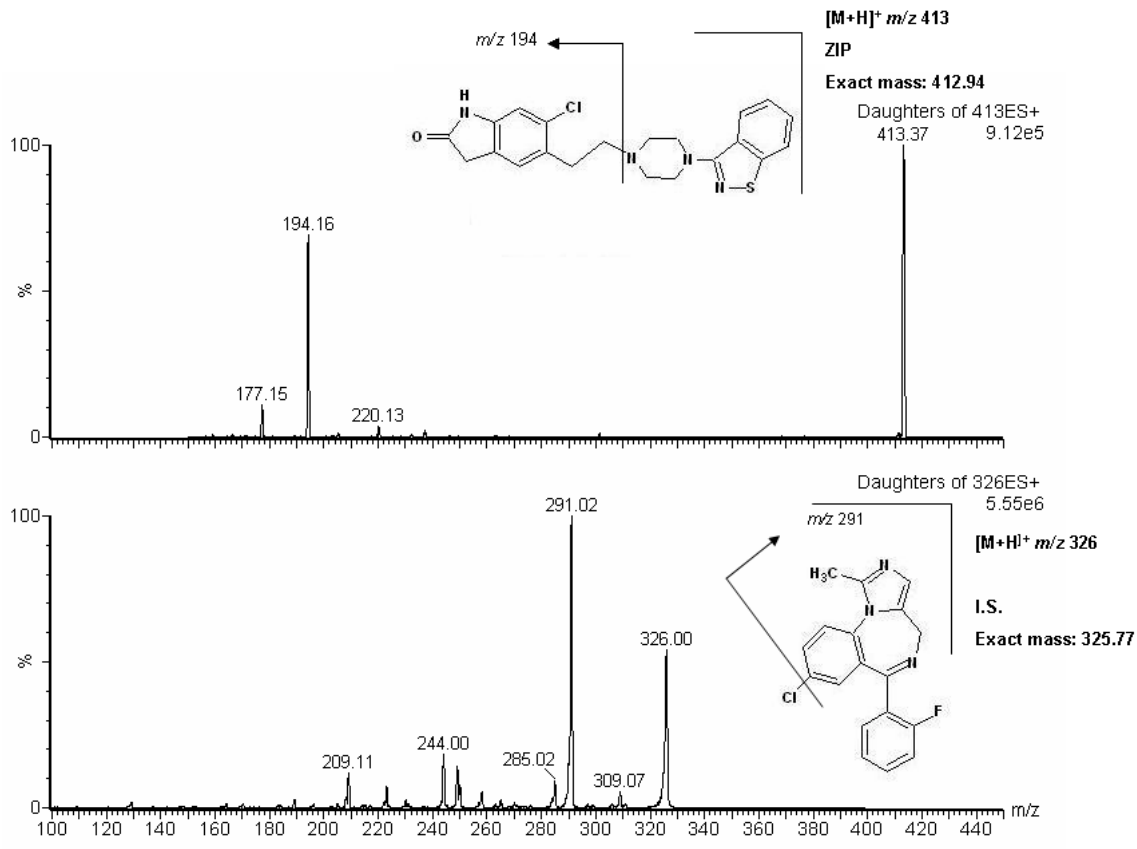

Figure 7.1. Product ion mass spectra of (A) ZIP, (B) I.S. and respective chemical structures.

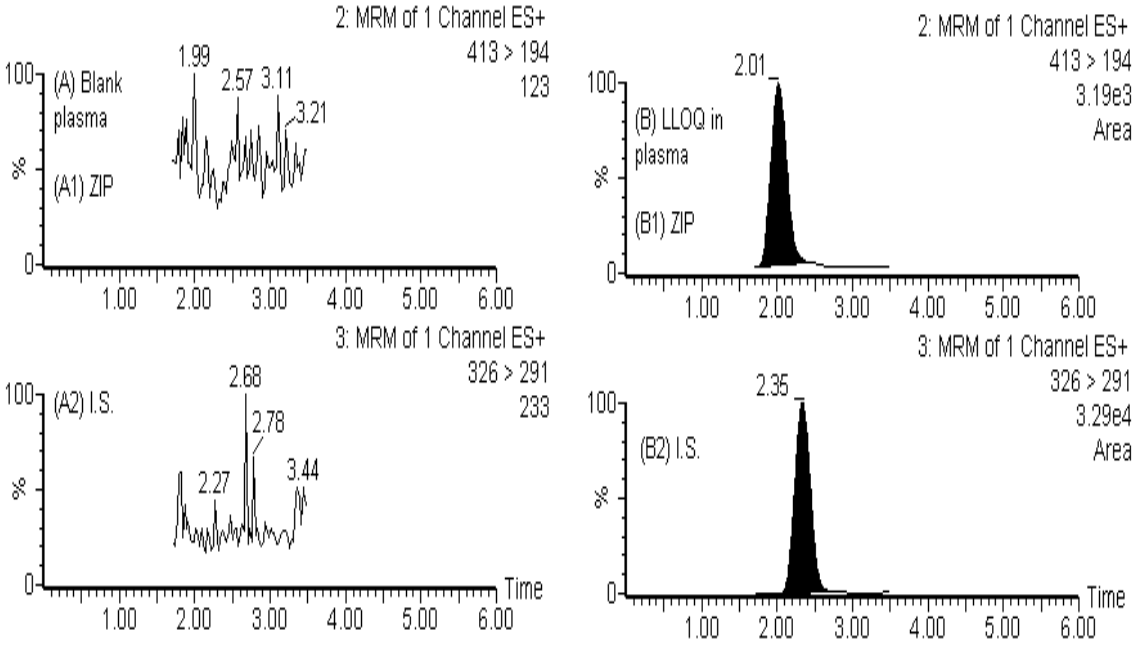

Figure 7.2. Representative chromatograms obtained from rat plasma, (A) blank rat plasma; (B) plasma spiked with LLOQ for ZIP at 0.2 ng/ml (B1) and I.S. at 4.0 ng/ml (B2).

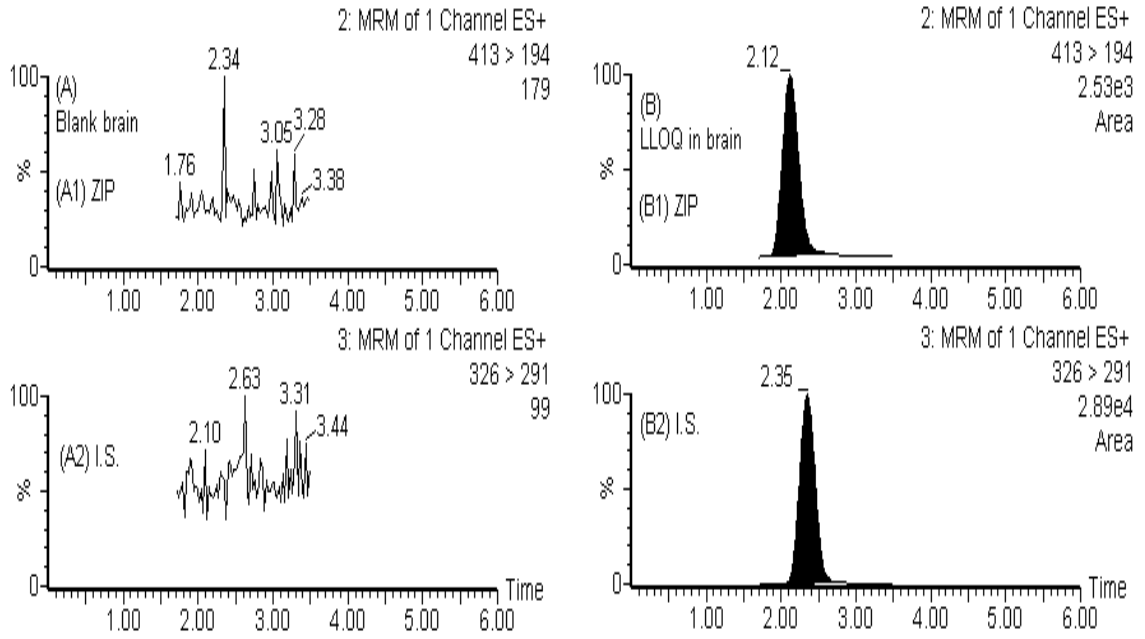

Figure 7.3. Representative chromatograms obtained from rat brain tissue, (A) blank rat tissue; (B) brain tissue homogenate spiked with LLOQ for ZIP at 0.833 ng/g (B1) and I.S. at 16.67 ng/g (B2).

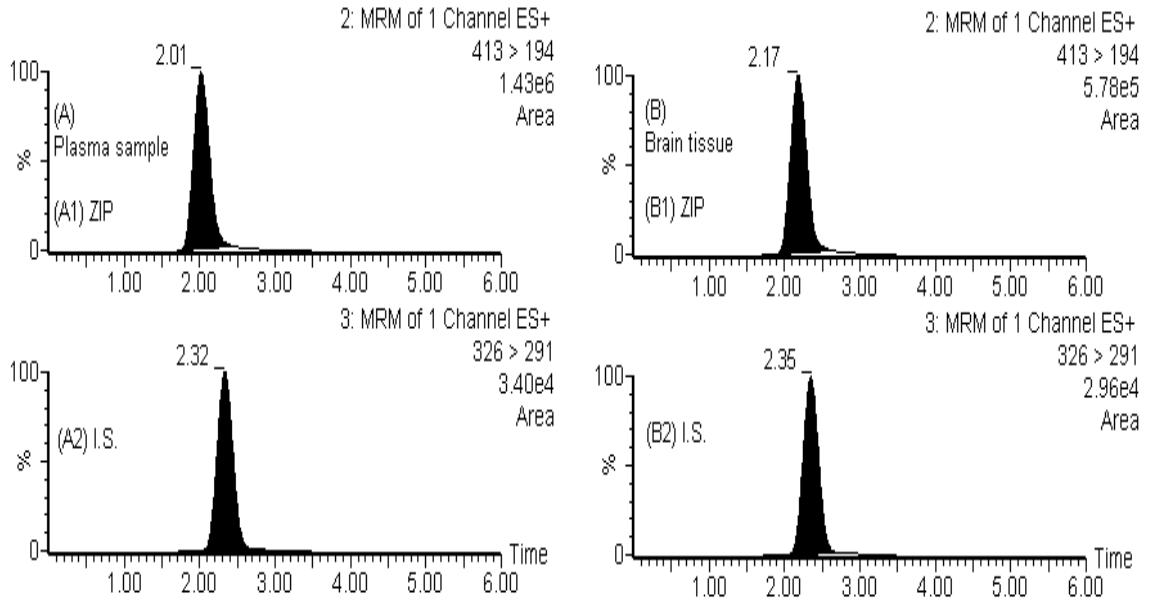

Figure 7.4. Representative chromatograms of plasma and brain tissue samples from rats following chronic treatment with ZIP for 15 days at 12.0 mg/kg/day: (A) the concentration of ZIP in the plasma sample was 105.08 ng/ml (A1); (B) the concentration of CPZ in brain tissue sample was 231.39 ng/g (B1).

Table 7.1
Statistical data for linearity including standard deviation (S.D.) (linear range 0.2-200.0 ng/ml for plasma and 0.833-833.3 ng/g for brain)

Biological matrix	R^2	Slope	Intercept
Plasma	0.9984 ± 0.00090	0.28714 ± 0.01294	0.00232 ± 0.003635
Brain tissue	0.9968 ± 0.00281	0.07146 ± 0.00467	0.01065 ± 0.004806

Table 7.2

The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the LC-MS/MS method used to quantitate ZIP in rat plasma and brain tissue

Matrix		Intra-day			Inter-day		
	Concentration added (ng/ml or ng/g)	Observed concentration (ng/ml or ng/g)	R.S.D. (%)	Error (%)	Observed concentration (ng/ml or ng/g)	R.S.D. (%)	Error (%)
Plasma	0.2	0.202 ± 0.009	4.68	3.70	0.204 ± 0.008	3.97	3.48
	0.6	0.606 ± 0.015	2.40	1.95	0.601 ± 0.041	6.89	5.39
	15	15.929 ± 0.398	2.50	6.19	15.789 ± 0.456	2.89	5.27
	150	144.883 ± 3.772	2.60	3.41	151.986 ± 6.056	3.98	3.60
Brain	0.833	0.833 ± 0.022	2.68	1.72	0.818 ± 0.054	6.59	5.01
	2.5	2.409 ± 0.027	1.10	3.65	2.638 ± 0.189	7.17	8.13
	62.5	63.63 ± 0.680	1.07	1.81	64.925 ± 3.835	5.91	5.31
	625	596.109 ± 27.426	4.60	4.62	596.862 ± 35.801	6.00	5.70

Table 7.3

Absolute recovery, relative recovery (%), and matrix effects (mean ± S.D.) of ZIP and I.S. in rat plasma and brain tissue at different concentrations (n = 5) using acetonitrile: methanol: 20 mM ammonium formate (pH 3.84 adjusted using formic acid) (48: 32: 20) as the reconstitution solution.

Matrix	Concentration (ng/ml or ng/g)	Absolute Recovery (%)	Relative Recovery (%)	Matrix Effect (%)	Type of Effect
Plasma	0.2	103.4 ± 3.28	98.9 ± 3.13	102.8 ± 1.42	2.8% enhancement
	0.6	99.4 ± 2.21	97.6 ± 2.17	102.1 ± 1.95	2.1% enhancement
	15	97.3 ± 1.65	99.3 ± 1.69	98.2 ± 0.48	1.8% suppression
	150	95.8 ± 1.02	96.6 ± 1.03	98.4 ± 2.22	1.6% suppression
I.S. (plasma)	4.0	101.9 ± 2.48	102.4 ± 3.25	101.5 ± 2.69	1.5% enhancement
Brain	0.833	82.8 ± 4.97	87.2 ± 5.24	95.9 ± 1.75	4.1% suppression
	2.5	81.0 ± 2.58	83.7 ± 4.24	94.8 ± 0.78	5.2% suppression
	62.5	86.4 ± 2.61	90.9 ± 2.74	95.2 ± 0.92	4.8% suppression
	625.0	85.0 ± 2.29	88.5 ± 2.38	95.5 ± 2.40	4.5% suppression
I.S. (brain)	16.67	94.5 ± 2.30	97.3 ± 2.97	96.5 ± 1.97	3.5% suppression

Table 7.4

Enhanced specificity of CPZ by multiple MRM transitions (413→194 for quantification; 413→177 and 413→220 for identification for ZIP in plasma and brain tissue).

Concentration (ng/ml in plasma / ng/g in brain tissue	0.2/0.833	0.4/1.666	1.0/4.165	2.0/8.33	20.0/83.3	200.0/833.3	Real samples plasma / brain
Peak area ratio in plasma (413→194 / 413→177)	n.d.	7.28	7.16	7.26	7.28	7.16	7.18
Peak area ratio in brain (413→194 / 413→177)	n.d.	7.20	7.24	7.29	7.29	7.26	7.25
Peak area ratio in plasma (413→194 / 413→220)	n.d.	n.d.	25.03	24.13	24.89	23.90	24.26
Peak area ratio in brain (413→194 / 413→220)	n.d.	n.d.	24.76	24.53	24.69	24.85	24.56

n.d.: not detected with 413→177 or 413→220 MRM transition

Table 7.5
Stability testing of ZIP in rat plasma and brain homogenate (n=5).

Matrix	Stability	Spiked conc. (ng/ml or ng/g)	Observed conc. ± S.D. (ng/g)	R.S.D. (%)	Relative error (%)
Plasma	Three freeze-thaw cycle	0.6	0.582 ± 0.0270	4.63	-3.00
		150.0	143.6 ± 0.649	0.45	-4.26
	Bench top (2h)	0.6	0.583 ± 0.0522	8.95	-2.87
		150.0	143.6 ± 3.614	2.52	-4.23
	Autosampler stability (24h)	0.6	0.586 ± 0.0078	1.34	-2.38
		150.0	153.7 ± 3.47	2.26	2.52
Brain	Three freeze-thaw cycle	2.5	2.55 ± 0.143	5.61	2.32
		625.0	665.4 ± 12.8	1.93	6.47
	Bench top (2h)	2.5	2.38 ± 0.047	1.97	-4.52
		625.0	582.7 ± 12.49	2.14	-6.76
	Autosampler stability (24h)	2.5	2.51 ± 0.106	4.22	3.91
		625.0	600.0 ± 17.28	2.88	-3.99

Table 7.6
Steady-state plasma and brain tissue concentrations of ZIP after the chronic treatment of ZIP in rats (n=3).

Matrix	Dose (mg/kg/day)	Steady-state concentrations (ng/ml or ng/g ± S.D.)	Concentration range (ng/ml or ng/g)
Plasma	12.0	148.2 ± 45.7	105.1-196.0
Brain tissue	12.0	238.1 ± 96.2	231.4-423.9

Chapter 8

DEVELOPMENT AND VALIDATION OF QUANTITATION OF A PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDE FROM RAT PLASMA USING CAPILLARY GEL ELECTROPHORESIS

Zhang G, Srinivasan K, Kavetskaia O, Duncan JN, Bartlett, MG. To be submitted to ***Anal. Biochem.***

Abstract

A highly selective, reproducible, robust capillary gel electrophoresis (CGE) method using ultraviolet detection was developed and validated for the quantitation of an intact phosphorothioate oligonucleotide (PS-ODN, 24-mer) and its numerous chain-shortened metabolites. A combined phenol-chloroform liquid-liquid extraction, strong anion-exchange column solid-phase extraction, ion-pair HLB solid-phase extraction method followed by dialysis with a 0.025 μm membrane was utilized to remove proteins and lipids in plasma. This sample treatment also lowers the ionic strength of the extract resulting in a significant improvement in the electrokinetic loading (5 kV, 5 s) of the analyte. Optimal electrophoretic separation was achieved at 10 kV using 7.0 M urea in a 10% polyacrylamide gel filled capillary (I.D. 75 μm, 22 cm effective length), 50 mM Tris-100 mM boric acid as the running buffer, and a temperature of 40 ºC. PS-ODN was determined using another phosphorothioate ODN (poly$(dT)_{40}$) as an internal standard. The method was validated over the range from 2.0-100.0 μg/ml and had precision (percent relative standard deviation) <8.4% and accuracy (percent relative error) <8.9%.

Keywords: Phosphorothioate; Oligonucleotide; Plasma; Capillary gel electrophoresis

1. Introduction

The development of oligonucleotide based drugs is moving forward rapidly. Members of the class of oligonucleotide drugs include antisense oligonucleotide, aptamers and siRNAs, several of which are in clinical development for various disease indications including cancer, infectious diseases, allergies and asthma [1-5]. In 1998, the FDA approval of fomivirsen (Vitravene^{TM}) for treatment of cytomegalo virus retinitis highlighted the progress made in moving antisense oligonucleotides from the laboratory to the market place. Pegaptanib (Macugen^{TM}), an anti-vascular endothelial growth factor (VEGF) aptamer, was recently approved by FDA (2004) for therapeutic use for the treatment of age-related macular degeneration, a progressive disease that is the leading cause of blindness in the elderly [6].

Therapeutic antisense oligonucleotides that are currently undergoing clinical development are predominantly phosphorothiate oligonucleotides (PS-ODN) where one oxygen is replaced by sulfur at a nonbridging position of the phosphodiester linkage (Figure 8.1.). PS-ODNs are usually sequences of 16-29 bases of single stranded DNA that hybridize to specific mRNA by Watson-Crick base pairing. These compounds can efficiently inhibit expression of a gene by reducing the translation of the target mRNA to the corresponding protein [7, 8]. The PS modification alone allows the antisense molecule to be much more resistant to nucleases and increases plasma protein binding, which, in turn, improves the distribution to tissues and prevents renal excretion. In addition, PS-ODN maintains the ability to trigger RNase H cleavage of the target RNA when bound as an RNA/DNA duplex [9]. The chemical

properties of PS-ODN are different from its phosphodiester counterpart. For example, a phosphorothioate has a lower pKa value than a phosphodiester due to differences in charge delocalization [10]. PS-ODN retention behavior on reversed-phase HPLC suggests that they are also more hydrophobic and therefore may exhibit more complex secondary structure [11]. The substitution of single oxygen to sulfur also creates a chiral center on the phosphate. The ability to measure PS-ODNs quantitatively and specifically in biological matrices is critical to evaluating the pharmacokinetic (PK), pharmacodynamic (PD) and toxicokinetic properties of PS-ODNs in support of their preclinical and clinical development. Quantitation of PS-ODN is required to establish exposure-response relationships and exposure-toxicity relationships, which are critical data for predicting human doses and clinical drug regimens [12]. The ideal bioanalytical method for measuring PS-ODNs would provide high selectivity, sufficient sensitivity, appropriate accuracy and precision, be robust, economical, and ultimately have high throughput capabilities. Therefore, PS-ODNs pose several unique analytical challenges. For example, one expected route of metabolism would likely involve the sequential removal of nucleotides from either the 3' or 5' terminus. The resulting family of oligonucleotides, shortened by one to several nucleotides, may still possess pharmacological activity. It is therefore critical to develop analytical methods that enable the detection and quantitation of intact oligonucleotide, as well as putative metabolic products which may differ by one or two nucleotides. Many different analytical methods have been reported for quantitation of PS-ODN in biological samples, such as HPLC-UV [13, 14],

radiolabeled methods [15], or hybridization techniques [16, 17]. Although these methods gave good approximations of oligonucleotide concentrations, none provided separation of the full-length oligonucleotide from a putative metabolite truncated by the loss of a single nucleotide unit. More recently, hybridization enzyme-linked immunosorbent assays [18, 19] have demonstrated very high sensitivity with little requirement for sample cleanup. These assays potentially quantitate drug related materials, which may include 3' and/or 5' chain-shorted metabolites. These cross-hybridization chain-shorted metabolites can result in the overestimation of the parent drug in vivo [12] and reduce the ability to develop clear PK/PD relationships. Also, the hybridization methods usually possess narrow dynamic ranges and reduced precision particularly at the lower concentrations. LC-MS/MS is an attractive choice to improve sensitivity and selectivity. LC-MS/MS also offers the ability to differentiate metabolites generated from 5' or 3'-deletion and has the potential for less rigorous sample preparation procedures. Several preliminary methods for PS-ODN quantitation in biological matrixes using LC-MS/MS with solid-phase extraction (SPE) or phenol/chloroform liquid-liquid extraction (LLE) have been reported in the literature [20-23]. Recently, Zhang et al. [24] described a LC-MS/MS method for quantitation of a 24-mer PS-ODN in plasma with LLOQ of 5.0 ng/ml. However, application of LC-MS/MS to PS-ODN is new, and reports using this technique in preclinical and clinical PK studies are only just beginning to appear in the literature. In order to accurately determine the metabolism and pharmacokintics of PS-ODN, capillary gel electrophoresis (CGE) is still the best analytical method for the

discrimination between full-length oligonucleotide from closely related metabolites. Several investigators have successfully used CGE to separate PS-ODNs up to approximately 25-nucleotide-long [25-34], however, not in a quantitative manner. In this paper we described a comprehensive and validated CGE-UV approach for analysis of a 24-mer PS-ODN, which uses liquid-liquid extraction followed by two SPE methods.

2. Experimental

2.1 Chemical and materials

The PS-ODN and internal standard (IS) (PS-poly $(dT)_{40}$) were obtained from Pfizer Inc. (Figure 8.2.). Tris base, boric acid, ammonium peroxidisulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED, 99.0%), acrylamide (electrophoresis grade, 99%), 3-(Trimethoxysilyl)propyl methacrylate, phenol, and chloroform were purchased from Sigma-Aldrich Inc. (St. Louis, MO). HPLC grade water, triethylamine (TEA), and ammonium hydroxide (28-30%) were obtained from J. T. Baker Inc. (Philipsburg, NJ). HPLC-grade acetonitrile, methanol, isopropyl ether and methyl chloride were purchased from Fisher Scientific (Pittsburgh, PA, USA). All the reagents used were of the highest (or HPLC) grade available. Strong anion-exchange SPE cartridge DSC-SAX (100 mg, 1.0 ml) was purchased from SUPELCO (Bellefonte, PA, USA). Oasis HLB (30 mg) SPE cartridges were obtained from Waters Inc. (Milford, MA). Filters (0.2 μm) and drop-dialysis cellulose acetate membranes of 0.025 μm pore size were obtained from Millipore (Bedford, MA).

Fused-silica capillary tubing (I.D. 75.0 µm, O.D. 365.0 µm) was obtained from Polymicro Technologies (Phoenix, AZ).

2.2 Capillary gel electrophoresis and HPLC-UV analysis

Capillary gel electrophoresis separations were accomplished with a Beckman MDQ capillary electrophoresis instrument (Beckman, Fullerton, CA) using a 29-cm silica capillary column (I.D. 75.0 µm) with an effective separation length of 22 cm. The separation runs were performed using buffer (50 mM Tris-100 mM boric acid, 7.0 M urea, pH 8.5 adjusted using hydrochloric acid). Samples were electrokinetically injected with a field strength of 5 kV for a duration of 5 s. Separation was achieved by operating at 40ºC using a circulating coolant with an applied voltage of 10 kV. The UV detector was set at 254 nm. Electropherograms were integrated, and the data were analyzed using Beckman's 32 Karat software (Beckman, Fullerton, CA).

To optimize the extraction procedure, an Agilent 1100 series HPLC system, consisting of a degasser, quaternary pump, autosampler, and a variable wavelength UV detector with a thermostatted column compartment (TC-50 controller, Wisconsin, USA) was used (Agilent, Palo Alto, CA, USA). For HPLC, the PS-ODN was separated using an XTerra^{TM} MS C18 (50 × 2.1 mm i.d., 2.5 µm) column. Mobile phase A consisted of 100 mM TEAA in water and mobile phase B was acetonitrile. The flow rate was set 0.20 ml/min. A 40 µL injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 5.0) (10.0 28.0) (17.0 40.0) (17.50 5.0) (28.0 5.0). The UV detector was set at 260 nm.

2.3 Preparation of gel-filled capillaries

Gel-filled capillary columns were prepared as described previously [29, 32] with the following modification. Briefly, the detection window on the capillary was created by removing the polyimide coating on the tubing using a hand held lighter. The capillary was washed with 1 M HCL, followed by a wash with 1 M NaOH which was left in the capillary for 1 h. The capillary was then flushed with methanol and treated with 1:1 v/v mixture of (3-(Trimethoxysilyl)propyl methacrylate with methanol. The sililating agent was left in the capillary for at least 8 h at room temperature of 25 °C. A solution of acrylamide (T=10.0%) in 50 mM Tris-100mM borate buffer containing 7.0 M urea was filtered through a 0.2 *μ*m filter and degassed under helium for 1 h. Polymerization of 1 mL acrylamide solution was initiated by addition of 3.0 µL of each 10% w/v ammonium persulfate and 10% w/v TEMED. The resulting solution was introduced in the capillary under pressure and left over 5 hrs at room temperature of 25 °C for complete polymerization with the ends of the capillary immersed in a Tris borate buffer. The CGE column was balanced using a low voltage of 3.0 kV for 30 min just before use.

2.4 Preparation of Stock, Working Standard and Quality Control (QC) Solutions

Individual ODN and IS stock solutions were prepared in deionized water to give a final concentrations of 1.0 mg/ml. Individual standard solutions with concentrations of 20.0, 50.0, 100.0, 200.0, 400.0, 500.0, and 1000.0 µg/ml were prepared by serial dilution with pure water. Precision and accuracy standards with concentrations of 2.0, 3.0, 15.0, and 80.0 ug/ml were also prepared in the same

manner. A 50.0 ug/ml I.S. standard solution was prepared with pure water from the 1.0mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept refrigerated when not in use and replaced on a weekly base. Fresh standard solution was prepared for each day of analysis or validation.

2.5 Preparation of Matrix Calibration and QC Samples

Samples for the calibration curves and QCs were prepared by adding 10.0 µl of each ODN standard and 10 µl of the 50.0 µg/ml I.S. standard solutions into 100 µl of blank plasma. This yielded calibration standard concentrations of 2, 5, 10, 20, 40, 50, and 100 µg/ml with an I.S. concentration in each sample of 5.0 µg/ml. The final concentration of QCs were 2.0, 3.0, 15.0 and 80.0 µg/ml.

2.6 Sample preparation

To 100 µL of rat plasma, 0.25 mL of a 5% ammonium hydroxide solution and 40 µL of chloroform/phenol, 1:2 (v/w), were added. These samples were vortexed for 2 min and centrifuged for 10 min at 14, 000 rpm. The aqueous solution (upper layer) was then transferred to a clean tube and washed using 0.8 mL of isopropylether: methyl chloride (80: 20) two times. Then 1.0 mL of loading buffer (10 mM Tris-HCl, 0.5M KCl (pH 9.0), 20% acetonitrile) was added to a new sample tube just before loading to the SAX-SPE cartridges. The SAX–SPE columns were pre-equilibrated with 1 ml acetonitrile on a vacuum manifold at a pressure of approximately 3 millibar. The acetonitrile wash was followed by 1 ml deionized H_2O wash and then 3 ml of the loading/running buffer. Samples, prepared as described above, were loaded onto the SAX–SPE column after which the column was washed with 0.7 ml × 2 of the loading/

running buffer. The oligonucleotide was eluted from the SAX–SPE column using 0.5 ml × 2 of the SAX elution buffer (10 mM Tris-HCl, pH 9.0, 0.5 M KCl, 1.0 M NaBr, 30% acetonitrile). The collected elute was evaporated for 40 minutes under a vacuum system to delete most of the acetonitrile. Then 1.0 ml of a 10 mM TEA solution was added and loaded to an Oasis HLB SPE cartridge. The HLB cartridges were pre-equilibrated with 1 ml acetonitrile, 1 ml deionized H_2O, and 1.0 ml of a 10 mM TEA solution sequentially. The samples were loaded onto the HLB cartridges and washed with 0.7 ml × 2 of 10 mM TEA and 0.3 ml × 2 of deionized H_2O sequentially. PS-ODN was eluted using 0.5 ml × 2 of freshly prepared 40% acetonitrile. The collected elute was evaporated almost to dryness (~2-3 µL remaining in the tube) in a Savant speed vac. Following this, 50.0 µL of dH_2O was added to the tubes. These tubes were vortexed for 1.0 min. The sample was further desalted by placing it on a Millipore 0.025-mm VS membrane floating on Nanopure deionized H_2O for 45 min.

2.7 Method validation

Plasma calibration curves were constructed using peak area ratios of PS-ODN to that of the IS and applying a weighted ($1/x^2$) least-squares linear regression analysis. Precision (expressed as percent relative standard deviation, %RSD) and accuracy (expressed as percent error) were calculated for the four QC samples (concentrations of 2, 3.0, 15.0, and 80.0 µg/mL). Five replicates of each QC point were analyzed every day to determine the intraday accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day

accuracy and precision. Stability of PS-ODNs during freeze-thaw (2 cycles), at room temperature (8 h), and in the autosampler (24 h) (*n*= 5) were also determined and reported.

3. Results and discussion

3.1 Method development

The CGE columns were, inner-wall coated capillaries used for eliminating electroosmotic flow and avoiding PS-ODN adsorption. The sililating agent was left in the capillary for at least 8 h to improve the coating of inner-surface and the column life time. The concentration of acrylamide was optimized from T = 8.0% to 15.0% for preparation of linear polyacrylamide (LPA) gel columns. Higher T values produced higher separation capacity; however, the column life times were decreased quickly. Finally, T = 10.0% was selected because it provided baseline separation between the full length PS-ODN and its chain-shortened metabolites and a good column life time (12 injections). The reproducibility of many types of capillary electrophoresis has not been ideal. Therefore, it was not surprising that there was no information about the reproducibility of CGE using coated capillaries in literature. The reproducibility of retention times of the PS-ODN and IS between three different CGE columns made at the same batch was quite good (Figure 3.).

PS-ODNs are highly bound to plasma proteins. In order to determine the level of PS-ODNs in a given biological matrix, the sample needs to be purified from other materials present in the matrix. CGE is unique among CE techniques because samles can not be introduced by hydrodynamic injection. Therefore, removing the salts and proteins, which are known to interfere with the amount of material applied

to gel filled capillary by electrokinetic injection [35], requires significant attention. Therefore, the sample preparation was critical in achieving acceptable sensitivity and reproducibility. A unique combination of phenol/chloroform LLE followed by SPE was found to solve this problem. The detailed method procedure was as follows. To 100 µL of rat plasma, 0.25 mL of 5% ammonium hydroxide solution and 40 µL of chloroform/phenol, 1:2 (v/w), were added. These samples were vortexed for 2 min and centrifuged for 10 min at 14 000 rpm. The aqueous solution (upper layer) was then transferred to a clean tube and washed using 0.8 mL of isopropylether: methyl chloride (80: 20) two times. Next, 0.6 mL of the 10 mM TEA was added to the new sample tubes. These tubes were then vortexed and the material was extracted on an Oasis HLB SPE cartridge (the cartridge was conditioned first with 1 mL of acetonitrile and then 1 mL of 10 mM TEA). The above samples were then loaded onto the SPE cartridge. The column was washed with 0.6 mL of 10 mM TEA followed by 0.7 mL of deionized water. Samples were eluted using 0.5 mL of acetonitrile/water, 40:60 (v/v). The eluant was evaporated to dryness under reduced pressure in a vacuum centrifuge and then reconstituted with 50 µL of water. Then, the reconstitution solution was dialyzed on a 0.025 µm floating desalting floating membrane filter (Millipore, Billerica MA) for 45 min. The extraction efficiency was greater than 88% using LC-UV method (Figure 4.). However, this method was not suitable for CGE because of high matrix effects and calibration curve was not linear (data not shown). Leeds *et al*. [29] directly used SAX SPE followed by C_{18} SPE method to extract PS-ODNs from plasma without also using LLE. This dual SPE method was not suitable

for our 24-mer PS-ODN and the recovery was very low. Finally, phenol/chloroform LLE followed by SAX SPE and ion-pair HLB SPE method was used to obtain cleaner sample extract (low matrix effect) and higher sensitivity. When using HLB SPE method, 10 mM TEA was used as an ion pairing reagent with the PS-ODN and the retention of PS-ODN on HLB cartridge was greatly improved compared with C_{18} cartridges without the ion pairing reagent (10 mM TEA). High concentrations of salts from the SAX SPE cartridges were separated from the PS-ODN using ion-pair HLB cartridge. This sample method obtained a cleaner extraction solution, improved the sensitivity, reproducibility, calibration curve linearity and recovery of the PS-ODN from plasma.

Increasing the run voltage decreases the analytical time, however, the life time of column was reduced. If the electrokinetic injection voltage and time was increased, the method sensitivity was improved but the peak shape was wider and separation capacity reduced. Finally, the run voltage was set 10 kV and the electrokinetic injection voltage and time were 5.0 kV and 5 s. In order to quantitate PS-ODN from plasma, an internal standard was used to reduce the discrimination of electrokinetic injection and matrix effects. Also, the internal standard played a key role in obtaining a linear calibration curve.

3.2 Linearity and sensitivity

Table 1 shows the calibration curves for each day of validation. The curves were linear (R^2 > 0.9955) over the range from 2.0-100.0 ug/ml for plasma. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations

for the calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for the PS-ODN. Table 1 contains the slope, intercept and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with accuracy within 20% and a precision < 20%, was 2.0 μg/ml for plasma as shown in Table 2. A signal-to-noise (S/N) > 10 at LLOQ was observed for the PS-ODN from the biological matrix.

3.3 Precision and accuracy

Precision and accuracy measurements were acquired at four QC points. The accuracy and precision data can be seen in Table 2. The values for the intra-day precision and accuracy were better than 8.2% and 8.9%. The inter-day precision and accuracy were determined by pooling all of the QC samples (n=15). The values for the inter-day precision and accuracy were better than 8.4% and 7.2% (Table 2).

3.4 Specificity

Representative chromatograms obtained from blank biological matrix and spiked with LLOQ standard (2.0 μg/ml) are shown in Figure 5. No interfering peaks from endogenous compounds were observed at the migration times of the PS-ODN or I.S. from blank plasma. A high separation capacity between PS-ODN and its chain-shortened metabolites was obtained.

3.5 Stability studies

Stability testing is very important for validated methods in biological samples. The stock solution was stable at the storage conditions (-20ºC) for one month (Data not shown). All the other stability studies were conducted at two concentration levels (3.0

and 80.0 µg/ml for plasma) with five determinations. Plasma extracts were stable in deionized H_2O in the CE autosampler (kept at 16ºC) for at least 24h, indicating that samples should be processed within this period of time (Table 3). The freeze/thaw stability tests indicated that the PS-ODN was stable in rat plasma for three freeze/thaw cycles. The results of bench-top stability indicated that spiked samples were stable for 2h. The accuracy (%) was from 88.9% to 104.0%, and RSD was from 0.6% to 8.1% (see Table 3).

4. Conclusions

In conclusion, a CGE-UV method for the determination of a PS-ODN and its chain-shortened metabolites from rat plasma using PS-poly$(dT)_{40}$ as an internal standard has been developed. The method was comprised of sequential LLE, SAX SPE, ion-pair HLB SPE and a membrane-desalting step to extract the PS-ODN from plasma. This method provided good selectivity between PS-ODN and its chain-shortened metabolites. LLOQ was 2.0 µg/ml using 100 µL of plasma. It is important to mention that the sensitivity of this CGE-UV method was not enough to determine the terminal elimination phase points of the drug at clinically relevant doses. Hence, our lab is currently investigating CGE-Laser-Induced-Fluorescence to improve the sensitivity for clinical applications.

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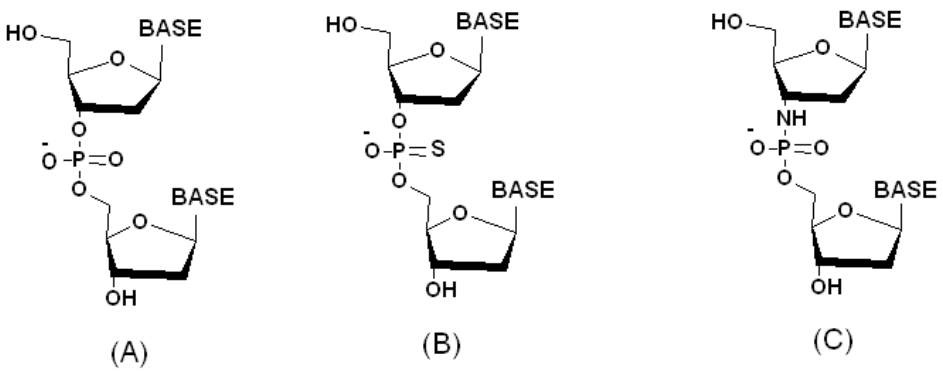

Figure 8.1. Different backbone chemical structure of oligonucleotides (A) phosphodiester, (B) phosphorothioate, (C) phosphoramidate.

Figure 8.2. Nucleotide sequences of PF-ODN and IS. Also basic structural elements of a phosphorothioate backbone oligodeoxynucleotide are shown.

SEQUENCES: PF-ODN 5'-TCGTGCTTTTGTTGTTTTCGCGTT-3' (MW: 7698)

IS 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3'

(MW: 12730)

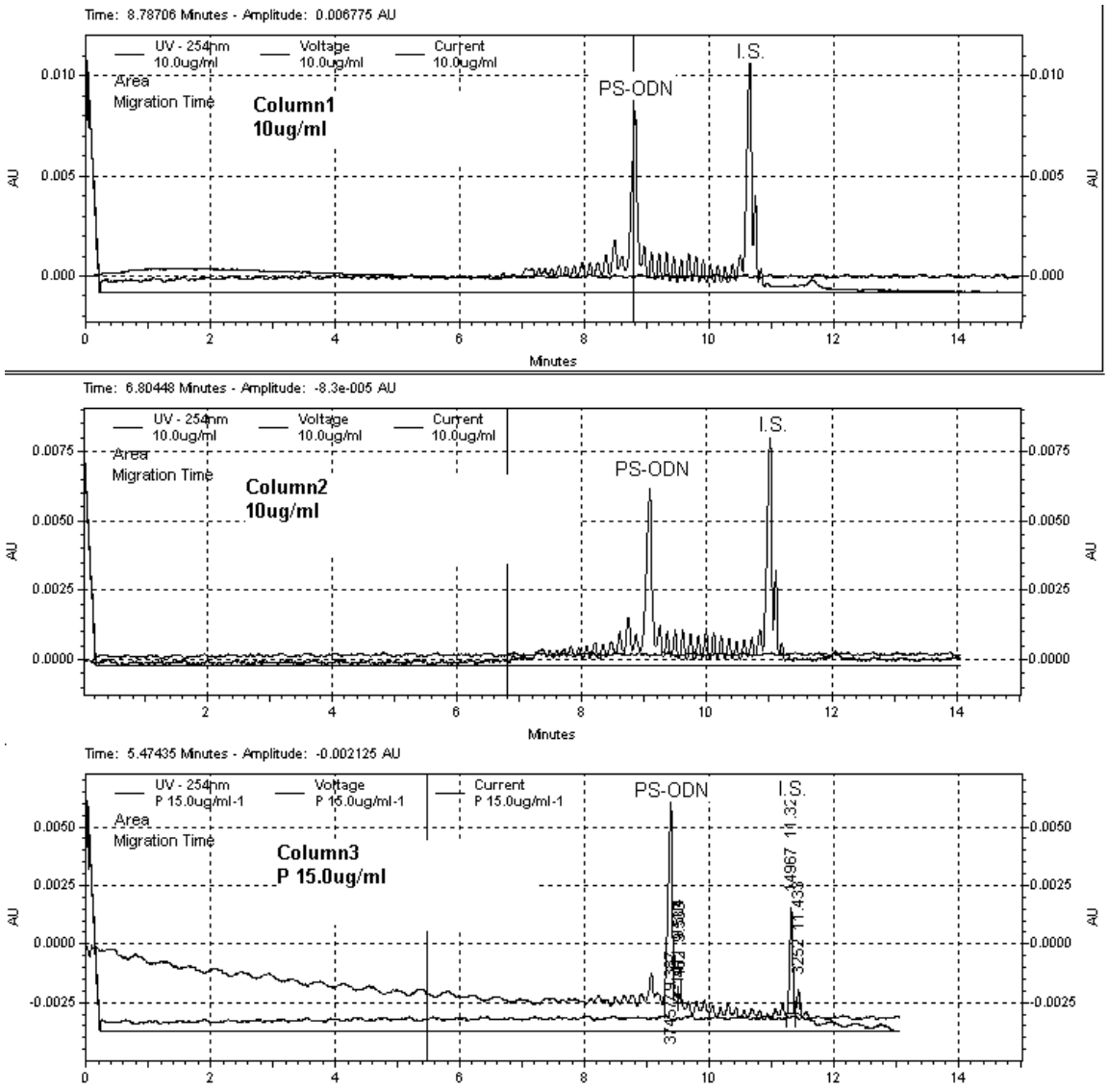

Figure 8.3. The reproducibility of retention time of PS-ODN and I.S. between three different CGE columns.

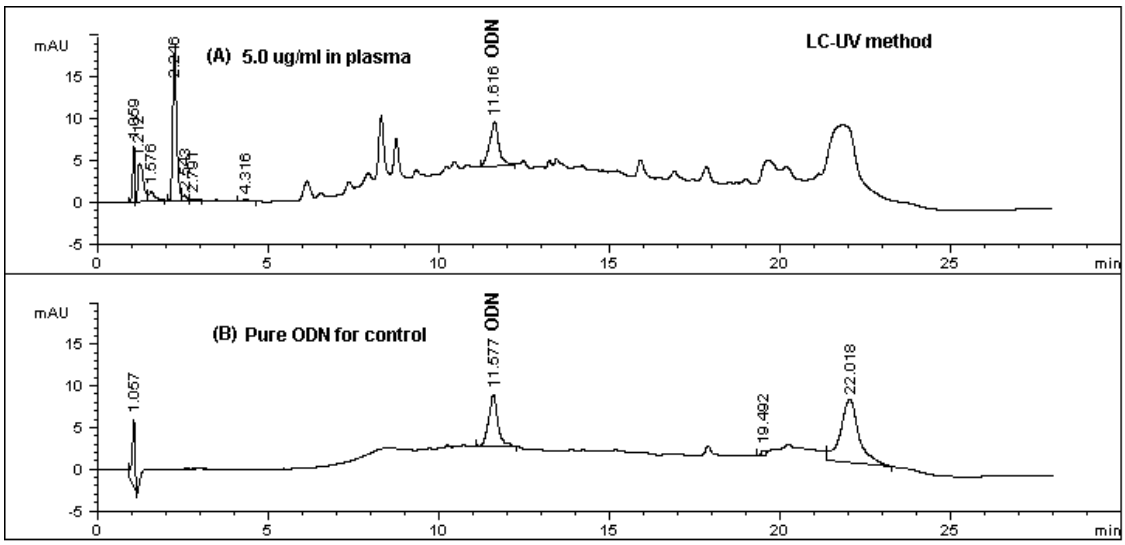

Figure 8.4. LC-UV method to evaluate the extraction efficiency of ODN in plasma (Absolute recovery: above 88%).

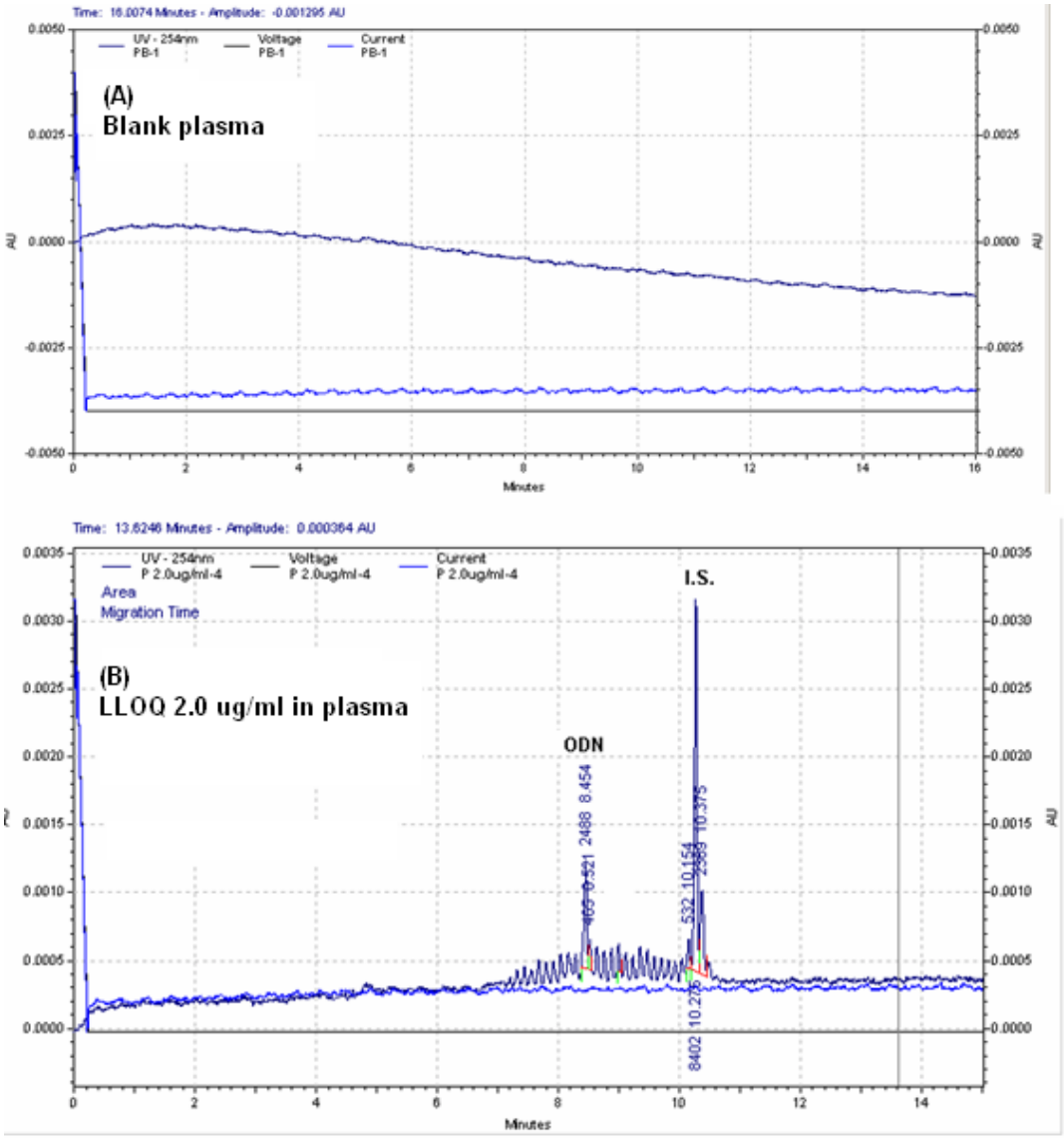

Figure 8.5. Representative chromatograms obtained from (A) blank rat plasma; (B) plasma spiked with LLOQ (2.0 µg/ml) concentration for all of the analytes and I.S. (5.0 µg/ml).

Table 8.1
Statistical data for linearity including standard deviation (S.D.) (linear range 2.0-100.0 ug/ml for plasma)

Biological matrix	R^2	Slope	Intercept
Plasma	0.99546 ± 0.00459	0.16251 ± 0.00799	-0.04683 ± 0.03012

Table 8.2
The intra-day (n=5) and inter-day (n=15) precision (% R.S.D.) and accuracy (% error) of the CGE-UV method used to quantitate ODN class drugs in plasma.

Intra-day (n=5)				Inter-day (n=15, 3 days)		
Conc. (μg/ml)	Observed conc. (μg/ml)	R.S.D. (%)	Error (%)	Observed conc. (μg/ml)	R.S.D (%)	Error (%)
2.0	2.12 ± 0.15	7.2	5.96	2.13 ± 0.14	6.6	7.04
3.0	3.19 ± 0.11	3.3	6.20	2.98 ± 0.25	8.4	6.73
15.0	15.30 ± 0.96	6.3	2.02	14.78 ± 1.01	6.9	5.90
80.0	87.11 ± 7.14	8.2	8.89	85.38 ± 4.55	5.3	7.18

Table 8.3. Stability of freeze-thaw cycle, bench top and autosampler (n=5)

	Free-thaw stability (two cycles)		Room temperature stability (2hrs)		Autosampler stability (24hrs)	
Nominal conc. (µg/ml)	3.0	80.0	3.0	80.0	3.0	80.0
Observed conc. (µg/ml)	2.72 ± 0.08	71.2 ± 2.24	3.08 ± 0.25	78.99 ± 0.44	2.89 ± 0.21	83.16 ± 3.56
(%) Accuracy	90.5	88.9	102.6	98.7	96.3	104.0
(%) R.S.D.	2.8	3.2	8.1	0.6	7.2	4.3

Chapter 9

CONCLUSIONS

Antipsychotic agents are used in psychiatric patients for the management of psychotic episodes as well as for other behavioral symptoms such as agitation. It is important to assess cognitive function after long term treatment with FGAs and SGAs. The bioanalytical methods presented here are simple, sensitive and specific for the determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol, ziprasidone and chlorpromazine in plasma and brain tissue. The HPLC-UV method using a single step liquid-liquid acid solution back extraction technique with wash procedure was optimized to provide a very clear background baseline for plasma with high recoveries for all of the analytes. Most of the antipsychotic drugs are highly hydrophobic. It is difficult to extract them from biological samples, especially from brain tissue. LC-MS/MS methods using a simple liquid-liquid extraction were optimized to obtain high recoveries and low matrix effect. In addition, ziprasidone and chlorpromazine are very highly hydrophobic antipsychotic drugs. The optimization of the reconstitution solution improved the recoveries and reduced the matrix effects greatly when using LC-MS/MS methods. All of the validated methods were acceptable with the current FDA requirement for bioanalytical methods. They were also successfully applied to real samples from rats dosed long term with SGAs or FGAs. In addition, the LC-MS/MS methods for brain tissue appear to be sensitive enough to determine the concentration of these antipsychotic drugs in different regions of the brain.

The bioanalysis of PS-ODN is full of challenges. It is difficult to extract them from biological samples because of their high polarity and high binding to plasma. A high separation capacity analytical method was necessary to determine the intact PS-ODN and its chain-shortened metabolites in biological samples. A highly selective, reproducible, robust CGE method was developed and validated for quantitation of a 24-mer PS-ODN in plasma. A combined phenol/chloroform LLE, SAX SPE and ion-pair HLB SPE followed by dialysis with a 0.025 μm membrane produced cleaner extraction solutions and improved the sensitivity.

APPENDIX

STRATEGIES FOR BIOANALYSIS OF AN OLIGONUCLEOTIDE CLASS MACROMOLECULE FROM RAT PLASMA USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Zhang G, Lin J, Srinivasan K, Kavetskaia O, Duncan JN. *Analytical Chemistry* 2007; **79:** 3416-3424. Reprinted here with the permission of publisher.

ABSTRACT

Quantitation of oligonucleotides in biological samples is a challenge using traditional liquid chromatography-electrospray tandem mass spectrometry (LC/MS/MS), even though they provide high throughput and selectivity. The method of choice for this class of compounds remains the hybridization based ligand-binding biological assays. These assays have good sensitivity but have narrow dynamic ranges, variable selectivity, and poor precision at low concentrations. Hybridization assays also require “capture and detection” probes, which can be prohibitively time consuming and expensive to develop in a pharmaceutical lead optimization scenario. In this paper we present the first literature report of a fully validated LC/MS/MS assay for an ODN (of 24 nucleotide sequence and 7692 amu, monoisotopic molecular weight) from rat plasma. Multiple analytical challenges were encountered during LC/MS/MS assay development of our model phosphorothioate backbone oligodeoxynucleotide (ODN) drug and strategies used to resolve of them will prove useful to scientists pursuing mass spectrometry (MS) based assays to quantitate such biologics. A list these challenges include: multiple charging and cation adduction (low assay sensitivity), oxidation of analyte on drying and high protein binding (low analyte recovery), affinity to bare silica (low chromatographic reproducibility and high carryover), non-specific binding to containers and affinity for metal components (low storage stability) and selection and custom synthesis of IS that does not interfere with analyte (potential cross-talk). In this paper, a buffer (7 mM TEA and 3 mM ammonium formate) : MeOH, 50:50 (v:v) was developed as ESI-

MS infusion solvent containing ODN and produced a sharp multiple charge distribution state and suppressed the sodium adduction. An ion pair LC/MS/MS method was developed to quantify this ODN in rat plasma. Sample extraction method combined a phenol/chloroform liquid-liquid extraction and a SPE Step, which improved the absolute recovery to > 70%. ODN and internal standard (IS) were separated using a Hypersil GOLD C18 50×2.1 mm column packed with 1.9 µm particles and quantified by tandem mass spectrometry using an electrospray source in negative ion mode. The method was validated in the linear range of 5-2000 ng/ml for ODN in rat plasma. This validated method had overall precision (% RSD) <10.1% and accuracy (% RE) < 11.4%.

INTRODUCTION

In recent years, oligonucleotides have been studied extensively as potential therapeutic agents for viral infections[1], inflammatory diseases[2], and cancers[3,4]. These oligonucleotides are sequences of 16 to 29 bases of single-stranded DNA (ssDNA) that hybridize to specific mRNA by Watson-Crick base pairing. These compounds can efficiently inhibit expression of a harmful gene and decrease the translation of the target mRNA into the corresponding protein[5,6]. Phosphorothioate oligonucleotides with oxygen replaced by sulfur at a non-bridging position of the phosphodiester linkage represent one of the most commonly used ODNs and seem to have favorable pharmacologic characteristics, such as nuclease resistance and the ability to recruit and activate RNase H, which make it suitable

for in vivo administration[7]. A phosphorothioate ODN, fomivirsen, was approved by the FDA in 1998 for treatment of cytomegalo virus retinitis.

The ability to measure ODNs quantitatively in biological matrices may prove important to evaluating the pharmacokinetic/pharmacodynamic (PK/PD) relationship in support of their preclinical and clinical development. Quantitation of ODNs and their metabolites is also required to obtain exposure-toxicity relationships. A reliable bioanalytical technique should be highly selective, sensitive, accurate, precise and robust. Early methods for quantification of oligonucleotides in biological matrices relied on ultraviolet (UV) detection such as HPLC-UV[8,9], CGE-UV[10,11]. But UV detection was insufficient to characterize the terminal elimination phase of the ODNs in plasma. CGE with laser-induced fluorescence was more sensitive but was found to have poor robustness[12].

More recently, Hybridization Enzyme-Linked Immunosorbent Assay[13,14] provided the ultra sensitivity assay with minimum sample clean-up but little was known about the metabolites fate in vivo due to poor assay selectivity. These assays quantitate all drug like material which may include 3' and/or 5' chain-shorted metabolites, and these cross-hybridization chain-shorted metabolites can result in overestimation of parent drug *in vivo*[15]. Also, the hybridization methods have narrow dynamic ranges and poor precision particularly at lower concentrations.

Electrospray ionization mass spectrometry (ESI-MS) has been proven to be a gentle and sensitive method for the sequencing, identification and characterization of oligonucleotides. But some challenges still need to be overcome for the qualitative and quantitative analysis of ODNs by MS methods, such as (1) limited ionization efficiency, (2) reduced sensitivity due to multiple charge distribution, (3) extensive adduction due to solution affinity of the polyanionic backbone for ubiquitous cations such as sodium or potassium ions in biological samples, which complicates the interpretation of the spectra[16,17,18] . Molecular weight measurement for unknown ODNs is obtained easily since ES-MS spectra contain a distribution of charges states which can be deconvoluted[19].

Liquid Chromatography (HPLC) has the advantage for analysis of ODNs because it has the ability to desalt, separate and characterize them. The disadvantages of HPLC based assays are (1) ability to achieve the required chromatographic separation in either reversed phase or ion exchange chromatography, (2) lack of suitable ion-pairing reagents required for both MS sensitivity and HPLC capacity. The use of ion pairing buffers, namely triethylammonium acetate (TEAA), enhances the separation but is not compatible with MS detection. Hube and Krajete[20] introduced a MS-friendly ion-pairing buffer, comprised of triethylammonium bicarbonate with an acetonitrile gradient for the analysis of up to 40 mer oligonucleotides but the sensitivity using this buffer was not found acceptable. Recently, Apffel *et. al.*[21,22] have described the use of hexafluoro-2-

isopropanol (HFIP) and triethylamine (TEA) for ion-pair reverse phase chromatographic separation of ODNs using ESI-MS detection with good assay sensitivity. This approach has found use in the analysis of chemically modified oligonucleotides using LC/MS[23].

Several preliminary methods for ODNs quantitation in biological matrices using LC/MS/MS with SPE or phenol/chloroform liquid-liquid extraction have been reported in literature[24,25,26]. Recently, the quantitation of an oligonucleotides (unvalidated assay with stability not unassessed) in biological matrices by LC/MS/MS has been reported[27], with promising results. However, most of these reported assays indicate low recovery and high matrix effect as challenges.

Our model compound is a novel oligonucleotide agent for treatment of lung cancer, called PF-ODN. It is a synthetic DNA molecule, 24 nucleotides in length (monoisotopic molecular weight of ~7692 amu) with a nuclease-resistant phosphorothioate backbone (see Figure 4 for sequences and structural elements). This work represents the first quantitative, fully validated mass spectrometry based assay for oligonucleotide class compounds reported in the literature. We also intend to provide scientists, through this paper, problem solving strategies while developing quantitative assays for ODN class compounds.

EXPERIMENTAL SECTION

Materials

The PF-ODN and a 23 mer PS oligonucleotide as internal standard were obtained from internal (Pfizer) sources. Hexafluoroisopropyl alcohol (HFIP, 99+%), triethylammonium acetate(TEAA), triethylammonium bicarbonate (TEAB), phenol and chloroform were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). HPLC grade method, acetonitrile, water, triethylamine and concentrated ammonium hydroxide (28-30%) were obtained from J.T.Baker Inc (Philipsburg, NJ, USA). All the reagents used were of the highest (HPLC) grade available. Rat Plasma in sodium heparin used for the preparation of controls and standards were obtained from the BioChemed Services (Winchester,VA, USA). 96-well extraction plates and Cartridges packed with Oasis HLB (10mg) sorbent were obtained from Waters Inc(Milford, MA, USA).The silanized glassware used was prepared inhouse.

Instrumentation

The system used was a Shimadzu two LC-10AD HPLC pumps (Columbia, MD, USA) and a Leap Technologies HTS PAL autoinjector (Carrboro, NC, USA). An Applied Biosystems MDS Sciex API 3000 and an API 5000 triple quadrupole mass spectrometers (Concord, Ontario, Canada) equipped with a Turbo Ion Spray source and operating in negative ion multiple reaction monitoring mode were used for all analyses. Data acquisition was performed with ABI/Sciex Analyst, software

version 1.1.

Preparation of Stock, Working Standard and Quality Control (QC) Solutions

Individual PF-ODN and IS stock solution were prepared in deionized water to give a final concentration of 1.0 mg/ml. Individual standard solutions with concentrations of 0.1, 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0 □g/ml were prepared by serial dilution with 7 mM TEA solution. Precision and accuracy standards with concentrations of 0.1, 0.3, 3.0, and 30.0 ug/ml were also prepared in the same manner. A 5.0 ug/ml I.S. standard solution was prepared with 7 mM TEA solutions from the 1.0mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept refrigerated when not in use and replaced on a weekly base. Fresh standard solution was prepared for each day of analysis or validation.

Preparation of Matrix Calibration and QC Samples

Sample for the calibration curves and QCs were prepared by adding 10.0 ul of each PF-ODN standard and 10 ul of the 5.0 ug/ml I.S. standard solution into 200ul blank plasma. This yielded calibration standard concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml with an I.S. concentration in each sample was 250 ng/ml. The final concentration of QCs were 5, 15, 150, 1500 ng/ml.

Liquid Chromatographic and Mass Spectrometric Conditions

Chromatographic separations were performed at a flow rate of 0.15 ml/min with a

Hypersil GOLD C_{18} column (Thermo, Waltham, MA, USA) 50×2.1mm, packed with 1.9 um particles. A binary gradient was used to perform the separation. Mobile phase A consisted of 1.7 mM TEA and 100mM HFIP (pH 7.5) in water and Mobile phase B was methanol. A 40 ul injection of each sample was loaded onto the column, separated and eluted using the following gradient: (minutes, % mobile phase B) (0, 8) (0.5, 8) (10, 28) (17, 40) (17.5, 8) (25.5, 8). The column temperature was maintained at 35□ using a column heater. The column elluent was directed into a Sciex API 3000 mass spectrometer (concord, Ontario, Canada). The LC flow was directed to the mass spectrometer in the time periods of 5.0-10.0 min and diverted to waste at other times using a six-port switching valve (VICI, Houston, TX, USA). The autosampler syringe and the injection valve were washed twice with 100ul of water and methanol separately to reduce carryover after each injection. The mass spectrometers were operated with negative-ion multiple reaction monitoring (MRM) mode and high resolution for Q_1 and low resolution for Q_3 to improve assay sensitivity. An Sciex API 5000 mass spectrometer was used for initial infusion at a flow rate of 20 $ul.min^{-1}$ in full scan (400 to 900 amu). For the compounds elicited multiple charge states, the most abundant charge state was the selected and fragmented in the mass spectrometer. The MRM transition was established based on the most abundant production. The MRM transition for PF-ODN was m/z 641>319 and the internal standard transition was m/z 811>319. Each transition was assigned a dwell time of 150 ms.

Sample Preparation

Plasma was prepared with phenol/chloroform liquid-liquid extraction and SPE. To 200 □l of matrix sample, 0.5 ml 5% ammonia solution and 0.1ml chloroform/phenol, 1:2 (v:w) were added to samples. The samples were vortexed for 2 minutes and centrifuge for 10 minutes at 14,000 rpm. The aqueous solution (upper layer) was removed and the bottom organic layer was discarded. 0.8 ml of the buffer (17.2 mM TEA and 200 mM HFIP, pH 8.5) was added to the samples. Thereafter, the mixed samples were extracted on a Waters (Milford, MA, USA) Oasis HLB Cartridge packed with 10 mg of stationary phase material.

The extraction tubes were conditioned first with 1 ml acetonitrile followed by 2 ml of the buffer (8.6 mM TEA and 100 mM HFIP, pH 8.5). Then the mixed samples were loaded and the SPE bed was washed with 0.3 ml of the buffer (8.6 mM TEA and 100 mM HFIP,PH=8.5) followed by 0.5 ml 100mM TEAB. Samples were eluted with 0.5 ml of methanol: 100mM TEA (60:40, v:v) and the eluant was evaporated to **almost** (approximately 2-3ul) dryness under a stream of nitrogen at 30□. Then 0.1ml of reconstituted solution, MeOH: 7mM TEA, 10:90 (v:v), was added and 40 ul aliquot was injected into the LC/MS/MS system.

Method Validation

Plasma calibration curves were constructed using the peak area ratios of PF-ODN to that of I.S., and applying a weighted ($1/x^2$) least squares linear regression

analysis. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples (5, 15, 150 and 1500 ng/ml). Five replicates of each QC points were analyzed every day to determine the intra-day accuracy and precision. This process was repeated three times over three day in order to determine the inter-day accuracy and precision. Absolute and relative recoveries, and matrix effects were calculated for PF-ODN samples (n=5) at 5, 15, 150 and 1500 ng/ml and 250 ng/ml I.S. samples (n=5) in plasma.

Absolute recovery was calculated as the peak area for PF-ODN in plasma sample spiked before extraction divided by the peak area of the neat sample of the same concentration. Relative recovery was calculated by dividing the peak area for a PF-ODN sample spiked before extraction by peak area for an equal concentration PF-ODN sample in the same matrix spiked after extraction. Matrix effects were calculated by dividing the peak area for a PF-ODN sample in plasma spiked after extraction by an equal concentration PF-ODN sample[28]. Stability of freeze-thaw (two cycles), room temperature (8hrs) and autosampler (24hrs) (n=5) was observed.

RESULTS AND DISCUSSION

Reduction of Cation Adduction in Solution

The first challenge for analysis of ODNs is to obtain a sharp multiple charge

distribution electrospray mass spectrum and deconvolution to match the exact mass of free acid ODNs. ESI-MS of infusion of oligonucleotides without chromatography have been somewhat hampered by ubiquitous cation adduction due to the high affinity binding of Na^+ and K^+ to the polyanionic backbone of the oligonucleotide[22]. Greig and Griffey[23] have demonstrated that the addition of strong bases such as triethylamine (TEA) or piperidine significantly suppresses adduct formation, while dramatically increasing sensitivity for electrospray ionization. We have tried different infusion solution for PF-ODN (7.5 µg/ml) including MeOH: H_2O (50:50) and MeOH: 0.1% formic acid (50:50), both of which could not produce the clear multiple charge distribution. MeOH: 7mM TEA (50:50) infusion solution produced a multiple charge distribution but the peaks were observed to be wide and clustered because of extensive sodium adduction (see Figure 1A). Also minor changes in buffer composition or pH caused shifts in the multiple charge-state distribution peak intensities, necessitating optimization of the MS detection conditions to achieve the needed sensitivity.

The infusion solution of MeOH: Buffer (7mM TEA and 3mM ammonium formate), 50:50 (v:v) produced sharp multiple charge distribution peaks and greatly suppress the sodium adduction (Figure 1B). It is observed that TEA and NH_4^+ can displace the sodium ions from the PF-ODN and $HCOO^-$ reduces the charge state of oligonucleotide negative ions[29]. So, MeOH: buffer (7 mM TEA and 3mM ammonium formate), 50:50 (v:v) is reported as the ideal infusion solution for our model PF-ODN and other compounds with similar chemical structure. But

presence of ammonium formate in the infusion solution was observed to decrease the sensitivity of the peaks in the charge-state distribution and hence is not recommended for use in a chromatographic mobile phase. Also, the LC/MS full scan (400-800 amu) with Mobile Phase A: 50mM HFIP and 8.6mM TEA, B: methanol including 50mM HFIP and 8.6mM TEA produced the sharper multiple charge state envelope (Figure 1. C) even though it still showed significant cation adduction.

Development of a Chromatographic System

Optimizing buffer composition

To get the most sensitivity and selective assay, concentration and nature of ion pairing agents used in mobile phase was found to be important. A mobile phase containing (1) 50 mM TEA, (2) 50 mM TEAB and led to wide peaks and low MS sensitivity upon ESI source optimization. The TEA/HFIP buffer system containing 1.7 mM TEA and 100mM HFIP, pH 7.5 performed much better displaying high sensitivity and good peak shape. It is believed that TEA ion pairs to the negatively charged phosphate groups of the oligonucleotides, which promotes retention and good peak shape. The HFIP aids desolvation of the solvent droplet, as it is travels through the ESI-MS source. Also during such desolvation the pH of the solvent droplet is reported to increase from 7.5 to 10 due to the evaporation of the more volatile HFIP, which leaves the more basic TEA-oligonucleotide complex in the droplet. The reduces the presence of other cation adducts thereby increasing

intensity of the unadducted analyte peaks monitored in the assay[22,26].

Analyte affinity/binding to HPLC column

It has been reported that, the exposed silanol groups on the reverse phase C18 column can irreversibly absorb the oligonucleotides[30]. Effect of particle size, column length, mobile phase flow-rate, and separation temperature on the resolution and sensitivity of oligonucleotides has also been reported[31]. In our experiments, C18 columns of different companies showed different sensitivity and peak shape for oligonucleotides, especially at the low concentration possibly due to different carbon load in these columns. The columns evaluated include Thermo Hypersil GOLD C18, Phenomenex Luna C18, Waters XTerra C18, and Varian Polaris C18-A columns. Among these columns Hypersil GOLD C18 column (50×2.1 mm, 1.9 µm particle) was found to provide the highest sensitivity, capacity and reproducibility for PF-ODN. It is important to note that preconditioning of the new column with PF-ODN (1 µg/ml) was found beneficial in improving chromatographic reproducibility. Once the new column was preconditioned, the column was found to be stable for more than 500 injections with reproducible peak response for low concentrations. In addition a low flow rate of 0.15 ml/min was found to significantly to improving assay sensitivity on a Sciex API-3000 mass spectrometer.

Non-specific Binding to Containers

Overcoming non-specific binding to storage containers (including injection inserts) at low concentration was found to be challenging for PF-ODN. The analyte peak response reduced rapidly (within 2-4 hours) in the polypropelene 96-well injection plates and in glass vial inserts for low concentrations (Figure 2 A). This problem was solved by using siliconized glass containers and autosampler vial inserts (Figure 2B). The non-specific binding problem was negligible for analyte in methanol:7 mM TEA, 1:9 (v:v) and peak responses were stable for the successive injections from the same container at low concentration for up to 12 hrs. The levels of analyte showed a downward trend, but still acceptable when compared to data from unsiliconized glass containers, when analyte was dissolved in water for upto 4 hours.

Optimizing MRM Conditions

The major product ions of most of the multiple charge state peaks of PF-ODN are at 95 (phosphate group), 125, and 319 Da. The signal from m/z 641→319 transition was observed to be the most sensitive transition for API 3000 instrument but the most response for API 5000 instrument was the transition m/z 641→95. Multiple charge state of IS also produced the same product ions as PF-ODN under MRM mode. In order to reduce possibility of cross talk between acquisition channels, the analytical transition for the IS also being m/z 811→319, baseline chromatographic separation between the PF-ODN and IS was accomplished.

Strategies for Recovery of PF-ODN from Rat Plasma

Oligonucleotides are highly bound to plasma protein, therefore, proper sample preparation is critical achieve acceptable recovery and reproducibility. Direct SPE method using different cartridges such as Waters Oasis MAX, Waters Oasis MCX, Waters Oasis HLB and Varian C_{18} were attempted. The analyte recoveries using these columns were found to be lower than 10%. Direct protein precipitation methods (using acetonitrile or methanol) generally resulted in poor recovery (5-10%). Combining protein precipitation and SPE methods also proved ineffective with recoveries lower than 30% and with poor reproducibility. Here we report a unique combination of phenol/chloroform liquid-liquid extraction and SPE method that provides high recovery (~ 70%). Details of the exact method can be found in the Experimental section.

Rationale for the steps in the extraction method:

The rationale for the success of this extraction method is presented below: The ammonia solution added to matrix sample helps maintain a basic condition and retains the drug in aqueous phase. Phenol is weak acid and when used in high concentrations aids displacing PF-ODN from plasma protein binding sites. The presence of chloroform further helps protein precipitation and release of analyte into aqueous phase. Unfortunately, it is believed that these step do not fully remove matrix related organic impurity from the sample. The SPE step helps with this in addition to removing residual phenol and buffer salts present in the sample.

SPE also helps pre-concentrate the plasma sample to improve assay sensitivity. Oasis HLB cartridges are made out of polymeric material and are not based on a silica support, which reduces adsorption of ODNs at low concentrations during sample extraction. Hence, it provides high recovery and reproducibility for oligonucleotides as reported in literature[17].

Stabilizing the analyte during evaporation to near dryness:

Shishkina *et. al.*[32] reported that the abasic sites in oligonucleotides are completely degraded during evaporation to dryness. Our experiments supported this observation and the elution solvent was found to be very important for the stabilizing PF-ODN during evaporation to near dryness. Different elution solution containing 100 mM TEAB, 100 mM TEAA and 17.2mM TEA/200 mM HFIP were attempted during SPE. It was found that in elution solvents acetonitrile: 8.6 mM TEA/100mM HFIP, 60:40 (v:v) and acetonitrile: water, 60:40 (v:v), PF-ODN was found to be undetectable after evaporation and reconstitution in mobile phase. Using acetonitrile : 100mM TEA, 60:40 (v:v) as SPE elution solvent, the evaporation to “almost” dry (~2-3ul) and reconsititution seemed to retain analyte stability. Our lab is currently investigating why this particular solvent is effective in stabilizing oligonucleotides during evaporation.

Assay Validation Results

Representative chromatograms of a blank plasma sample, a blank plasma sample spiked with PF-ODN at the LLOQ (5ng/ml) and I.S. are shown in Figure 3. No interferences from endogenous substances with analyte or I.S. were detected. Although I.S. has cross-talk in PF-ODN MRM channel, there is no interference due to baseline resolution of PF-ODN and IS.

Correlation coefficients of triplicate calibration curves were above 0.99. The calibration curves were linear over the concentration ranges 5.0-2000ng/ml The lower limit of quantitation (LLOQ), defined as the lowest concentration of analyte with accuracy within ±15% and a precision <15%, was 5 ng/ml for determination of PF-ODN in rat plasma. The signal over noise ratio > 15 at LLOQ (5 ng/ml) was observed. The absolute recoveries of PF-ODN at concentrations of 5.0, 15, 150, 1500ng/ml (n=5) were in the range of 72.4-85.6% and relative recoveries in the range of 69.5-82.7% (shown in Table 1). The intra-day (n=5) precision and accuracy for PF-ODN (spiked concentrations: 5, 15, 150 and 1500ng/ml) were in the range of 4.30-7.60% (%RSD) and 4.7-11.4% (%Error), respectively. The inter-day (n=15) precision and accuracy for PF-ODN ranged from 7.33-10.1% (%RSD.) and from 6.2-11.0% (%Error), respectively. These intra- and inter-day precision and accuracy data are shown in Table 2. Calculated matrix effect values (ion suppression or enhancement) were low (0.6-6.1%). This proved that the sample preparation procedure provided very clean extract solution.

The assay indicated stability of samples in matrix at -20 degree C (4 days) of two freeze-thaw cycles, bench top (8 hours) and processed samples at room temperature (24 hours) of PF-ODN at 15 and 150 ng/ml in plasma. This data is shown in Table 3.

Selectivity of the Assay

Different lots of rat plasma were extracted and analyzed using the LC/MS/MS assay detailed in the experimental section. The matrix control samples showed no significant peak in either the analyte or the internal standard MRM channels, indicationthat the method is highly selective. Also four key 3' and 5' deletion metabolites of PF-ODN were injected along with the parent drug and internal standard using the above method (see Figure 5). All the metabolites were found to be baseline resolved from PF-ODN. Even though some of the metabolites coeluted with the internal standard, it was found that at the concentrations of the IS used in the assay, there was no observed interference between the IS and metabolites that were tested.

CONCLUSIONS

We have successfully developed and reported an LC/MS/MS assay (5-2000 ng/ml dynamic range) for quantitation of an oligonucleotide from plasma using traditional LC/MS/MS approaches. This paper also provides future biochemist and analytical

scientists with guidance on what challenges to expect when they attempt such approaches for their class of compounds (see Figure 6). Ligand binding biological assays have distinct advantage of sensitivity and lack of need for sample extraction/purification. Despite this, the difficulty in developing the biological assays combined with their lack of assay selectivity should encourage scientists to consider developing mass spectrometry based assays. We believe that this work will is the first step to considering chemical assays for quantitating antisense-DNA, RNAi, phosphorylated proteins from biological matrices.

We have shown in this paper that extensive adduction with ubiquitous cation and limited ionization efficiency of oligonucleotides, though challenging, can be overcome. A novel sample preparation scheme combining phenol/chloroform liquid-liquid extraction and SPE demonstrated good recovery for our test oligonucleotide. Chromatographic schemes using unique buffer and HPLC column combinations were demonstrated. Though our assay does not possess the sensitivity required to measure the elimination phase of the drug in clinically relevant doses, it is believed that by using higher sample volume during extraction and by combining multiple MRM transitions (from many the peaks in the charge state distribution) the sensitivity can be enhanced. Preliminary work does indicate possibility of developing quantitative LC/MS/MS assay at concentrations a order of magnitude lower than what is reported here, using the above approach. Stability assessment of PF-ODN in rat plasma through freeze thaw (2 cycles), bench top (8

hrs) and autosampler stability (24hrs) were found to be acceptable. Future work in our lab will include evaluation of polymer columns high flowrate monolithic approaches and utilization of automated online sample extractions.

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FIGURES

Figure 1(A) Infusion of PF-ODN, 7.5 µg/ml in buffer (0.07mM TEA and 0.5mM HFIP) : methanol=50:50 (peaks cluster and broaden, a lot of Na adducts);

(B) Infusion of PF-ODN, 7.5 µg/ml in buffer (7mM TEA and 3mM ammonium formate) : methanol, 50:50 (sodium adducts suppression and sharp peaks)

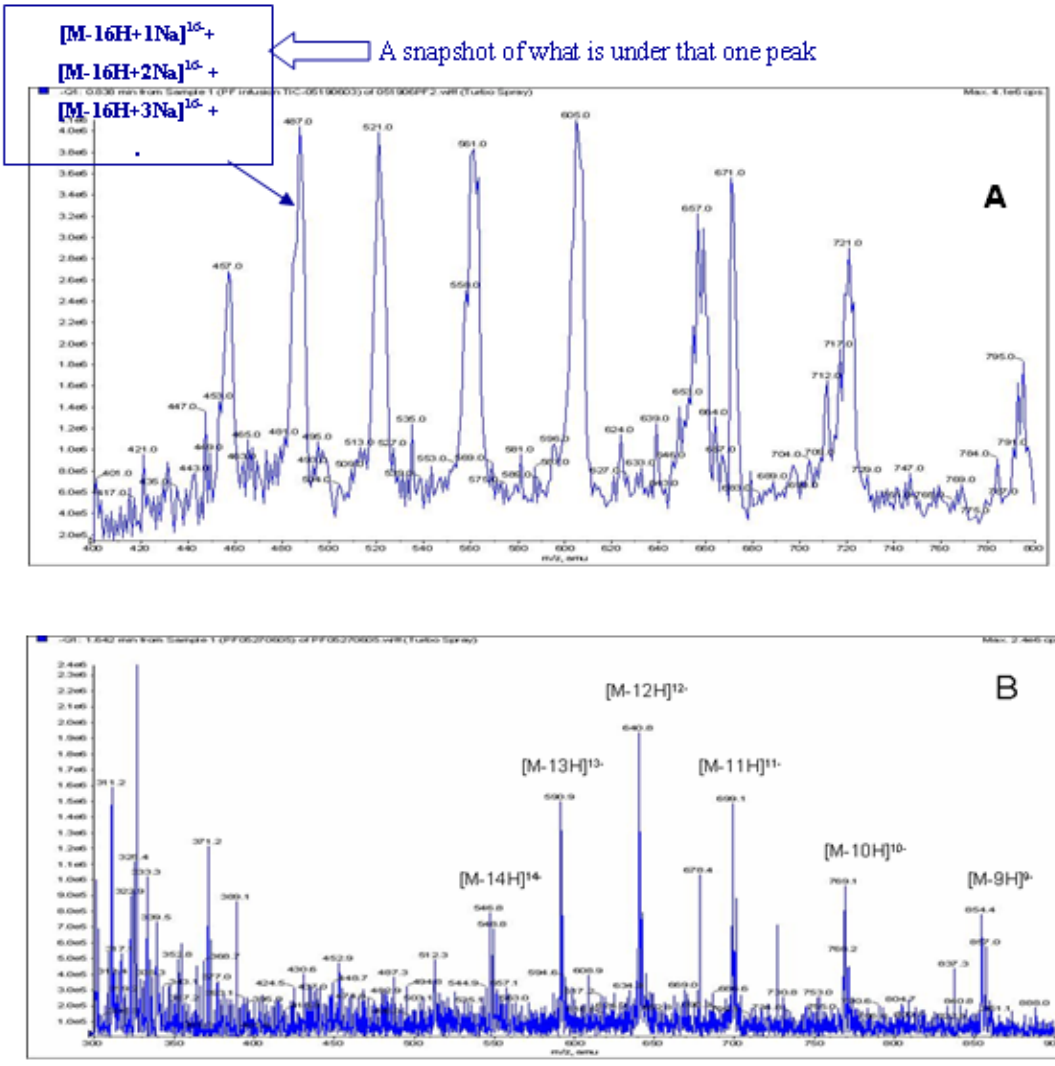


Figure 1(C) LC-ESI-MS scan from m/z 400-800 amu, Waters XTerra MS C18, 50×2.0mm, 5µm, Mobile Phase A: 50mM HFIP and 8.6mM TEA, B: methanol including 50mM HFIP and 8.6mM TEA. Gradient starts at 20% B and at 10 min it reaches 50%, flow rate: 0.3ml/min.

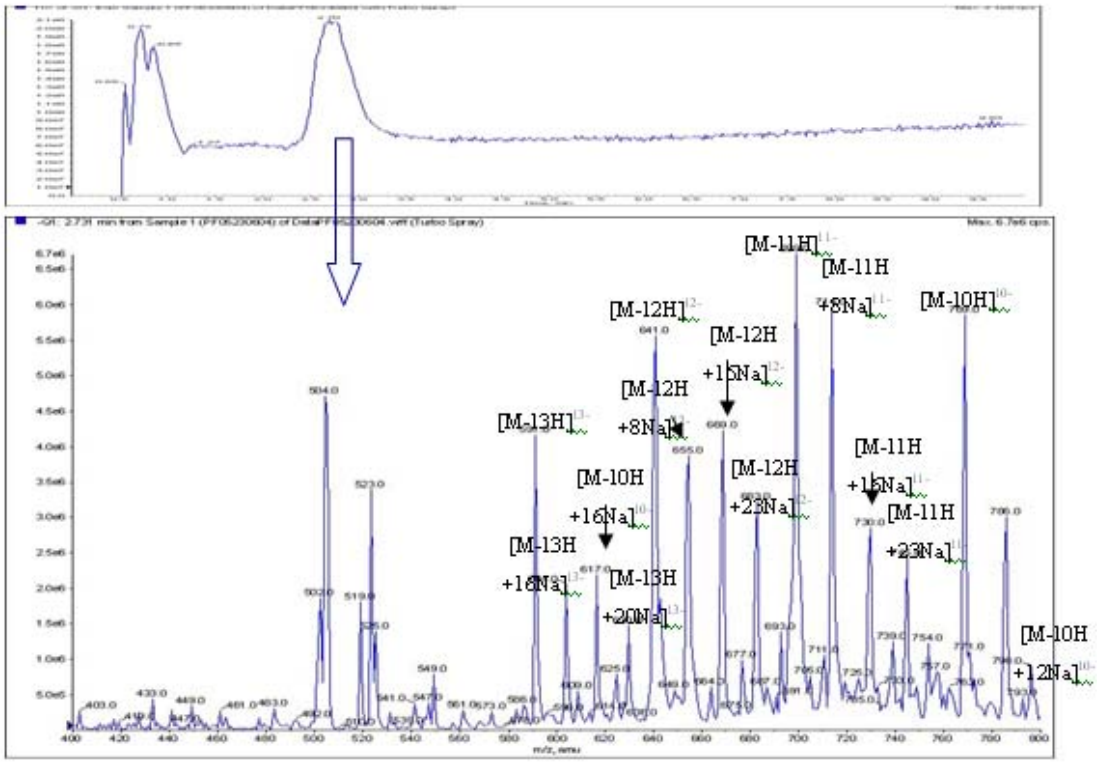


Figure 2. Non-specific binding experiments; Waters XTerra MS C18, 50×2.0mm, 5µm, Mobile phase A: 50mM HFIP and 8.6mM TEA in water; B: methanol including 50mM HFIP and 8.6mM TEA. Gradient starts at 10% B and at 10 min it reaches 40%, flow rate: 0.3ml/min.

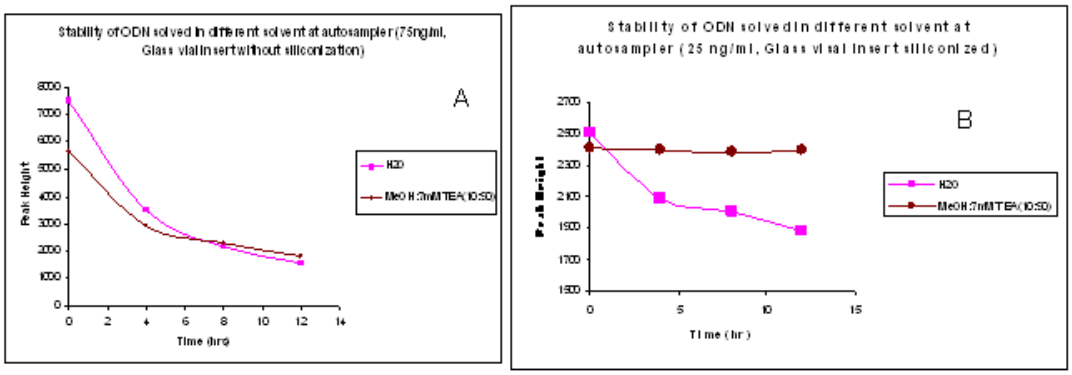


Figure 3. Chromatograms of LLOQ (5 ng/ml) and blank plasma by ion pair LC/MS/MS.

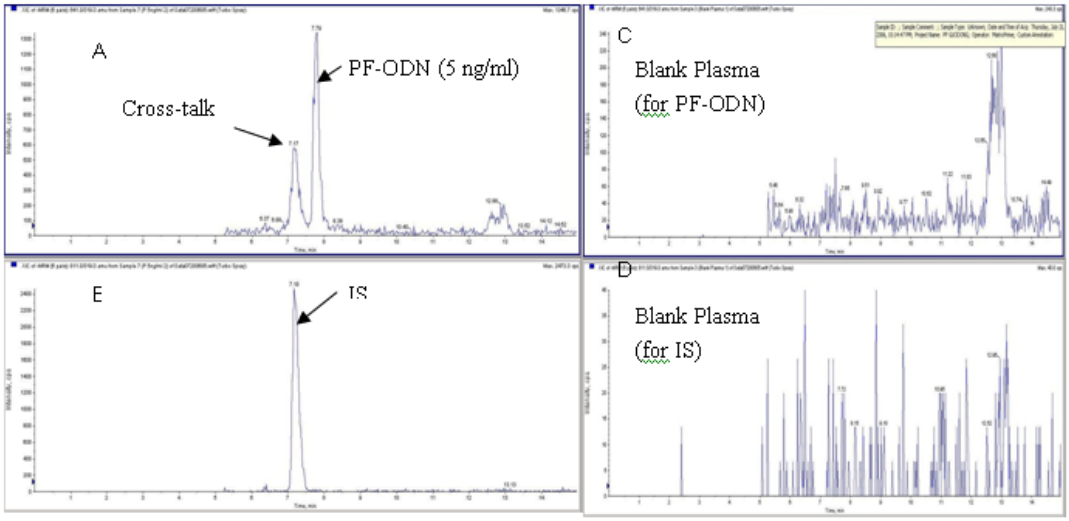


Figure 4. Nucleotide sequences of PF-ODN and IS. Also basic structural elements of a phosphorothioate backbone oligodeoxynucleotide are shown.

SEQUENCES: PF-ODN 5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'

IS 5'-TCGTGGTTAGCCGGCCGTTCGGC-3'

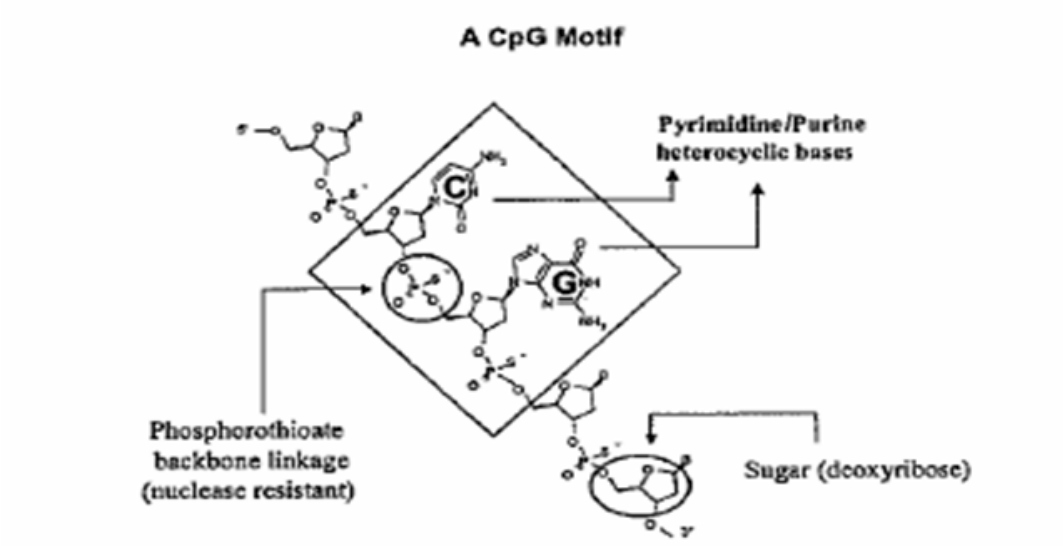
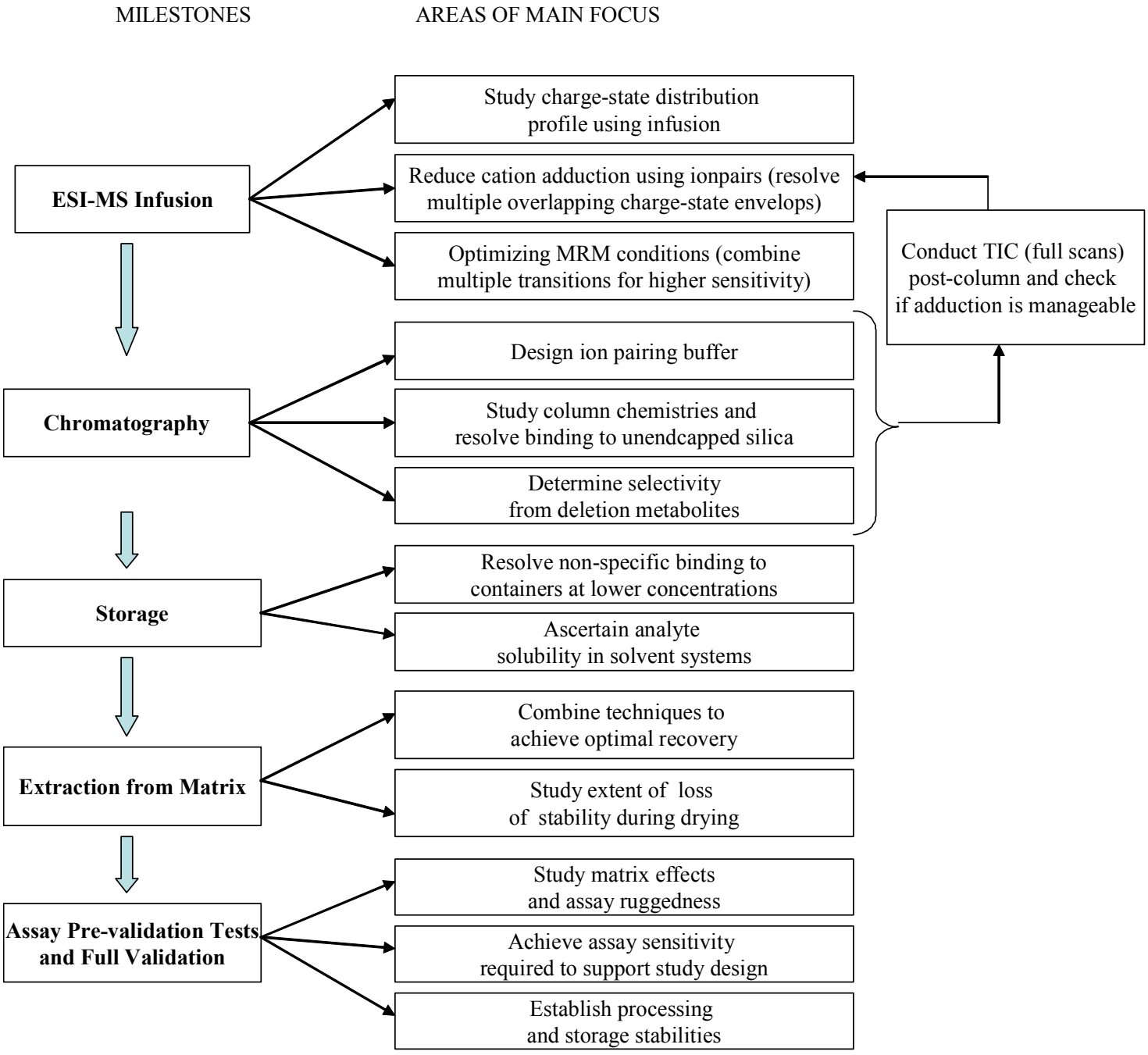


Figure 5. Flow chart describes the recommended strategy (in chronological sequence) for developing mass spectrometry based assays for oligonucleotide class compounds, including the major challenges/milestones that may have to be achieved for successful outcomes.



TABLES

Table 1. Absolute recovery, relative recovery (%), and matrix effects in rat plasma (n=5)

Concentration (ng/ml)	Absolute Recovery	Relative Recovery	Matrix Effect (%)	Type of Effect
5	85.6±4.73	82.71 ±4.57	103.5	3.5% enhancement
15	72.4±2.07	69.5 ±1.99	104.1	4.1% enhancement
150	76.2 ±2.69	81.1 ±2.86	93.9	6.1% suppression
1500	73.5±1.62	78.1 ±1.72	94.2	5.8% suppression
IS (250ng/ml)	67.2 ±5.91	66.8 ±5.87	100.6	0.6% enhancement

Table 2. Precision (%CV) and Accuracy (% Error)

Intra-day (n=5)				Inter-day (n=15, 3 days)		
Conc. (ng/ml)	Observed conc. (ng/ml)	R.S.D. (%)	Error (%)	Observed conc. (ng/ml)	R.S.D (%)	Error (%)
5	5.03±0.38	7.60	6.2	4.93±0.50	10.09	6.2
15	15.45±0.96	6.23	6.1	16.13±1.18	7.33	9.3
150	156.02±6.71	4.30	4.7	162.87±13.39	8.22	9.2
1500	1329±86.48	6.51	11.4	1338.6±114.3	8.54	11.0

Table 3. Stability of freeze-thaw cycle, bench top and autosampler (n=5)

	Free-thaw stability (two cycles)		Room temperature stability (8hrs)		Autosampler stability (24hrs)	
Nominal conc. (ng/ml)	15	150	15	150	15	150
Observed conc. (ng/ml)	14.7±0.98	163.2±20.3	16.6±1.5	158.8±8.5	13.3±0.80	155.0±10.1
(%) Accuracy	97.9	108.8	110.7	105.8	88.4	103.3
(%) R.S.D.	6.7	12.4	9.5	5.4	6.0	6.5