DEVELOPMENT OF AN LC-MS METHOD FOR URSOLIC ACID AND ITS PRODRUG FROM PLASMA

by

YI CHANG JEON

(Under the Direction of Michael G. Bartlett)

ABSTRACT

Ursolic acid is a pentacyclic triterpenoid that has anti-cancer activities. Oral administration of ursolic acid is difficult because of its low bioavailability. Therefore, a prodrug of ursolic acid has been synthesized through esterification to increase its bioavailability. Ursolic acid and its prodrug were studied to sensitize bladder cancer cells that are made resistant to conventional chemotherapy drugs like gemcitabine and cisplatin. In this study, an LC-MS method was developed to accurately quantify ursolic acid and its prodrug in rat plasma as part of a pharmacokinetic study.

INDEX WORDS: Ursolic acid, Prodrug, Bladder cancer, UPLC, Mass spectrometry, LC-MS, LC-MS/MS

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DEDICATION

I wholeheartedly dedicate my thesis work to my father and mother, who made countless sacrifices to raise my older sister and me in the United States in hopes to provide us with better education and life.

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CHAPTER 1

LITERATURE REVIEW

Bladder Cancer

The National Institutes of Health estimates that there will be more than 80,000 new cases of bladder cancer by the end of 2022 in the United States. Deaths caused by bladder cancer are more prevalent in men, accounting for almost 75% of all bladder cancer cases (*Key Statistics for Bladder Cancer*, 2022). However, women often present with more advanced stages of bladder cancer and have lower survival rates than men (Dobruch et al., 2016). Not only is there a disparity in the prevalence of bladder cancer between sex, but there is also a disparity between race; the presence of bladder cancer among white males is twice that of black males (*Cancer Facts & Figures 2022*, 2022). Age also plays an important role. People 55 years or older constitute 90% of bladder cancer cases and on average are diagnosed with bladder cancer when they are 73 years old (*Key Statistics for Bladder Cancer*, 2022). Risk of bladder cancer can be reduced by maintaining a healthy weight and minimizing or quitting the use of smoking tobacco and alcohol (Dobruch & Oszczudłowski, 2021). Quitting smoking is especially beneficial as carcinogens in tobacco smoke were responsible for nearly half (47%) of all bladder cancer cases in the United States (*Cancer Facts & Figures 2022*, 2022).

Urothelial carcinoma, cancer in the urothelial cells of the bladder, comprises approximately 90 to 95% of all bladder cancer cases (Martin et al., 2016). The other 5 to 10% are non-urothelial bladder cancer types and primarily consist of squamous cell carcinoma (2%), adenocarcinoma (1%), and small cell carcinoma (<1%) (Chalasani et al.,

2009). Urothelial carcinoma can be further separated into non-muscle-invasive bladder cancer or muscle-invasive bladder cancer (Woldu et al., 2017).

Common tests to diagnose and confirm the presence of non-muscle-invasive bladder cancer are cystoscopy, urinary cytology, urine-based biomarkers, and transurethral resection of the bladder tumor. Although cystoscopy is an effective diagnosis tool for bladder cancer, there are also drawbacks. Since the medical procedure require the insertion of a cystoscope into the urethra to detect cancer cells inside the bladder, cystoscopy is considered an invasive method and can cause patients significant discomfort (Łaszkiewicz et al., 2021). Severe side effects include burning sensation during urination, hematuria, and blood clots (Matulewicz et al., 2017).

A non-invasive alternative to cystoscopy is urinary cytology. Cells collected from a urine sample are examined under a microscope for the presence of bladder cancer cells, but it is not as effective as cystoscopy. It has relatively high sensitivity for high-grade urothelial carcinoma (84%), but severely lacks the sensitivity for low-grade urothelial carcinoma (16%) (Yafi et al., 2015). Therefore, urinary cytology is most effective when used as a supplement to cystoscopy.

In the past few years, FDA-approved urine-based biomarkers have become commercially available. Although sensitivity and specificity vary depending on the biomarker, they typically offer higher sensitivity, but lower specificity than urinary cytology (Oliveira et al., 2020). A study comparing ImmunoCyt test to urinary cytology found that the sensitivity of the test and urinary cytology was 84 and 44% respectively and 75 and 96% for specificity (Mowatt et al., 2010). In the near future, urine-based biomarkers may potentially replace urinary cytology (Schmitz-Dräger et al., 2008).

Transurethral resection of bladder tumor (TURBT) can function not only as a diagnosis tool, but also as a treatment for early stages of bladder cancer. In TURBT, a resectoscope is inserted into the urethra either under a general or regional anesthesia to remove abnormal tissue samples detected inside the bladder for further testing (*Bladder Cancer Surgery*, 2019).

Diagnosis results are often organized by stages which are differentiated by the progression of the disease. Stage 0 describes bladder cancer that is only present in the transitional epithelium, also known as urothelium (*Treatment of Bladder Cancer, by Stage*, 2021). When bladder cancer grows towards the hollow center of a bladder, it is referred to as non-invasive papillary carcinoma (Ta), or stage 0a. If bladder cancer grows in the opposite direction deeper into transitional epithelium, it is called flat non-invasive carcinoma (Tis), or stage 0is (*Treatment of Bladder Cancer, by Stage*, 2021; *What Is Bladder Cancer?*, 2019).

Both stage 0a and 0is are first treated with TURBT in combination of fulguration, which uses heat generated from electric current to eradicate tumors (*NCI Dictionaries*; *Treatment of Bladder Cancer, by Stage*, 2021). Next steps in treatment vary depending on the grade and the type of stage 0 tumor. Intravesical chemotherapy is typically performed several weeks after TURBT for a low-grade Ta tumor (Porten et al., 2015). As for high-grade Ta tumors and Tis tumors, intravesical Bacillus Calmette-Guérin is performed within 24 hours of TURBT (*Treatment of Bladder Cancer, by Stage*, 2021).

Bacillus Calmette-Guérin immunotherapy, or BCG was originally used to prevent tuberculosis and had been used since the 1970s for bladder cancer (Lobo et al., 2021). Lamm et al. reported that patients who received BCG immunotherapy had lower rates of

tumor recurrence than patients that received only surgical therapy (17% vs 42%) (Lamm et al., 1980). Using BCG has also shown to lower the chance of tumor recurrence more than mitomycin C, a chemotherapeutic agent (Böhle et al., 2003; Malmström et al., 2009; Shelley et al., 2004).

If bladder cancer spreads to the lamina propria of the bladder, it is classified as stage I, or stage T1 (Yun et al., 2016). TURBT is still given as the initial treatment and a second one a month later whether it is a low-grade or high-grade T1 bladder cancer. (Nepple & O'Donnell, 2009). Afterwards, urologists must choose between intravesical BCG and cystectomy depending on the severity of the stage T1 bladder cancer.

Intravesical BCG is ideal for low-grade T1 bladder cancer even though the recurrence rate after intravesical BCG is 20% to 40% among patients with non-muscle-invasive bladder cancer because the risk that the cancer will progress farther is low (de Jong Florus et al., 2021). Although intravesical BCG can also be used for high-grade T1 bladder cancer, its use can be risky. van der Meijden et al. reported 20% of patients had to stop intravesical BCG therapy due to side effects such as cystitis, hematuria, general malaise, skin rash, and fever (van der Meijden et al., 2003). Time spent on intravesical BCG could have been used to perform radical cystectomy from the very start and lower the risk of high-grade T1 bladder cancer progression (Bochner, 2009).

Radical cystectomy procedures differ slightly by sex, but in general involve the removal of the bladder. The prostate is removed in men while reproductive organs are removed for women (Aminoltejari & Black, 2020). Because of the removal of the bladder, urinary diversion is required to redirect the flow of urine and the options range from ureterosigmoidostomy, conduits, continent cutaneous diversions, and orthotopic

neobladders (Lee et al., 2014). As radical cystectomy is a life-altering surgery, careful considerations must be made beforehand.

Bladder cancer is classified as stage II once the cancer has breached the muscle tissue of the bladder (*NCI Dictionaries*). Stage II is separated into T2a and T2b based on the severity of the breach. Survival rate for T2a is higher than T2b 5 years after treatment (70.6% compared to 65%) because the cancer hasn't spread farther into the muscle layers (Boudreaux Kelly et al., 2009). Bladder cancer is beyond stage II if cancer has already spread to the paravesical fatty tissue and/or to adjacent tissues, organs, or lymph nodes (16).

From stage II and onwards, TURBT becomes ineffective as a treatment procedure and is only used as a diagnosis tool (Richterstetter et al., 2012). Instead, radical cystectomy, radiation, and chemotherapy are the suggested treatment procedures. Clinical trials have shown previously that the combination of chemotherapy and radiotherapy instead of solely chemotherapy after radical cystectomy increased the locoregional recurrence-free survival rate from 69% to 96% (Zaghloul et al., 2018).

Chemotherapy drugs are often given in combinations. Common combinations of chemotherapy drugs include: 1) gemcitabine and cisplatin (GC) and 2) methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC). GC has slightly lower survival rate than MVAC at 13% compared to 15.3% based on a clinical trial conducted on patients with locally advanced or metastatic transitional cell carcinoma of the urothelium (von der Maase et al., 2005). However, GC is preferred because it has lower toxicity than MVAC. MVAC was associated with higher cases of grade 3 and 4 mucositis (22% compared to 1%), grade 3 and 4 neutropenia (82% compared to 71%), neutropenic fever (14%

compared to 2%), neutropenic sepsis (12% compared to 1%), and alopecia (55% compared to 11%) (von der Maase et al., 2000).

Unfortunately, subclonal mutation of bladder cancer cells with chemotherapy resistance can proliferate and cause conventional chemotherapeutic drugs to be ineffective (Jamal-Hanjani et al., 2015). A heavily expressed gene in bladder cancer called GHET1 upregulates the ABCC1 gene to induce gemcitabine resistance (Li et al., 2019). Xiong et al. has shown that the KNSTRN gene activates AKT phosphorylation, which in turn inhibits FOXO1 protein expression to inhibit gemcitabine chemosensitivity (Xiong et al., 2021).

Given that the median survival rate for patients with stage IV bladder cancer treated with chemotherapy is only around 13 months (Flannery et al., 2018), it is vital to search for ways to make chemotherapy more effective. One potential solution would be to resensitize bladder cancer cells to chemotherapy using ursolic acid.

Ursolic Acid

Triterpenoids describe compounds that consist of 30 carbon atoms organized into 6 isoprene units that are more abundantly present in dicotyledonous plants (Şoica et al., 2021). Triterpenoids can be classified by the number of rings in their chemical structures, which can range from zero to five rings (Du et al., 2014). Two principal triterpenoid groups, tetracyclic and pentacyclic, are biosynthesized by the cyclization of the linear triterpene, squalene (Du et al., 2014; Ghante & Jamkhande, 2019). The two groups can be further divided by their derivatives such as dammarane and euphane groups for tetracyclic derivatives and ursane, oleanane, and lupine groups for pentacyclic derivatives (Şoica et al., 2021).

Recently, research into triterpenoids has exponentially grown as triterpenoids have various biological activities such as, but not limited to, anti-diabetic, hepaprotective, cardioprotective, neuroprotective, anti-obesity, anti-inflammatory, antioxidative, anti-osteoarthritic, antimicrobial, antiparasitic, and most importantly, anti-cancer activities (Nguyen et al., 2021; Olech et al., 2021). Triterpenoids, particularly ursolic acid, have been investigated as alternative cancer treatments to chemotherapy and radiotherapy (Chudzik et al., 2015).

Ursolic acid is a pentacyclic triterpenoid with a molecular formula of $C_{30}H_{48}O_3$ and a molecular weight of 456.7 g/mol (NCBI, 2022). First identified in the 1920s, ursolic acid is detected in various fruits, vegetables, and herbs, but is most commonly found in epicuticular waxes, especially in apple fruit peels (Luo et al., 2017). Other natural sources of ursolic acid include, but are not limited to, rosemary leaves, coffee leaves, sage leaves, oleander leaves, lavender leaves, eucalyptus leaves, and thyme leaves (Jäger et al., 2009).

Like other triterpenoids, ursolic acid also presents various biological activities. Ursolic acid shows anti-diabetic properties by acting as an insulin receptor activator when studied in Chinese-hamster ovary cells expressing human IR cells and adipocytes (Jung et al., 2007). It also shows anti-obesity properties by increasing β-oxidation and the uptake of skeletal muscle free fatty acid (Chu et al., 2015). Most importantly, ursolic acid shows anti-cancer effects for many different cancer types. The list includes skin cancer, colon cancer, breast cancer, bladder cancer, cervical cancer, pancreatic cancer, ovarian cancer, and liver cancer. Seo et al. have shown that ursolic acid inhibits tumorigenesis and the proliferation of cancer cells and promotes autophagy (Seo et al., 2018).

Ursolic acid can be used to sensitize cancer cells that become resistant to chemotherapy. Colon cells under hypoxia were sensitized using ursolic acid by inhibiting HIF-1α and therefore downregulated the multidrug resistance gene 1 (Shan et al., 2016). For pancreatic cancer, combining ursolic acid with gemcitabine suppressed MicroRNA-29a, which is responsible for gemcitabine resistance in pancreatic cancer cells, by 68% (Prasad et al., 2016). Ursolic acid can be effective in sensitizing cells resistant to not only chemotherapy drugs, but also for other anti-cancer therapeutics. In triple-negative breast cancer, ursolic acid upregulated death receptors 4 and 5 used by anti-cancer therapeutic, recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL), to induce apoptosis and downregulate the anti-apoptotic protein caspase 8 inhibitor (c-FLIP$_L$) (Manouchehri & Kalafatis, 2018). Ursolic acid also has sensitizing effects on liver cancer cells, particularly the cell line HepG2/DDP, which is a cisplatin-resistant hepatocellular carcinoma cell line. Combinations of ursolic acid and cisplatin have been shown to lower the expression of NF-E2-related factor (Nrf2) and its substrates, HO-1, NQO1, and GST (Wu et al., 2016). Inhibiting Nrf2 is critical because of its role in enhancing resistance in cancer cells to chemotherapeutic drugs like cisplatin, doxorubicin, and etoposide when upregulated (Wang et al., 2008).

Only a few studies focus on ursolic acid and its application to bladder cancer in the past two decades. One study shows that ursolic acid decreases cell proliferation of T24 bladder cancer cells by suppressing the Akt/NF-κB pathway to promote apoptosis (Gai et al., 2013). T24 bladder cancer cells dosed with 50 μmol/L of ursolic acid had cell proliferation decrease nearly 70% when compared to the control group by downregulating anti-apoptotic NF-κBp65 and B-cell lymphoma 2 (Bcl-2), while upregulating pro-apoptotic

caspase-3 (Gai et al., 2013). Besides the Akt/NF-κB pathway, ursolic acid also promotes apoptosis by activating c-Jun N-terminal kinase (JNK) signaling either through AMPK or ER stress responses (Zheng et al., 2013).

A few ursolic acid derivatives have been studied in the past for the inhibition of bladder cancer. One ursolic acid derivative that has been synthesized is isopropyl 3*β*-hydroxyurs-12-en-28-oat (UA 17). Combinations of UA 17 with cisplatin induced apoptosis by upregulating the phosphorylated state of p38 mitogen-activated protein kinase (p-p38) and downregulating Bcl-2 (Lin et al., 2014).

Prodrug

One reason for the lack of ursolic acid studies on bladder cancer may be due to its intrinsic properties. Ursolic acid is a BCS class IV drug, meaning it has low solubility and permeability; therefore ursolic acid has very low oral bioavailability (Ren et al., 2021). Oral delivery of drugs is generally recommended because of high patient compliance and cost-effectiveness, but it is particularly challenging to formulate for BCS class IV drugs like ursolic acid (Savjani et al., 2012). No matter how effective ursolic acid may be in treating bladder cancer, it would be of little use if it cannot enter the bloodstream.

One solution to increase the bioavailability of ursolic acid is to synthesize an ursolic acid derivative to function as a prodrug. Prodrugs refer to pharmacologically inactive drug substances that are converted to their pharmacologically active drug form either by metabolic or physicochemical reactions, or both (Wu, 2009). Because prodrugs can provide many benefits besides increasing bioavailability, such as site-specific targeting, decreased toxicity, and prevention of rapid drug metabolism, they make up around 10% of all marketed drugs in the past decade (Markovic et al., 2020; Najjar & Karaman, 2019).

In the case of ursolic acid, its prodrug must have better solubility and permeability than ursolic acid. This can be achieved by esterification of ursolic acid to attach a carbon chain and a methylpiperazine to the ursolic acid. The addition of the alkyl moiety will increase the lipophilicity of the overall compound, thus improving passive permeability (Markovic et al., 2020). The methylpiperazine moiety attached at the end of the carbon chain will increase the solubility since it already possesses high aqueous solubility (Vignaroli et al., 2013).

Quantitative Pharmaceutical Analysis

Two analytical techniques commonly paired together for identification and quantitation of drug compounds in plasma are liquid chromatography and mass spectrometry. Liquid chromatography's origin can be traced back to the early 1900s when a Russian botanist, Mikhail Semyonovish Tsvet, separated plant pigments by pouring the sample and petroleum ether into a glass tube filled with calcium carbonate and aluminia. The result was colored bands representing the different plant pigments (Sack, 2016). From its rudimentary beginning, liquid chromatography has evolved and presently is found in many different varieties such as, thin-layer chromatography, paper chromatography, column chromatography, and high performance liquid chromatography (Coskun, 2016). No matter the different types, all liquid chromatography has these components in common: 1) a liquid sample, 2) a liquid mobile phase, and 3) a stationary phase. In essence, a sample in solution is transported through a stationary phase with the help of a mobile phase. The constituents of the sample are then separated depending on their different affinities either towards the stationary phase, or the mobile phase.

Constituents with higher affinity towards the stationary phase will take longer to elute than constituents with higher affinity towards the mobile phase.

The most widely used form of liquid chromatography is high performance liquid chromatography. It differs from other types of liquid chromatography in that it utilizes pressure to move the mobile phase through the stationary phase. Because HPLC relies on pressure rather than gravity, flow rates of the mobile phase can therefore be manually altered. Basic components of HPLC consist of: 1) mobile phase reservoir, 2) degasser, 3) solvent delivery system, 4) sample injector, 5) HPLC column, and 6) detector. Before the mobile phase goes through the stationary phase, the HPLC column, it must first pass through the degasser and the solvent delivery system. The function of a degasser is to remove any dissolved gases from the mobile phase as outgassing can cause issues leading to pump failure, erratic flow rates, and undesired background peaks in chromatograms (Dolan, 2014). The pump, also known as, the solvent delivery system, is what generates the pressure to maintain a constant flow rate of mobile phase. Sample injectors put samples into the HPLC system before reaching the HPLC column. Modern HPLC instrument store samples in an autosampler, which automates the injection process and controls the sample environment. The HPLC column contains the stationary phase in an HPLC system, responsible for separating samples into their components. HPLC columns are often inserted into a column oven to maintain constant column temperature, as column temperature can positively affect pressure and the overall chromatogram. Higher column temperatures can lead to lower pressures and decreases in retention time, increasing column life and decreasing chromatographic runtimes (Li, 1999). After the constituents have eluted from the column, they enter the detector, where

they are shown as chromatogram peaks in workstations connected to the system. Common detectors paired with HPLC are the mass spectrometer, UV/VIS detector, photodiode array detector, fluorescence detector, and refractive index detector.

The maximum pressure that HPLC systems can typically handle is 5,800 psi. However, there is another liquid chromatography technique that can handle up to 15,000 psi. It is an upgraded version of HPLC called UPLC®, which stands for Ultra Performance Liquid Chromatography and was first developed in 2004 by an analytical laboratory instrument manufacturing company called Waters Corporation (Fountain, 2011). Since the name, UPLC®, is trademarked by Waters Corporation, other vendors started naming their instruments that are similar to UPLC® as UHPLC, or ultra-high performance liquid chromatography.

The need for an LC system that can manage higher pressure is to accommodate the use of columns with lower particle sizes. The van Deemter equation shows that lower particle size leads to lower height equivalent to a theoretical plate (HETP), increasing column efficiency (Nováková et al., 2006). Advantages of lower particle sizes are improved sensitivity, increased resolution, shorter run times, and therefore lower mobile phase consumption (DeStefano et al., 2014). However the tradeoff is that columns with lower particle size lead to higher back pressure which exceeds the maximum pressure that HPLC systems can handle (Xiang et al., 2006). With UPLC® capable of handling up to 15,000 psi, columns with particle sizes that are sub-2 µm can be safely used while HPLC can typically only handle particle sizes that are 3 to 5 µm.

Aside from particle sizes, there are many diverse types of columns used for different applications. Commonly used ones are: 1) normal-phase columns, 2) reversed-

phase columns, 3) ion-exchange columns, and 4) size exclusion columns. The primary difference between normal-phase and reversed-phase columns is the polarity of the associated stationary and mobile phases. In a normal-phase column, the stationary phase is polar while the mobile phase is non-polar, or at least less polar than the stationary phase. In a reversed-phase column, the stationary phase is non-polar whereas the mobile phase is polar. Non-polar constituents will be more strongly retained in a reversed-phase column and elute earlier in a normal-phase column.

Ion-exchange columns differ from both normal and reversed-phase columns in that they are based on electrical charges, not on polarity. In normal and reversed-phase columns, polar stationary phases retain polar analytes longer and non-polar stationary phases retain non-polar analytes longer. However, in an ion-exchange column, the opposite is true. An anion exchange column will have a positively charged stationary phase to attract and retain negatively charged analytes while cation exchange columns will have a negatively charged stationary phase to attract and retain positively charged analytes.

Size-exclusion columns, as the name suggests, separate molecules based on their size. Size-exclusion columns are packed with polymer beads with ranging pore sizes. Contrary to techniques like western blot, larger analytes elute earlier in size-exclusion chromatography because molecules that are larger than the pores of the particles cannot pass through them; therefore, they are excluded from the polymer beads and quickly pass through the column in between the particles.

Out of all the different types of columns, the reversed-phase column is the most widely used, making up for more than 90% of all HPLC and UPLC applications (Žuvela

et al., 2019). Among the reversed-phase LC columns, the most commonly used one is a C18 column (Zhang & Hu, 2010). C18 refers to the number of carbon atoms in chains attached to the silica bed of the column. Other linear alkylsilanes used for reversed-phase LC columns are C8 and C4, having 8 and 4 carbons, respectively. Since C18 column's carbon chains are longer than that of C8 and C4's, analytes, especially non-polar analytes, are more likely to interact with them. Therefore, non-polar analytes will be retained longer than they will on C8 and C4 columns.

Once the analytes are separated by the column in the LC system, they enter the detector of choice to be analyzed. As stated previously, the mass spectrometer is a popular option to be paired with an LC system. The basic components of a mass spectrometer include the sample inlet, ionization source, mass analyzer, and the ion detector. The sample inlet introduces the analytes separated by the LC system into the mass spectrometer where they are selectively ionized into anions or cations by the ionization source. Then the ionized molecules are accelerated into the mass analyzer to be separated based on their mass-to-charge ratios. Once separated, they are sent to the detector which converts the ions into electrical current measurable by a workstation (Siuzdak, 2004).

There are many different types of mass spectrometers based primarily on different ionization methods and different mass analyzers. Commonly used ionization methods are electron ionization (EI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), chemical ionization (CI), and matrix assisted laser desorption ionization (MALDI). The electron ionization method is more often used for GC-MS than in LC-MS because the electron ionization method requires analytes to already be in the gas phase.

Inside of an electron ionization chamber, an electron beam is created by running an electric current of 70 eV through a filament. Electrons are accelerated into an electron collector at the opposite end of the source. When neutral analytes in the gas phase are introduced into the ionization chamber and pass through the electron beam, the accelerated electrons eject one or more electrons from the analyte. Therefore, neutral analytes are turned into cations and often fragment due the excess energy transferred by the electron beam. Electron ionization is referred to as a hard ionization technique because large amounts of energy are used to ionize the analytes. Because of the many fragments electron ionization can produce, it is a good method to identify unknown compounds. However, analytes need to be 600 Da or lower because large molecules are difficult to vaporize.

Besides the hard ionization technique, there are also soft ionization techniques. These techniques use less energy to ionize analytes and therefore leads to lower quantities, but bigger fragments; CI, APCI, MALDI, and ESI are all considered soft ionization techniques. Chemical ionization is also a great technique to pair with a GC if the molecular ion is more of interest than the fragment ions. It works in similar ways as electron ionization, but instead of analytes encountering the electrons, reagent gases such as methane, ammonia, or isobutane are ionized instead. Ionized reagent gases react with a reagent gas that has yet to ionize to form a product that will transfer a proton to the analytes, ionizing them (Gates, 2014a). It is possible to have negative chemical ionization, but usually only for analytes with high electronegativity such as halogenated compounds.

Unlike EI and CI, MALDI is useful for nonvolatile compounds that have high molecular weights, such as biomolecules (oligonucleotides, peptides, proteins, and carbohydrates). MALDI MS is often used as a standalone, but over the past decade, MALDI has been paired with LC systems more often (Weidner & Falkenhagen, 2011). In MALDI, analytes are co-crystalized with a matrix compound in one of the many individual wells on a target plate. The ratio of analyte to matrix deposited onto the well can vary, but it is typically 1:1 (*Bruker Guide to MALDI Sample Preparation*, 2012). The matrix compound typically is a weak organic acid that has high UV absorbance. Once the analyte and matrix compound are co-crystalized, laser irradiation desorb and ionize the sample. The high matrix UV absorbance absorbs most of the laser radiation, which will vaporize the sample and protect the analytes from fragmentation.

Two ionization methods that are paired regularly with LC are APCI and ESI and they are both atmospheric pressure ionization techniques that nebulizes LC eluent. Ionization in electrospray ionization begins when the LC eluent goes through the inlet capillary, or the spray needle. High voltage is applied to the inlet capillary and a sample plate entrance to the mass analyzer. This supply of voltage (3 to 5 kV) results in an electric field gradient in the atmospheric pressure region. The charge of the voltage between the inlet capillary and sample plate determines whether the ions will be positively or negatively charged. Positively charged ions will gather at the tip of the inlet capillary to create a Taylor cone if the inlet capillary is positively charged while the sample plate is negatively charged. Negatively charged ions will be formed if the charges of the inlet capillary and the sample plate are flipped. Streams of droplets ejected from the tip of Taylor cone are nebulized and evaporated with the assistance of heated nitrogen gas in

the ion evaporation region to separate the solvent from the ionized analyte. Ionized analytes then pass through the sample plate towards the mass analyzer. Just like MALDI, ESI is useful for ionizing biological molecules with the benefit of not requiring a matrix. However, its tradeoffs are that it loses sensitivity when buffers are used, and nonpolar molecules are poorly ionized. The latter can be fixed by using atmospheric pressure chemical ionization technique (Pitt, 2009).

Unlike in ESI, eluent from the LC is not ionized as it passes through the inlet capillary in APCI. Instead, heat (120 to 550 °C) is applied near the end of the inlet capillary to facilitate the desolvation and vaporization of the eluent. Once the solvent and analyte are nebulized, solvent is ionized by a corona discharge at atmospheric pressure (760 torr). The atmospheric pressure increases the collision of ionized solvent and analyte, which in turn ionizes the analyte through proton transfer. The main difference between ESI and APCI is that analytes are ionized in the liquid phase in ESI and in the gas phase in APCI.

After the analytes are ionized, they are sent to the mass analyzer to be separated by their mass-to-charge ratios. The mass analyzer that is important for the scope of this paper is the quadrupole. Quadrupole mass analyzers consist of four rods that are parallel around a central axis. DC and RF voltages are applied to each quadruple so that each rod has the opposite polarity of the adjacent rods. Analyte ions travel through the quadrupole rods towards the detector but in oscillation due to the oscillating electric fields generated by the quadrupole. Voltages applied to the rods can be changed in a quadrupole mass analyzer so that only the desired analyte ion has a stable trajectory towards the detector. Ions with larger or smaller mass-to-charge ratios will be unstable through the quadrupole rods and end up not reaching the detector (Gates, 2014b).

A mass analyzer can also be coupled with one or more mass analyzers to be used for tandem mass spectrometry. The most common setup is called a triple quadrupole and as the name suggests, three quadrupoles are lined up. The first (Q1) and the third (Q3) quadrupoles are mass filters that behave in the same manner described in the previous section. The middle (q2) quadrupole, also known as the collision cell, is responsible for collision-induced dissociation (CID) to fragment the ions that arrived from Q1. The fragmented ions are then accelerated into Q3 to further be filtered.

While the only quantitative mass spectra scan mode that a single quadrupole mass spectrometer can perform is selected ion monitoring (SIM), a triple quadrupole mass spectrometer can also provide selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) scan mode. The MRM scan mode only allows specific precursor ions to accelerate through the collision cell. Resulting fragmented ions are once again filtered in Q3 so that only the desired product ions can reach the detector. MRM allows for higher selectivity and sensitivity than SIM because ions are filtered out twice, rather than once.

Previous Studies on Quantitation of Ursolic Acid in Plasma Using LC-MS

LC-MS is the standard for the quantification of ursolic acid in plasma because it offers greater sensitivity and selectivity relative to other detectors such as UV. One of the earliest validated methods for ursolic acid in plasma used an LC-MS system consisting of a single quadrupole MS analyzer with an APCI source in negative ion mode using selected ion-monitoring (SIM) (Liao et al., 2005). Similar MS instrumentation and conditions are still used today but paired with an UPLC system. The upgraded LC system allowed for greater sensitivity of ursolic acid. The lower limit of quantitation of ursolic acid for the most recently developed method using the same MS instrumentation was 1 ng/mL

while it was 10 ng/mL previously (Stebounova et al., 2018). Another common technique used is the triple quadruple mass spectrometer in ESI negative ion mode. Multiple reaction monitoring (MRM) scanning mode is often used to gain the full benefit from the triple quadrupole mass spectrometer (Tan et al., 2010; Xia et al., 2011).

The internal standards typically used for ursolic acid analysis are either glycyrrhetinic acid or betulinic acid. They are often used because both are triterpenoids like ursolic acid and are structurally similar. Glycyrrhetinic acid elutes earlier than ursolic acid in the studies referenced above. This is because the extra ketone functional group present in glycyrrhetinic acid makes the compound more polar than ursolic acid and therefore it is not retained longer in the reversed-phase liquid chromatography system.

Isocratic elution was used in all previous method developments, but the composition of the mobile phase varied. Some of the mobile phases used were methanol and water (95:5, v/v) (Liao et al., 2005), methanol and 5 mM ammonium acetate (85:15, v/v) (Stebounova et al., 2018), acetonitrile and 10 mM ammonium formate (90:10, v/v) (Xia et al., 2011), and acetonitrile and 20 mM ammonium acetate containing 0.1% formic acid (95/5, v/v) (Tan et al., 2010). An interesting thing to note about the latter two mobile phases is that Xia et al. stated that they had better peak response and shape without the use of 0.1% formic acid while Tan et al. stated its addition enhanced the peak.

Liquid-liquid extraction was a common choice for extracting ursolic acid from plasma (Tan et al., 2010; Xia et al., 2011; Zhao et al., 2015). The non-polar characteristic of ursolic acid simplifies the transfer to the organic layer (ethyl acetate or methyl tertiary-butyl ether) from plasma. These studies using liquid-liquid extractions typically had a relative recovery of ursolic acid around 70%.

CHAPTER 2

QUANTITATION OF URSOLIC ACID AND ITS PRODRUG IN RAT PLASMA BY LC-MS

Introduction

The first step in the pharmacokinetic study of ursolic acid and its prodrug is the development of a quantitative method that can accurately determine the concentration of ursolic acid and its prodrug in plasma. Several sample preparation methods have been established for the extraction of ursolic acid in plasma including solid-phase extraction, supercritical fluid extraction, and liquid-liquid extraction being the most prominent (Huang et al., 2007; Zhang et al., 2022; Zhao et al., 2015). Although protein precipitation has also been used previously, its use has been limited because it creates a gradual increase in the LC back pressure due to buildup of impurities in the column and because of the long solvent drying times when compared to liquid-liquid extraction. However, even liquid-liquid extraction was not ideal because the differences in the physicochemical properties of the analytes (ursolic acid, the prodrug, and the internal standard) made the simultaneous extractions unreliable. In this research for the development of an LC-MS method for ursolic acid and its prodrug from plasma, protein precipitation procedure has been modified to provide a reliable, fast sample extraction method.

Materials and Methods

Chemical and Reagents

Ursolic acid (UA) and its prodrug (UA 4) were obtained from the Lokeshwar Laboratory in Augusta University. β-glycyrrhetinic acid (IS) was purchased from BioVision Inc. (Waltham, MA). LC-MS grade ammonium formate was purchased from MilliporeSigma (MILWAUKEE, WI). LC-MS grade water and methanol were also obtained from MilliporeSigma (Allentown, PA). The chemical structures of UA, UA 4, and internal standard are shown in Figure 1.

Instrument and Conditions

Waters ACQUITY UPLC H-Class PLUS System (Milford, MA) was equipped with a Waters ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, I.D., 1.7 µm) (Milford, MA) for chromatographic separation. Column and autosampler temperature were maintained at 30 °C and 4 °C respectively. Mobile phase A consisted of 5 mM ammonium formate in LC-MS grade water while mobile phase B consisted of 5 mM ammonium formate in LC-MS grade methanol. The flow rate was left constant at 0.3 mL/min and the gradient elution program was as follows: 0-1 min, 80% B; 1-3 min, 80-95% B, 3-6 min, 95% B; 6-6.1 min, 95-80% B; 6.1-9 min, 80% B.

A Waters Xevo TQ-S micro triple quadrupole mass spectrometer with a Z-spray ESI probe (Milford, MA) was used to detect the eluted analytes and MassLynx v.4.2 software was used to process the data. ESI positive ion mode was used with the following conditions: capillary voltage 1 kV, cone voltage 25 V, desolvation temperature 450 °C, desolvation gas flow 800 L/h, cone gas flow 50 L/h, and source temperature 120 °C. Multiple reaction monitoring mode was applied for the method: *m/z 457.3* → 411.5 for UA,

m/z 639.5 → 183.25 for UA 4, and *m/z 471.25* → 95.1 for IS. The collision energy was 15 V for ursolic acid, 50 V for both UA 4 and IS. Confirmation ion-transitions were set to: *m/z 457.3* → 95.0 for ursolic acid, *m/z 639.5* → 70.2 for UA 4, and *m/z 471.25* → 135.1 for IS.

Solutions and Standards

Stock solutions of ursolic acid, UA 4, and β-glycyrrhetinic acid were prepared in methanol with final concentrations of 0.25 mg/mL, 0.5 mg/mL, and 0.5 mg/mL respectively and were stored at -80 °C. Ursolic acid and UA 4 stock solutions were combined with methanol to create the first standard working solution with UA and UA 4 concentrations of 50000 ng/mL. This standard working solution was used to prepare the remaining working solutions with UA and UA 4 concentrations of 2000 ng/mL. Calibration standard solutions of 80, 200, 300, 500, 1000, 1600, and 4000 ng/mL and quality control (QC) working solutions 240, 1200, and 3200 ng/mL were prepared using the two standard working solutions and methanol. The second standard working solution was used to prepare calibration standard solutions and quality control working solution that are 500 ng/mL or lower; the rest were prepared using the first standard working solution. β-glycyrrhetinic acid (IS) stock solution was diluted to 100 ng/mL with methanol and the working solution was stored at 4 °C.

Spiked Samples

20 µL of calibration standard solutions or QC working solutions were spiked into 380 µL of blank Sprague Dawley rat plasma to yield calibration standard concentrations of 4, 10, 15, 25, 50, 80, and 200 ng/mL and QC samples with concentrations of 12, 60, and 160 ng/mL.

Sample Preparation

Protein precipitation extraction was used with methanol acting as the precipitating agent. 100 µL of spiked plasma sample was precipitated with 200 µL of cold IS working solution and was vortexed for 5 minutes. The sample was set aside for 20 minutes at 24 °C and was centrifuged at 21,130 x g at 4 °C for 15 minutes. 140 µL of supernatant was transferred and mixed with 35 µL of water. The sample was then vortexed for 1 minute and centrifuged at 21,120 x g at 4 °C for 10 minutes. The upper 140 µL of the sample was transferred for injection.

Method Validation

The method was validated following the U.S. FDA guideline on bioanalytical method validation (*Bioanalytical Method Validation*, 2018); selectivity, calibration curve, accuracy and precision, recovery, and stability (freeze-thaw, bench top, and autosampler stability) were assessed.

Seven-point calibration curves ranging from 4-200 ng/mL were analyzed by plotting the peak area ratios (analyte/IS) against the associated concentration points. Both UA and UA 4 calibration curves used a $1/x^2$ weighted linear regression model.

Accuracy and precision for intra-day (n = 5) and inter-day (n = 15) were assessed by analyzing LLOQ, LQC, MQC, and HQC (4, 12, 60, and 160 ng/mL) samples of UA and UA 4. Method validation for inter-day accuracy and precision had to be conducted with three independent runs of five replicates at each QC levels. Accuracy and precision were displayed as relative error and relative standard deviation, respectively.

For recovery, three replicates of LQC and HQC spiked samples, post-extraction spiked samples, and standard solutions for UA and UA 4 were analyzed. Recovery was

expressed in relative recovery (spiked sample/post-extraction spiked samples), absolute recovery (spiked samples/standard solutions), and matrix effects (post-extraction spiked samples/standard solutions).

Three different stability studies were conducted for LQC and HQC samples of UA and UA 4. For the autosampler stability study, five replicates of LQC and HQC samples were analyzed after 12 hours inside the autosampler at 4 °C. Three replicates of LQC and HQC spiked samples were left on a bench top at 24 °C for 4 hours and then extracted and analyzed for the bench top stability study. For the freeze-thaw stability study, blank rat plasma samples were spiked at the LQC and HQC concentrations for UA and UA 4. Three replicates of LQC and HQC spiked samples were extracted right after preparation and were analyzed. Remaining spiked samples were stored at -80 °C and were thawed every 24 hours for a total of 3 cycles. Three replicates of the LQC and HQC spiked samples were extracted and analyzed for each cycle.

Results and Discussion

Method Development

Various combinations of mobile phase compositions have been used based on previous pharmacokinetic studies involving ursolic acid (Stebounova et al., 2018; Tan et al., 2010; Xia et al., 2011). Combinations that have been tried were 5 mM of ammonium formate in acetonitrile, 5 mM of ammonium formate with 0.1% formic acid in acetonitrile, and 5 mM of ammonium formate in methanol. Acetonitrile was abandoned because there was severe peak tailing for UA 4 when acetonitrile was used in mobile phase B. Formic acid was also abandoned because it lowered the peak response of UA.

Several extraction techniques were evaluated to determine the best method for the quantitation of UA and UA 4 using LC-MS. Because UA and UA 4 are lipophilic, liquid-liquid extraction was the first choice. Preliminary data for liquid-liquid extraction using methyl tert-butyl ether (MTBE) showed similar UA relative recovery of 79%. However, relative recovery of UA 4 was poor at 35%. Protein precipitation was used next to determine whether it could increase the relative recovery of UA 4. Preliminary data showed increases in both relative recovery of UA and UA 4 compared to that of liquid-liquid extraction at 85% and 88%, respectively.

Prior to the final validated method, another method with a different calibration curve range had already been validated using an earlier variation of protein precipitation. The calibration curve ranges for UA and UA 4 were from 15-200 ng/mL and 0.5-200 ng/mL respectively. The main differences between the earlier variations of protein precipitation from the final version were that: 1) 40 µL of plasma was used instead of 100 µL, 2) the precipitating agent was acetonitrile at 24 °C, not methanol at 4 °C, 3) samples were centrifuged at 2000 x g at 24 °C instead of 21,120 x g at 4 °C, and 4) the supernatant was dried under vacuum and reconstituted with 100 µL of 1:4 water: methanol instead of forgoing the drying step completely.

Issues using the earlier variations of protein precipitation was that as more samples were injected through the column, the back pressure increased and resulting chromatographic peaks showed increased broadening with each run. This method was abandoned because it was not optimal for larger studies where hundreds of samples must be injected. Using the linear equation derived from the UA calibration curve of 15-200 ng/mL, the lowest concentration of UA detected at 8 hours after dosing was 6.5 ng/mL.

Although the concentration cannot be accurately calculated using the UA calibration curve, it was still clear that the lowest detected concentration was still below the LLOQ set by the calibration curve. Therefore, the calibration curve range had to be altered to account for the low concentration of UA expected.

Liquid-liquid extraction was pursued despite having poor UA 4 recovery to prevent the increase in back pressure. Compared to protein precipitation, liquid-liquid extractions typically results in relatively cleaner extracts (Rainville, 2013). When liquid-liquid extraction was used for method validation, another challenge appeared. The chromatographic peak response of the internal standard fluctuated randomly between each injection and therefore gave inaccurate results. Different approaches have been used to prevent the internal standard response variability: 1) the internal standard was replaced with another ursolic acid derivative, 2) ethyl acetate was used as the organic extraction solvent instead of MTBE, 3) the internal standard was spiked into the organic solvent prior to mixing with plasma, 4) liquid-liquid extraction was combined with protein precipitation. However, none of these approaches stabilized the variability in the internal standard response. This variability may be caused by the ketone group present in β-glycyrrhetinic acid, which makes the internal standard less non-polar. The polarity of the internal standard could have affected its transfer over to the organic layer during liquid-liquid extraction. Once again, the liquid-liquid extraction method had to be abandoned.

Protein precipitation would have been the ideal extraction technique to use for UA, UA 4, and IS if it did not increase the back pressure. To alleviate the back pressure, the UPLC column was replaced with Thermo Scientific BDS Hypersil C8 HPLC column (2.1 mm x 50 mm, I.D., 5 µm) (Waltham, MA) due to its larger particle size. Even though the

HPLC column did help maintain the back pressure, the drawback was broader peaks, which was not ideal.

The last attempt made for the use of protein precipitation was forgoing the drying process. In previous protein precipitation experiments, dried UA, UA 4, and IS particles were observed after the drying step. These solid particles were then reconstituted and sonicated. It was hypothesized that the UA, UA 4 and IS particles were not dissolving back into solution and were clogging the column, leading to the increase in back pressure. Forgoing the drying process prevented the buildup of back pressure and peak abnormalities.

Selectivity and Specificity

MRM channels of a LLOQ run can be seen in Figure 2. All three peaks appear symmetrical and well-spaced out from each other with no overlap. The IS eluted the earliest at 1.97 min due to its ketone functional group making it more polar than UA and UA 4. The next analyte to elute was UA at 3.80 min and UA 4 was last at 4.56 min. Figures 3, 4, and 5 show the chromatograms of UA, UA 4, and IS in a LLOQ run compared to chromatograms of a blank plasma run within the same retention time range. Given that no significant peaks were present in blank plasma runs when the analytes are supposed to elute, the method was selective.

Linearity and Sensitivity

Table 1 shows the averaged calibration curve during the freeze-thaw stability cycles along with the R^2 value using $1/x^2$ weighted linear regression model. Averaged R^2 values of 0.99 for both UA and UA 4 indicated that the calibration curve from 4-200 ng/mL

showed good linearity. Although LLOQ UA peak response was significantly lower than that of UA 4, the signal-to-noise ratio of UA was still at least 35.

Precision and Accuracy

Precision and accuracy of UA and UA 4 are shown in Table 2. Both precision and accuracy for intra-day and inter-day passed as they were all under 15% of the nominal concentrations.

Recovery

Table 3 shows that the relative recovery of UA and UA 4 is roughly 70%, a significant improvement for UA 4 when compared to liquid-liquid extraction. UA and UA 4 have opposite effects for absolute recovery. While UA has an absolute recovery of around 37%, UA 4 has absolute recovery that is over 100%. This can be explained by the matrix effect. Since UA has a matrix effect that is lower than 100%, there is suppressed ionization of UA. Alternatively, ionization of UA 4 is enhanced because the matrix effect is higher than 100%.

Stability

LQC and HQC samples of UA and UA 4 that were stored in the autosampler at 4 °C were stable for at least 12 hours as shown in Table 4. LQC and HQC samples of UA and UA 4 were also stable when left on the bench top at 24 °C for 4 hours before protein precipitation. Three cycles of freeze-thaw were conducted, and the samples were stable throughout all three cycles, given that accuracy was lower than 15%, just like the other two stability studies.

Conclusion

An LC-MS method has been developed for the pharmacokinetic study of UA and its prodrug, UA 4. Forgoing the drying process in protein precipitation made it possible to extract UA and UA 4 reliably and quicker than before. Most importantly, it did not cause the back pressure of the LC system to increase. The next step in determining UA and prodrug's effect on bladder cancer is conducting an animal study. Rats should be orally dosed with the prodrug and their plasmas should be extracted at different time intervals following the same extraction protocol described in this research. The resulting data will show how much of UA 4 is turned into UA to treat bladder cancer.

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TABLES

Table 1 Calibration curves of UA and UA 4 in plasma (n = 4).

Analyte	Slope	Intercept	R^2
UA	0.0840 ± 0.0062	0.0506 ± 0.0490	0.9945 ± 0.0038
UA 4	4.2303 ± 0.1841	2.9898 ± 3.3246	0.9905 ± 0.0061

Table 2 Accuracy and precision of UA and UA 4 in rat plasma (n = 5 for intra-day) (n = 15 for inter-day).

Analyte	Nominal conc. (ng/mL)	Intra-day			Inter-day		
		Measured conc. ± S.D (ng/mL)	Precision (RSD (%))	Accuracy (RE (%))	Measured conc. ± S.D (ng/mL)	Precision (RSD (%))	Accuracy (RE (%))
UA	4 (LLOQ)	3.91 ± 0.17	4.47	-2.22	4.15 ± 0.25	6.14	3.74
	12 (LQC)	13.68 ± 0.33	2.40	14.00	13.24 ±0.44	3.32	10.30
	60 (MQC)	65.27 ± 1.25	1.91	8.79	67.54 ± 2.00	2.96	12.56
	160 (HQC)	155.42 ± 2.09	1.34	-2.86	163.56 ± 6.58	4.02	2.23
UA 4	4 (LLOQ)	4.00 ± 0.13	3.31	-0.01	3.94 ± 0.21	5.26	-1.43
	12 (LQC)	12.84 ± 0.47	3.65	7.02	12.36 ± 0.78	6.28	2.96
	60 (MQC)	59.04 ± 0.72	1.23	-1.60	62.81 ± 5.30	8.43	4.68
	160 (HQC)	145.19 ± 5.44	3.74	-9.26	156.96 ± 12.83	8.17	-1.90

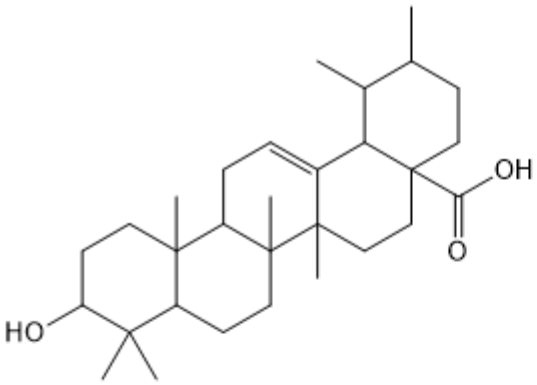
Table 3 Recovery (n = 3) of UA and UA 4.

Analyte	Nominal conc. (ng/mL)	Relative Recovery (%)	Absolute Recovery (%)	Matrix Effect (%)
UA	12 (LQC)	68.53 ± 2.42	37.24 ± 1.32	54.34 ± 0.74
	60 (MQC)	67.03 ± 1.41	35.69 ± 0.75	53.25 ± 0.74
	160 (HQC)	70.28 ± 0.78	37.47 ± 0.42	53.32 ± 0.78
UA 4	12 (LQC)	76.80 ± 4.05	123.01 ± 6.48	160.16 ± 10.38
	60 (MQC)	71.60 ± 3.27	115.32 ± 5.26	161.06 ± 5.55
	160 (HQC)	76.60 ± 1.13	110.37 ± 1.6	144.09 ± 0.57

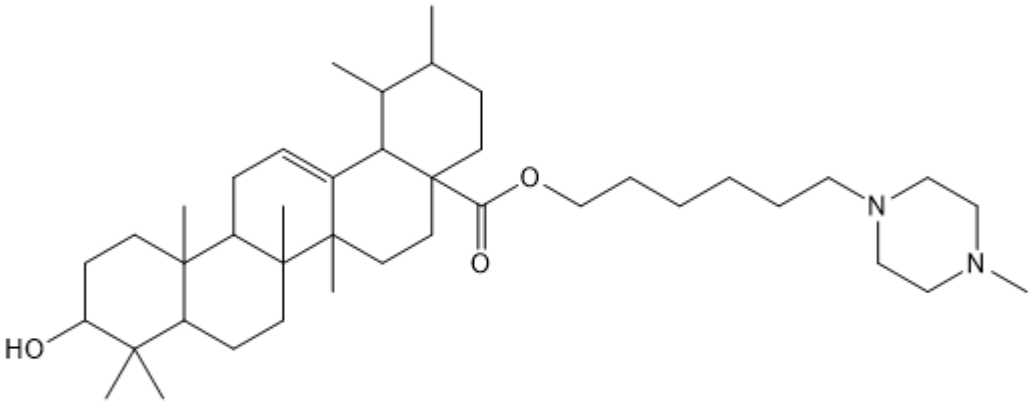
Table 4 Stability studies of UA and UA 4: autosampler stability (n = 5), bench top stability (n = 3), and freeze-thaw cycle 3 stability (n = 3).

Analyte	Stability	Nominal conc. (ng/mL)	Measured conc. ± S.D (ng/mL)	Accuracy (RE (%))
UA	Autosampler stability	12 (LQC)	12.73 ± 0.53	6.09
		160 (HQC)	165.03 ± 0.82	3.14
	Bench top stability	12 (LQC)	13.06 ± 1.10	8.81
		160 (HQC)	148.89 ± 3.15	-6.94
	Freeze-thaw stability	12 (LQC)	12.80 ± 0.10	6.65
		160 (HQC)	164.87 ± 1.98	3.04
UA 4	Autosampler stability	12 (LQC)	13.20 ± 0.87	10.02
		160 (HQC)	163.15 ± 2.43	1.97
	Bench top stability	12 (LQC)	13.67 ± 0.52	13.90
		160 (HQC)	148.68 ± 3.24	-7.07
	Freeze-thaw stability	12 (LQC)	12.30 ± 2.21	2.46
		160 (HQC)	140.66 ± 8.36	-12.09

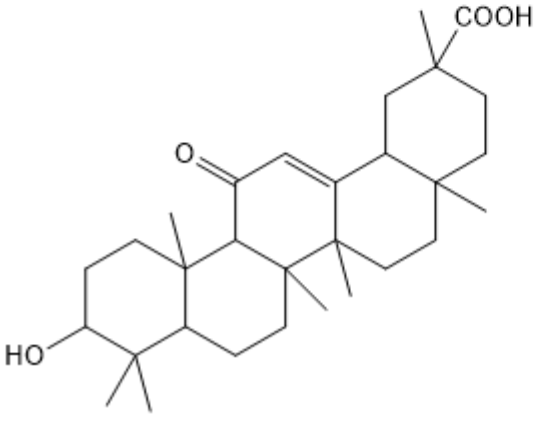
FIGURES



Ursolic Acid



UA 4



β-Glycyrrhetinic Acid

Figure 1 Chemical structures of ursolic acid (MW = 456.71 g/mol), UA 4 (MW = 639.02 g/mol), and β-glycyrrhetinic acid (MW = 470.68 g/mol).

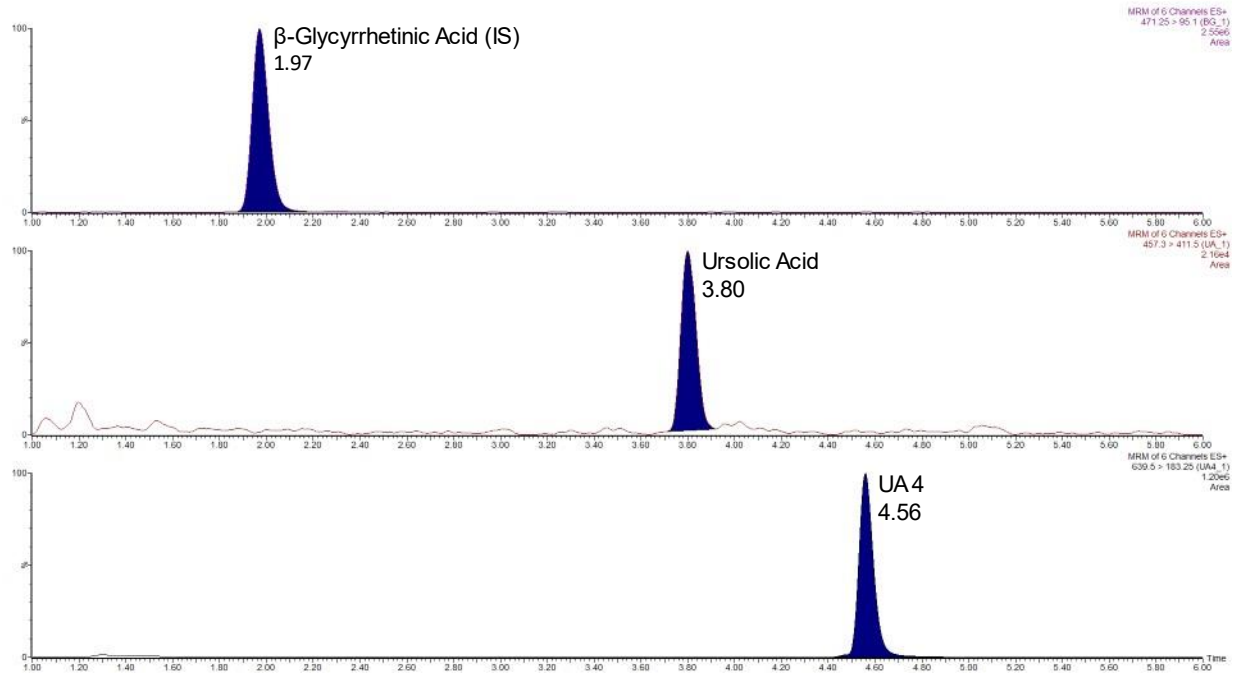


Figure 2 Representative MRM chromatograms of β-glycyrrhetinic acid (IS), UA, and UA 4 at 4 ng/mL (LLOQ).

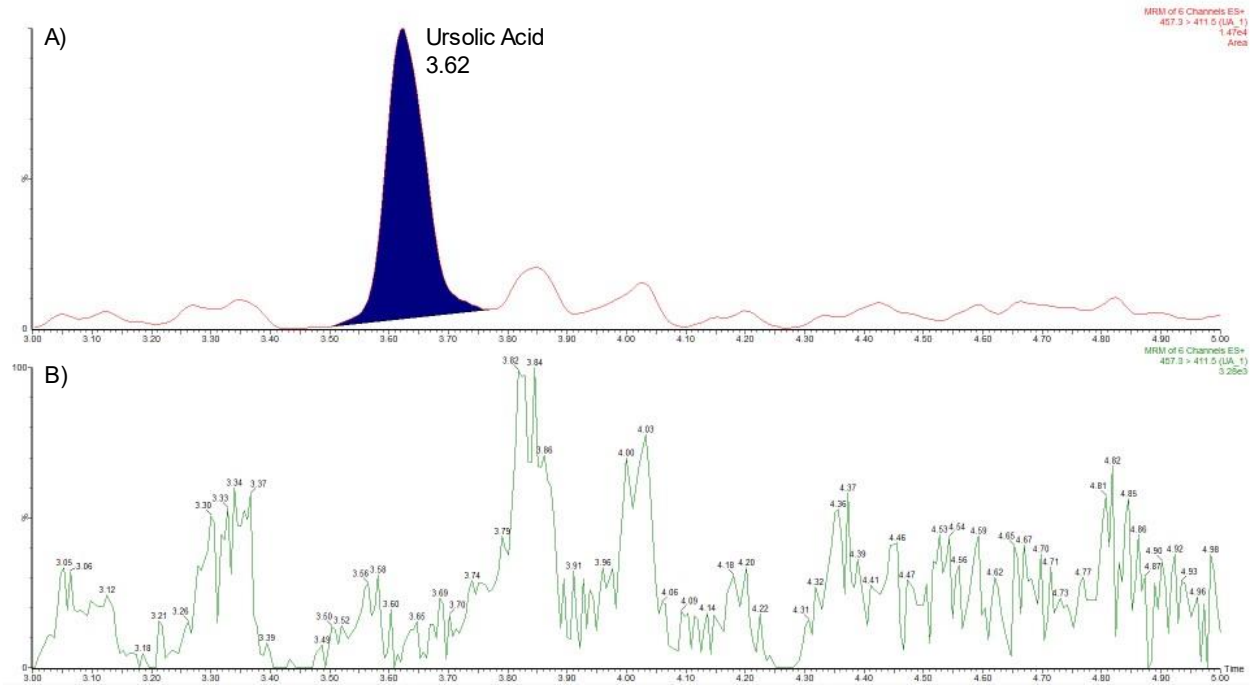


Figure 3 A) Ursolic acid at 4 ng/mL (LLOQ) compared to B) Blank plasma.

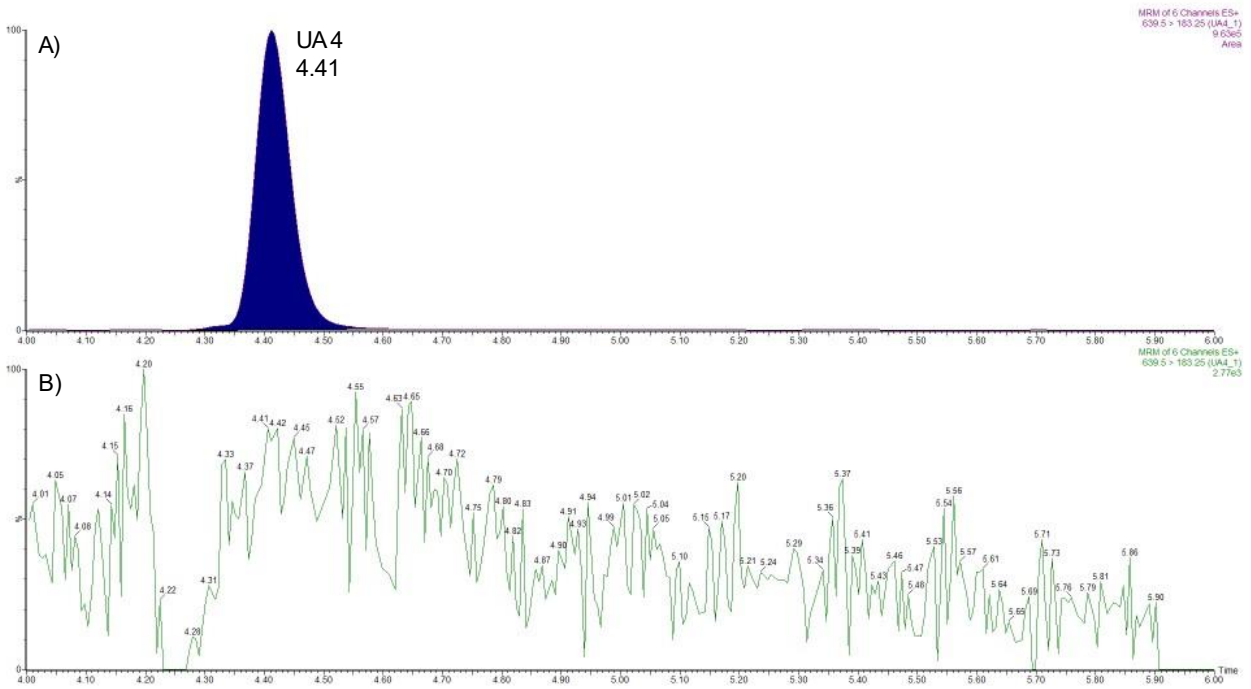


Figure 4 A) UA 4 at 4 ng/mL (LLOQ) compared to B) Blank plasma.

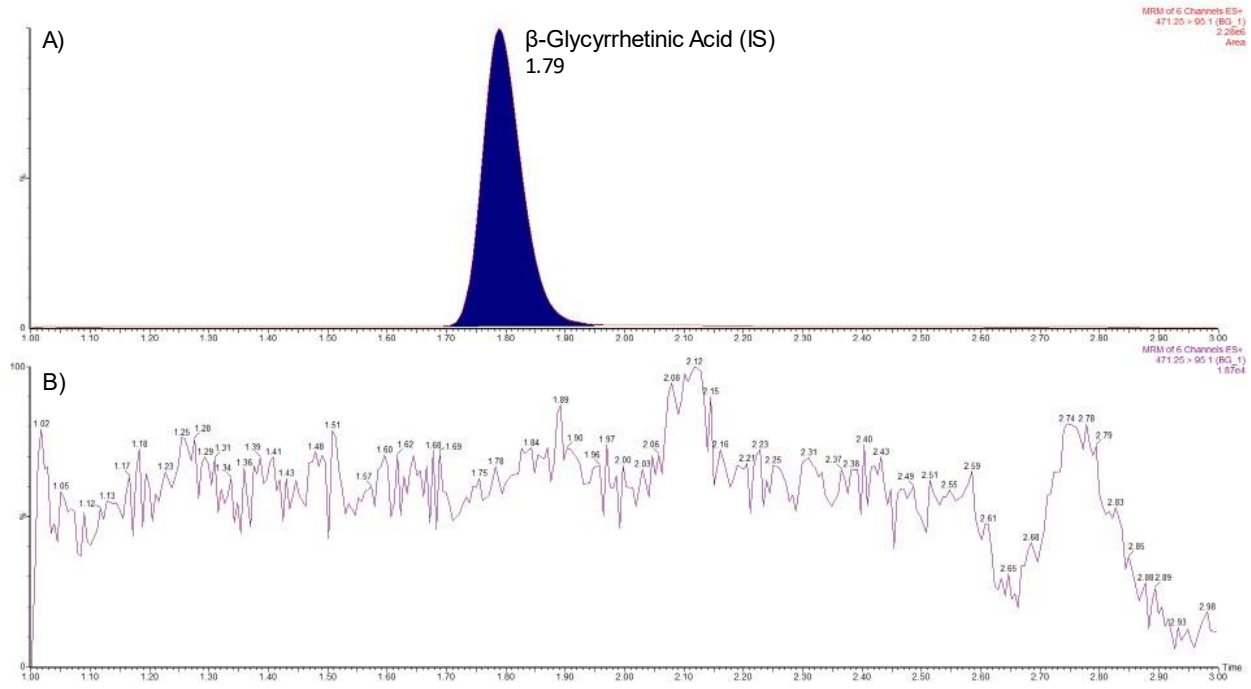


Figure 5 A) β-glycyrrhetinic acid (IS) compared to B) Blank plasma