JASON L. BOYD
Applications in Pharmacokinetic Modeling
(Under the direction of F. DOUGLAS BOUDINOT)

The viewpoints and methodologies of pharmacokinetic modeling in toxicology and pharmaceutics are divergent. ln order to appreciate both approaches, each method was used to model the kinetics of various compounds.

The pharmacokinetics of the nucleoside analog (2S, 4R)-1-2-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (lOddU), a potent in vitro antagonist of Epstein-Barr virus replication, were investigated. Total (mean (± rsd)) and renal clearance after iv administration were 1.44 (0.588) and 0.69 (0.46) L/h/kg in six male Sprague-Dawley rats. Volume of distribution was 0.643 (0.619) L/kg. MRT (h), λ_z (min^{-1}), and fraction of dose excreted in urine were 0.510 (0.527), 1.08 (0.714), and 47.8%.

lohexol is a radio contrast agent eliminated solely by glomerular filtration. ln order to support development of a clean, simple, and precise means of determining glomerular filtration rate in dogs, the plasma clearance of iohexol was compared with urinary creatinine clearance at different levels of obesity (lean, fattened, and obese). Absolute plasma clearance of iohexol increased with obesity as expected; body weight adjusted plasma clearance of iohexol did not increase significantly. A three point method sampling in the terminal phase overpredicted urinary creatinine clearance slightly.

The nucleoside analogs, 3'-azido-3'-deoxythymidine and (-)-2',3'-dideoxy-3'-thiacytidine (AZT, 3TC) are potent inhibitors of feline immunodeficiency virus (FIV) for which lymph tissues serve as viral sanctuaries. The lymphatic disposition of AZT and 3TC in cats were determined. Lymph tissues included were tonsil, thymus, submandibular, bronchiolar, sternal, and mesenteric lymph nodes. Mean overall lymph tissue concentrations of AZT and 3TC were 8.13 (0.79) and 7.74 (0.66). Tissue to plasma concentration ratios were 0.36 (0.76) and 0.44 (0.51) for AZT and 3TC.

Bromodichloromethane is a hepatic carcinogen present in chlorinated drinking water. A physiologically based pharmacokinetic model was developed to simulate concentrations in plasma and tissues of the Japanese Medaka fish (Orynchias latipes). lncidence of hepatocellular adenoma in male medaka exposed at three levels (0, 1.5, 15 mg/L) correlated very well (r^2=1.00) with weekly averaged simulated area under the concentration versus time curve.

INDEX WORDS: Nucleoside analog, lOddU, AZT, 3TC, Zidovudine, Lamivudine, lohexol, Bromodichloromethane, Medaka, “Physiologically based pharmacokinetic model”, FlV, EBV, “Feline immunodeficiency virus”, “Epstein-Barr virus”

APPLICATIONS IN PHARMACOKINETIC MODELING

by

Jason L. Boyd

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JASON L. BOYD

Approved:

Major Professor: F. Douglas Boudinot
Stuart Feldman

Committee: Randy Manning
Peter Ruenitz
Ragubir Sharma
Anand Vidyashankar

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2003

With unceasing love and affection to Paulette, Charlie, Joel.

To the host within the host. Turn the light around.

VOCATUS ATQUE NON VOCATUS DEUS ADERIT.

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CHAPTER 1

INTRODUCTION

Pharmacokinetics is the discipline of pharmacology which characterizes the absorption, distribution, metabolism, and excretion of substances in the animal body. Pharmacokineticists seek to discover and/or identify transporters, metabolizing enzymes, and sequestration phenomena, in general or in relation to a specific chemical, thereby applying the discovered pathways into the mathematical description of chemical fate in animals. Also intrinsic to the discipline of pharmacokinetics is pharmacodynamics, the relationship between pharmacological activity, concentration, and time. Experimentally, pharmacokinetics deals with choice of animals for study, surgical technique, analytical method development, and biological matrix preparation for analysis. Theoretically, pharmacokinetics deals with the development of models to predict chemical concentrations and pharmacological effects. Toxicokinetics applies pharmacokinetic and pharmacodynamic principles to the design and interpretation of studies involving drug safety and toxicity (Shargel and Yu, 1999); outcomes of toxicokinetic studies are in the form of such things as safety levels, effect levels of various types, and prediction of biomarkers which serve as surrogates for toxic effects.

Modeling itself increases predictive power in any science. The development of a perfect model precludes the need to test, other than virtually. Since the advent of pharmacokinetic modeling, a variety of model types and techniques have been used to describe the behavior of drugs and chemicals in the body. The diversity of types of pharmacokinetic models has arisen from concomitant development in parallel but seldom intercommunicating fields, as well as for historic reasons which include but are not limited to limitations on computational and statistical prowess.

DIFFERENT TYPES OF PHARMACOKINETIC MODELS

The most fundamental division of models used in any science is that between the mechanistic model and the empirical model (Thakur, 1991). Mechanistic, or as they are sometimes called, systems analytical, pharmacokinetic models seek to describe the

disposition of a chemical in the body using as many components as are likely to affect the chemical's disposition; when enough data are collected, they serve as powerful tools, both descriptively and predictively. The drawback to many mechanistic models is that the abundance of terms in the model precludes statistical means of assaying goodness of fit (Hovorka & Vicini, 2001). Empirical models seek to describe data in a purely mathematical sense and without regard to underlying processes.

Another distinction in modeling is that of model dependant and model independant modeling. Model independant means not that there is no model but refers to indepenance of assumptions such that a small number of parameters (or a single parameter) is inferred with a distribution dependant on study design and not on the origin (or model) of the data (Aarons L etal, 2001 p. 116-7; Sheiner & Steimer, 2000). Models of any type may be "descriptive" or "predictive". Descriptive models are pertinent only to the set of circumstances from which its data were obtained, such as a specific study design or subpopulation. Predictive models, because they incorporate measurement of baseline features of the modeled system, are able to extrapolate across and beyond the varying levels of these factors (Sheiner & Steimer, 2000). The preferred model is one which is mechanistic because its parameters are relevant and interpretable, predictive because it incorporates baseline or normal features, and model-independant because assumption is minimized.

Classical pharmacokinetics

Pharmacokinetics today is dominated by several general schools of technique: classical, physiologically based (pharmacokinetic models, PBPKs), population, and clinical. Classical pharmacokinetics typically uses two techniques, compartmental and noncompartmental. The compartmental pharmacokinetic model when parameterized in its most basic form is:

$$C = \sum_{1}^{i} [C_0 e^{-k_i t}]$$

such that *i* is the number of compartments modeled, and k_i is the rate term associated with that compartment. Taking this empirical model and making it one step more mechanistic to reflect physiology, k_i would then be interpreted as Cl_i/V_i with Cl_i and V_i being the clearance and volume terms associated with the *i*th compartment. As a differential equation, when the value of *i* is two, its form is:

$$dC_1/dt = C_2(Cl_d/V_2) - C_1((Cl_d+CL_e)/V_1)$$
$$dC_2/dt = C_1(Cl_d/V_1) - C_2(Cl_d/V_2)$$

where Cl_d, Cl_e, and V_i represent distributional or intercompartmental clearance, elimination clearance, and volume of the *i*th compartment. Area is calculated the same for either parameterization and is simply the integral of the regressed concentration versus time curve; it may also be calculated algebraically. Noncompartmental models derive their pharmacokinetic parameters via statistical moment theory, by first calculating the terminal elimination rate, the area under the plasma (or blood) concentration versus time curve (AUC) and other integrals of the concentration versus time curve (AUTC, AUT2C, etc); physiological parameters such as clearance and volume are calculated from mathematical treatment of the previously derived parameters. Noncompartmental models are sometimes called 'modelless models' because they do not attempt to build a specific structural model (a graphical representation of the pharmacokinetics). Actually, they are not truly without a model; instead of a structural model, they instead assume that pharmacokinetic processes (absorption, distribution, metabolism, and excretion) are linear, such as the area under the concentration versus time curve (AUC) being linearly related to dose.

The first parameterization of the compartmental model, that with slopes and intercepts only, is certainly an empirical model as its terms are not physiologically interpretable. In its reparameterization, it is mechanistic; other terms may be added to this model which may describe nonlinearities of metabolism or other physiologies. They are considered model independant because extrapolation of inferred parameters may be done across experimental conditions. (because the parameters depend on study design and not the origin of the data). And because a full model may incorporate mathematical descriptions of nonlinearities and other physiological phenomena, they are predictive. Though the noncompartmental method is mechanistic, its parameters are model dependant because they only apply to the experimental conditions from which the data come. Also, as its predictive ability is limited, it remains mainly descriptive.

Physiologically based pharmacokinetics

Physiologically based pharmacokinetic (pbpk) models are more mechanistically oriented than classical or even population pharmacokinetic models. In the basest sense, physiologically based pharmacokinetic models simply elaborate on a simpler pharmacokinetic model in a way that describes physiological and anatomical processes. In this interpretation a more elaborate submodel may be linked to a single compartment of a simpler pharmacokinetic model; an example might be the description of metabolism in a peripheral compartment, which would correspond to the liver, of a classical two or three compartment model. Another example is the combination of a two or three compartment mammilary model describing disposition and elimination to a caternary model describing transit through and subsequent absorption from a variety of different gut compartments (Witcher etal, 1996). Most of the preceding models are considered to be physiologically detailed compartmental models and not "physiologically based pharmacokinetic models."

Many physiologically based pharmacokinetic models are built using estimations or approximations of mean tissue/organ volumes, mean cardiac outputs, fractional blood flows, represented structurally with visceral organs and tissues in parallel with each other connected in circuit with the heart and lungs. This circuit, although representative of anatomy, is directly borrowed from electrical engineering (Mapleson, 1961). These models are usually blood flow rate-limited models in which disposition is related to blood flow. In opposition, compartmental models do not consider blood flow except in the comparative interpretation of fitted parameters (eg. renal clearance in relation to renal blood flow, etc). Most classical models instead assume that distribution in a compartment is instantaneous and, thus, well mixed (Gibaldi & Perrier, 1982); and that the mixing is limited by diffusion rather than perfusion. However, classical models which are concerned with specific tissue or organ distribution are described in relation to blood flow. Hereafter in this paper the term, "physiologically based pharmacokinetic model," will refer only to these "circuit" style models of drug behavior.

The number of phenomena which affect the chemical in question may be a few or nearly infinite, limited only by available information of the chemical, its properties, and the mathematical description of the physiology and tissue in question. An example of such a detailed description may be found in the interfacial phenomena involved in the chemical's interaction with various layers of cells inside subcompartments of an organ (Erickson & McKim, 1990), or mathematical descriptions of physiological events such as complex endocrinological feedback inhibition (Yu KO etal, 2002). A limitation in complex models is that the number of state variables, that is, variables which are typically fixed and enter a host of differential equations, are so numerous that statistical validation has historically been precluded (Bois, 1999; Hovorka & Vicini, 2001).

A first step in such simulation has been made as told in the FDA transcript for Posicor (mibefradil; FDA, 1997), the T-type Ca++ channel blocker, which causes arrhythmiae at higher doses due to L-type Ca++ channel activity. A three dimensional physiological simulation of the heart and torso was used to demonstrate the similar

resulting effects that different (shortening or prolonging) changes in action potentials can produce on the electrocardiogram. Other complex pbpk models of the heart include arrhythmiae in relation to transporter protein expression (Winslow RL etal, 1998b) and of myocardial ischaemia (Chen FFT, etal, 1998), the latter of which has led to the reconsideration of the model of cellular ischemic/reperfusion arrhythmiae (Noble etal, 1999).

Physiologically based pharmacokinetic models have been used in clinical trials to demonstrate both safety and efficacy, the earliest example of which being that of the pharmacokinetics and pharmacodynamics of circulating tissue-type plasminogen activator (Tiefenbrunn etal, 1986); this simulation allowed the evaluation of prospective dosing regimens for t-PA in clinical trials (Holford etal, 2000; Noe etal, 1987).

Single parameter models

Single parameter pbpk models are those which investigate only one parameter with the goal that the parameter be later incorporated into a more complex model (Grass & Sinko, 2002). A common single parameter model experiment is the in vitro or in vivo tissue/blood partition coefficient which is often added to a more complex model of the chemical's pharmacokinetics in an animal. Other examples of single parameter models are fraction of dose absorbed into the hepatic vein or the percent absorbed into Caco-2 cells (Grass & Sinko, 2002).

Multiparameter models

Multiparameter pbpk models are more complex and include several tested parameters. An example of such a model is the CAT (compartmentalized absorption and transit) model (Yu & Amidon, 1999; Yu, 1999; Bolger, 2000) which describes

gastrointestinal absorption and transit of drugs by describing the GI tract with different compartments (Witcher etal, 1996). The iDEA Simulation System is an ambitious example of a complex multiparameter modeling system (Grass, 1997; Timony etal, 2000; Norris etal, 2000) consisting of QSAR relationships of entire industrial corporate libraries of drugs of multiple chemical classes whose purpose is to provide a reliable means of simulating a hypothetical chemical's absorption profile from the human gut.

Linked and combined models

In addition to single parameter and multiparameter models are combined models which combine two or more independantly validated models in order to make a more comprehensive model, and linked models in which a fully physiologically specified model is linked to one which is not fully specified physiologically, for example linking the aforementioned CAT model to a classical pharmacokinetic model, or a QSAR-PBPK linked model (Grass & Sinko, 2002). Norris etal (2000) determined the limitation on fraction of ganciclovir absorbed in dogs was due to solubility as opposed to permeability based on pbpk modeling techniques; the success of this study adds to the credibility of the iDEA absorption database (Timony etal, 2000) which is composed of preclinical and clinical data from currently or previously marketed drugs as well as failed drug candidates data from numerous corporate drug library data bases regarding drug pharmacokinetic profiles and whose goal is to provide a rationale for better drug development, especially in the area of drug absorption. The database is an approach that bridges in vivo data with QSAR like data. Most physiologically based pharmacokinetic models are either linked or combined models. The technique of linking and combining models is not restricted to physiologically based pharmacokinetic modeling though, as classical pharmacokinetic models also make use of linking and combining both single and multi-parameter models.

Pharmacodynamics

Pharmacokinetics and pharmacodynamics are the two major subclasses of pharmacology. Where pharmacokinetics deals with what the body does to a chemical, pharmacodynamics deals with what the chemical does to the body (Holford & Sheiner, 1982). Though these two disciplines have traditionally been treated separately, the two are now routinely regarded as one process and this trend will assuredly stay (Derendorf etal, 2000). Pharmacodynamics deals with dose-response relationships, especially at the site of action. The main difference between pharmacokinetics and pharmacodynamics is the issue of linearity. Pharmacokinetic process are less likely to be saturated primarily because the pharmacokinetics of a compound depend so heavily on physiochemical processes. Active processes like metabolism and active distribution/excretion processes (eg., secretion in the renal tubules or MDR (multi-drug resistant) transporter related distribution) are saturable, yet marketed therapeutics are generally used in the linear range of pharmacokinetics. However the pharmacodynamic relationships of many drugs are decidedly nonlinear (Derendorf etal, 2000). The protein- or receptor- mediated nature of the pharmacology of most drugs entails saturation and sometimes complex and nonlinear 2nd messenger and DNA synthesis reactions.

Besides therapeutic effects, other effects, toxic or not, are related to concentration of the compound. Clinical trials and environmental risk assessment both attempt to correlate concentration of a drug or chemical to unintended effects. Either direct effects or biomarkers can be related to concentration. An obvious example is the effect of anesthetic concentration in plasma and level of induction or obtundation. A biomarker is a “specific pharmacological effect that does not necessarily confer a therapeutic benefit of a drug on a given disease process or provide any therapeutic benefit to a patient” (Derendorf etal, 2000); they help to explain clinical outcomes, both therapeutic and toxic.

Pharmacodynamic effects may be explained empirically through the Emax model which states

$$E = E_0 + E_{max} * C / (EC_{50} + C)$$

where E_0 is the baseline effect, a function $E_0=f(x,t)$ that may either be a simple average value or a complex function, E_{max} is the maximum effect, EC_{50} is the concentration at which the effect is 50% of the maximum, and C is obviously the concentration. In order to explain effects more mechanistically the sigmoidal Emax model is often used. It states

$$E = E_0 + E_{max} * C^n / (EC_{50}^n + C^n) ,$$

where n is a scaling factor which may relate to number of binding sites. Obviously in complex cases involving transcription, 2nd messenger effects, or pharmacodynamic tolerance or induction in general, the Emax model would fail to describe the effect relationship and other disease specific models will come to fore. For models involving long term exposures such as cancer induction or teratology, other surrogates for concentration are used such as the area under the concentration versus time curve in either plasma, blood, or some effect tissue.

A variant of the pharmacodynamic model is the toxicokinetic model. The toxicokinetic model is one which ties a pharmacokinetic model to a toxic response. The breadth of the model may be such that a single endpoint is related to the compound's pharmacokinetics or may incorporate hundreds of biomarkers and other endpoints as is the case with preclinical toxicokinetic studies with drug candidates. Toxicokinetic models may employ either classical or physiologically based pharmacokinetic methods, and may or may not be clinically and/or population based. The term toxicokinetics is also sometimes used in the toxicology community to refer to a pharmacokinetic model of any toxicant whether or not it relates pharmacokinetics to toxic response.

STATISTICAL MODELS

"Population pharmacokinetics" refers to pharmacokinetics in a diverse population and therefore involves expected values of parameters and deviations from those parameters. In classical and physiologically based pharmacokinetics the mean or other expected value of pharmacokinetic parameters are modeled and deviations of those parameters are reported, yet the variance itself is not modeled in these branches of pharmacokinetics. So, where the mean has traditionally been modeled by the scientist, deviations have been modeled by the statistician (Sheiner & Steimer, 2000). Population pharmacokinetics includes modeling of variance or covariance of parameter estimates as well as the expected value. In order to understand population pharmacokinetics it is important to understand the historical method of reporting population parameters and the statistical concepts behind the so called discipline of "population pharmacokinetics." The modern statistical community is traditionally divided into two camps, Frequentist and Bayesian. The Frequentist views parameters as fixed and random, with fixed parameters being those which are a part of the structural model, typically the mean of some physiological expression, and random effects being variances (and covariances of the physical or structural parameters). The Bayesian views all parameters in the model as being random yet with model output, that is distributions of parameters, as being fixed.

The traditional way of reporting pharmacokinetic parameters for a compound involves reporting the mean and variance when derived from the Two Stage method (Peck etal, 1993) in which pharmacokinetic parameters from each animal separately derived in an experiment (stage I) and then reporting the means and variances of those pharmacokinetic parameters (stage II). This type of statistical model is useful in early investigation scenarios in determining pharmacokinetics in an animal for the first time. It is easy to do, intuitive, and does not require exceptional amounts of statistical training.

The disadvantage of using the two stage model is that, due to the extremely high level of pharmacokinetic variability in the human population, owing mainly to active processes such as metabolism and active transport and excretion phenomena, it is unable to estimate individual pharmacokinetic parameters unless the sampling set is very large and inclusive of appropriate subpopuli; also, predictions of population pharmacokinetic parameters tend to be upwardly biased (Peck etal, 1993). Such rich data is expensive and difficult to acquire and generally financially preclusive in clinical trials which are hallmarked by their paucity of data relative to the population (Sheiner etal, 1977; Sheiner, 1984; Steimer etal 1984).

The differences between the Frequentist and Bayesian paradigms are hotly debated. That Bayesians and Frequentists have different outlooks, goes without saying. An example of the difference in their perspectives is

> "Frequentist approach to deciding when to quit watching a football game:
> Of all games which ended in a tie or with your team losing, what proportion had your team leading by 10 points with 12m to go in 4th quarter?
> Must consider sample space.
> Bayesian approach: at each moment can estimate the probability that your team will ultimately win based on the time left and the point spread"
> (Harrell, 2000).

The Frequentist approach to statistics is the so-called classical approach; it is the approach usually taught in most statistics classes. The Frequentist defines probability as "the frequency with which different values of statistics (arising from sets of data other than those which have actually happened) could occur for some fixed but unknown values of parameters" (Harrell, 2000; Box & Tiao, 1973). The Frequentist approach has some advantages in that parameters are simply thought of as constants, p-values are easily computed, prior belief is not needed to run or fit a model, and robust nonparametric tests are available without modeling. The most notable advantage to Frequentist modeling

is the fact that computationally it is less intense than Bayesian. Disadvantages include lack of use of available knowledge, the usual and often mistaken use of //=0.05, controversy of use of one and two tailed tests, misinterpretation of p-values and confidence intervals, p-values can only be used to reject a hypothesis but not to support one, multiple methods for arriving at p-values, issues with use of multiple comparisons, among others (Harrell, 2000).

Prior knowledge is regarded as an important component in probability; therefore belief, or opinion, is ranked by Bayesians. Among the Bayesians are several camps. Subjectivism, as proposed by Bruno de Finetti (1937), states that "probabilities only represent degrees of rational belief and [one's] belief function is rational just when it is a probability function, no further constraints need be satisfied" (de Finetti, 1937; Williamson and Corfield, 2001). An Objectivist Bayesian believes however that such a belief function does not obey the mathematical laws of probability and that it must satisfy constraints before being regarded as rational (Williamson and Corfield, 2001). The Objectivist camp is further divided into Logical and Empirical schools of thought. The Logical Objectivist believes that probability is truly objective and that two people with the same knowledge cannot arrive at different opinions about some event and remain perfectly rational. The principle idea of Logical Bayesianism is that "a probability $p(b|a)$ is the degree to which *a* partially entails *b*, and also the degree to which a rational agent should believe *b*, if she knows *a*" (where an agent is a betting agent of the Dutch Book variety; Williamson and Corfield, 2001; Keynes, 1921). The Empirical Objectivist Bayesian takes a midway stance between the Subjectivist and Empirical Objectivist and believes that degrees of belief should be calibrated to objective frequencies, where they are known. Furthermore, the Empirical warns that "Bayesian probabilities are single-case, defined over sentences or events, whereas frequencies are general-case, defined over classes of outcomes, and there may be no way of ascertaining which frequencies are to be calibrated with a given degree of belief" (Williamson and Corfield, 2001).

Advantages to using Bayesian inference at least from a pharmaceutical and clinical perspective are that it results in the most preferable probability to clinicians (the posterior probability); additionally, these probabilities can be interpreted out of context more readily than p-values (Harrell, 2000). It also allows for measuring support for hypotheses rather than simple rejection of them (Schervish, 1996).

Estimators

The goal of pharmacokinetic modeling is to determine both an appropriate model and subsequently to estimate the pharmacokinetic parameters. *True* values of parameters are impossible to know because of measurement error in the experimental data. Thus an estimate of precision and accuracy of parameter estimates is used in order to provide interpretability to the parameters.

The least squares method of estimating parameters is simple and intuitive and based on the principle of minimizing the squared differences between measured observations and predictions (Hovorka & Vicini, 2001); it is the most commonly used method of parameter estimation in pharmacokinetics. Under Gaussian assumptions, that is independant and identically distributed errors, $N(0,\sigma^2)$, the LS method is the same as the maximum likelihood method.

The least squares estimator (for an individual model) is described as follows:

$$SS \equiv \sum_{i=1}^{N}\left[\frac{y_i - y_{ipred}}{\sigma_i}\right]^2$$

where SS is is the "sum of squares of weighted residuals" and is χ^2 distributed, y_{ipre} is the predicted y at time, i, and is a function, $f(t_i,\theta)$, of t_i, time, and θ, a vector of M number of parameters, $(y_i - y_{ipred})$, represents residuals, and $(y_i - y_{ipred})/\sigma_i$ denotes standardized, or normalized, residuals. The value of the SS reflects a good fit when its

value is near its degrees of freedom, ν ($\nu = N - M$, with N being the number of observations and M being the number of parameters); values above ν denote a poor model or not enough parameters and values below ν denote too many parameters. Linear models are guaranteed a unique solution (only one set of parameter values can achieve the minimization of SS. Nonlinear models, that is, models which are nonlinear in their parameters, may have multiple solutions, called local minima, with only one global minimum being the set of parameter values which produces the best SS overall (Bates & Watts, 1988). A model is nonlinear when at least one of its partial derivatives with respect to any parameter is dependant on at least one other parameter. The reason most fully characterized classical compartmental pharmacokinetic models are nonlinear is because behavior of xenobiotics is typically mediated by metabolism and active processes in disposition and excretion which are saturable; the mathematic description of the saturable process introduces nonlinearity into the model because its partial derivatives in one parameter are dependant on at least one parameter. (It is worth noting however that though a full characterization of the pharmacokinetics of a compound is generally nonlinear, most currently marketed drugs and many environmental toxicants have exposures in the linear range of pharmacokinetics.)

The maximum likelihood method expounds on the least squares method by considering the conditional probability of the occurrence of the residuals given the parameter vector; this exercise is called likelihood. The maximum likelihood estimator of a model for an individual is given by the equation,

$$L = \prod_{i=1}^{N} \frac{1}{\sigma_i \sqrt{2\pi}} \exp\left[-\frac{1}{2}\left(\frac{y_i - y_{ipred}}{\sigma_i}\right)^2\right],$$

which minimizes

$$\sum_{i=1}^{N} \left(\frac{y_i - y_{ipred}}{\sigma_i}\right)^2 + 2\sum_{i=1}^{N} \ln(\sigma_i),$$

and when ϕ_i are independant from predictions, this minimizer is equivalent to the least squares estimator. The right hand side of the summation is important then in indicating model errors.

If y_{ij} is the jth observation in the ith individual and y_i is the vector of observations for the ith individual, then the specification of the population problem would be,

$$y_i = f_i(\phi_i) + R_i(\phi_i)^{1/2} e_i,$$

where $f_i(\phi_i)$ is a function of the (vector of) parameters (ϕ_i) for the ith individual. The function, $f_i(\phi_i)$, may be linear or nonlinear, and incorporates both, (d) of the fixed (a) and random (b) effects $(f = d_{ij}(a, b_i))$. Typical parameterizations are $f_i = a(1+b)$ or $f_i = ae^b$. R is a known positive definite continuous matrix-valued function describing variance of data measurement error, and e_i is the vector of independant multivariate random (noise) vectors, each with zero mean and covariance given by $R_i(\phi_i)$, whose square root can be obtained through orthogonal decomposition (such that $R = L^T L$). The e_i and ϕ_i are mutully independant.

The log-likelihood of the data in a population is given as

$$L(F) = \sum_{i=1}^{M} \ln p(y_i \mid F),$$

where F is a population distribution (of data sets), and with the density of y_i given F specified as:

$$p(y_i \mid F) = \int p(y_i \mid \phi) dF(\phi),$$

which states that the probability of the observed data, given the population distribution, is equal to the cumulative density of the probability of the measurement at time i given the parameters, integrated over the function of the parameters.

Bayesian estimators consider the probability of the posterior parameter vector, as does the maximum likelihood estimator, and the probability of the initial parameter vector, more accurately known as the prior probability. The Bayesian estimator, the posterior probability,

$$\Phi(\theta) = p(\theta \mid y) = \frac{p(y \mid \theta)p(\theta)}{p(y)},$$

where $p(\theta)$ is the prior probability of the parameters, $p(y)$ is the prior probability of the measurements, and $p(y \mid \theta)$ the conditional probability of the measurements given the parameter vector, which is also analogous to the maximum likelihood, ***L***. Because $p(y)$ is independant of the parameters,

$$\Phi(\theta) \propto p(y \mid \theta)p(\theta).$$

The Bayesian estimator returns a probability distribution for the vector of parameters; parameter estimation using Bayesian methods then are much more demanding than the LS estimation problems, which return a parameter vector, or a *point* estimate (Hovorka & Vicini, 2001). The estimation of $\Phi(\theta)$ is a difficult task since analytical (closed form) solutions of it are difficult to find; rather they are estimated by numerical solution to the differential equations of the model (Hovorka & Vicini, 2001). The advantage of using Bayesian methods is the interpretability of its posterior distribution.

Application

Where the two stage method takes obviously two steps to determine population pharmacokinetic parameters, mixed effects modeling takes one. This type of modeling can be implemented in either a Frequentist (LS or ML) manner or a Bayesian. In the step, data from all individuals including dependant variables and covariates are used to

simultaneously fit pharmacokinetic parameters to structural and statistical models such that a global minimum in least squares or extended least squares is accomplished, resulting in a global maximum for likelihood of the derived pharmacokinetic parameters.

Maximum likelihood is the current method of choice among classical pharmacokinetic scientists for basic pharmacokinetic studies. Fixed effects such as volumes, clearances, and other physiological parameters, as well as random effects (inter- and intra-individual variability, as well as uncertainty) may be determined in this way.

The practice in classical pharmacokinetics includes using linked and combined models, similar to those used in physiologically based pharmacokinetic modeling, in which two similar models, both of whose parameters have been determined by at least two separate instances of maximum likelihood are used. This practice is done because maximum likelihood models are limited as to the number of parameters it can fit; so parameters are often fit using one model specification, then fit using another specification, then tied together to make a meta-model. This type of model building is ideologically Bayesian because it uses prior information which is not simultaneously fit (maximized). Bayesian methods are used at least in part in many pharmacokinetic models, especially those which draw information from previous studies. Another obvious example is the typical development of a PBPK model whose parameters, such as Vmax and Km for metabolism, transport, or elimination processes, partition coefficients, cardiac outputs, etc which were determined from a host of different experiments. Most pbpk studies use only mean values as opposed to using distributions, which are normally reported in studies, and analyze aggregate data which is misleading at best (Bois, 1999; Sheiner & Ludden, 1992; Sheiner, 1984), because little statistical treatment is given. Also, the PBPK typically does not state posterior probabilities or distributions for all output, only for one or a few parameters of interest. Thus, because of the two preceding reasons, typical PBPK models, as obvious in a cursory review of the literature, are not

Bayesian. Neither are pbpk models Frequentist since no likelihood function is maximized; indeed, likelihood functions in the Frequentist sense (and based on residuals) have little to no meaningful interpretation in PBPK models owing to the large number of parameters and equations, and consequent mathematical massaging.

Recent use of Markov Chain Monte Carlo simulation methods has been demonstrated in PBPK models of environmental contaminants (Johanson etal, 1999; Bois, 1999; Bois etal, 1999; Bois, 2001). Markov Chain Monte Carlo simulation is a technique (often used in a Logical Objective Bayesian framework) utilizing the Markov Property, which states that the state of X at time, t+1, depends only on the state at time, t, (or, $P[X_n = j \mid X_{n-1} = i] = p_{ij}^n$). The stochastic process utilizing this property is called the Markov Chain. If the process samples randomly from a (set of) distribution function(s) to produce simulated data, it is a Markov Chain Monte Carlo simulation (Thompson, 2000, p. 24-8, 71-6). It is Bayesian because it requires prior information and Logical Objective because the Markov condition does not hold for Subjective or Empirical Bayesianism (Williamson, 2001).

WHICH METHOD TO USE?

The fact that the pharmaceutical industry generally uses maximum likelihood (Frequentist) methods does not make it always correct, even for the problems it uses them on. Nor does the fact that the "physiologically based" modeling camp uses a partial Bayesian strategy to model complex phenomena make them any more correct. Between the various Frequentist and Bayesian methods of estimations and model fitting, the obvious question an unindoctrinated scientist will naturally ask is "which one is better?"

In situations in which parameter distributions are not known, and the number of parameters in relation to number of dependent variables is small, maximum likelihood

will give both reliable estimates of parameter distributions and a resulting estimate of goodness of fit to be used in choosing between appropriate models. Situations using prior information such as parameters maximized in previous estimation procedures indicate Bayesian methods. Why? Because maximum likelihood does not formally consider prior information (previously maximized parameters) and only deals with simultaneously fitting of all parameters. The previously estimated parameter distributions are not the true parameter distributions, nor can any estimation give the true distributions of parameters, only estimates of them; thus, the practice of fixing parameters in partial fitting, may lead to erroneous final parameter estimates, because local minima are difficult to find with partial fitting in maximum likelihood contexts, for all parameters used in the model (Bois, 1999).

This situation, that of fixing some parameters, based on the results of fittings of previous experiments, and fitting others is the “linked” or “combined” model described previously. Because this situation uses prior information, it is ideologically Bayesian. In addition, the state variables (those numerous, fixed variables which enter into differential equations) used in these large-number-of-parameters models are almost invariably gotten from the literature; thus, nearly all of them have been determined from previous experiments in a range of unextrapolable conditions and by a large number of independant investigators whose methods are almost certainly dissimilar. In this sense, it is easy to see the value of *not* fixing parameter values since they, in addition to not being the *true* values, are complicated by interoccasion and inter-investigator variability (Bois etal, 1990; Karlsson & Sheiner, 1993). High dimensional mechanistic physiological models in which a very large number of state variables in relation to the number of dependant variables can give reliable estimates of parameter distributions when using Bayesian estimation methods (Sheiner & Steimer, 2000).

The Bayesian method of MCMC simulation gives a modeler the ability to model a larger number of parameters in relation to number of dependant variables than

maximum likelihood methods because both the parameter (its mean and distribution) and probability, both prior and posterior, are used to give an overall probability of the model and its parameter vector; the data serving to filter the prior information to give a posterior distribution (Bois, 1999). Both current computer hardware and current operating systems are robust enough to handle the computationally intensive task of MCMC simulation. Many software packages are technically capable of handling mixed effects modeling of both (Frequentist & Bayesian) types as well as Monte Carlo simulations (including Bootstrap and MCMC simulation). The knowledge of statistical methodology, which is not widespread especially in the physiologically based community, and the programming expertise required to do any of these is both substantial and solely on the shoulders of the end-user. Despite the nearly decade old existence of freeware MCMC simulation software, this modeling technique in the pharmacokinetic communities, whether pharmaceutical, toxicological, physiologically based, regulatory, academic or otherwise, is limited in its literature presence to a handful of investigators and a maximum of 20 papers. Among reasons for this absence are probably both lack of knowledge of this technique or even its existence.

OTHER PHARMACOKINETIC MODELS

In addition to classical (compartmental and noncompartmental), physiologically based, pharmacodynamic, and population models is the vast field of clinical pharmacokinetic models. Clinical models may incorporate techniques from all the aforementioned techniques but involve several issues which commonly arise only in clinical testing. Covariate modeling involves use of a covariate, a measurement which is not the dependant variable (either concentration or effect), and maybe used to describe baseline conditions in the subject. Covariate modeling is useful in drug development scenarios; however, is much more important in clinical trial simulation. By establishing a relationship between a library of compounds and some covariate, a new

compound in that library may be simulated on the covariate. The deviation model is extremely important for clinical trials as it considers the frequent deviations from the planned initiation of a trial, compliance during the trial (eg, patient forgot a dose), and termination (eg, patients drop out or must be excluded).

SIMULATION

The role of simulation in any scientific industry is "to improve the process and efficiency of research and development" (Johnson, 1998). Simulation is "the generation of pseudodata on the basis of a model, a database, or the use of a model in the light of a database" (Thompson, 2000). It may be used to understand complex processes before manufacture, scale up, and production (Johnson, 1998). There are two types of simulation, iconic and mathematical. Iconic simulation uses scale models such as a miniature scale model boat in a similarly scaled model lake; iconic simulations are done by experimental scientists. Preclinical pharmacokinetic studies may be regarded as iconic in that animals are used (for ethical and budget purposes) to represent humans. Animals however are not true iconic models since each species has a different set of metabolizing enzymes and transport proteins with different (drug to protein) affinities and capacities than analogous proteins and enzymes in humans (or other species). Mathematical simulation is computer based and is often called in silico, in numero, or virtual simulation; mathematical simulation is accomplished by modelers.

For the pharmaceutical industry simulation is especially important for clinical trials, for the sake of both safety and the extraction of the highest level of information from trials; pharmacokinetic simulation is becoming equally important in pharmacokinetic derivation of NOAELs, LOAELs, and BMDs (no/low observed adverse effect levels, bench mark dose (the upper 95% confidence interval of the dose affecting five percent of the population (IRIS, 1999)) of environmental toxicants. The end goal in both cases is the generation of an effect incidence in a large and disparate population

based on a mechanistic and probably complex model. Monte Carlo methods of simulation are frequently used to generate "pseudodata" into which noise (attributable to measurement error) has been introduced. Two types of Monte Carlo are typically used in pharmacokinetic simulations. The first uses synthetic data sets and the second relies on data resampling. The first method fits pharmacokinetic parameters then large numbers of synthetic, noisy data sets are generated from the parameters by adding synthetic measurement errors to model predictions (Press etal, 1992). The second method uses a data resampling method, the Bootstrap, in which parameters are generated with LS or ML methods then points are randomly removed and replaced with replicates for the remaining data to generate synthetic data (Efron & Tibshirani, 1993). Other means of data resampling, such as the Jacknife, have been used as well (Bonnate, 1997; Holder etal, 1999; Efron, 1992).

"For simulation studies, mechanistic models are encouraged. Such models are expected to extrapolate to new situations better than empirical models, and exploring the study design properties in a simulation project inevitably requires extrapolation beyond current data" (Holford etal, 1999).

Changing world

The pharmaceutical industry has traditionally used the Build and Test Paradigm (Johnson, 1998). After much research and hypothesizing about an intended molecular target, a medicinal chemist synthesizes a chemical. The chemical is characterized as to its physiochemical properties first, then some basic in vitro pharmacology work is done with some analog of the intended target. If all goes well, the drug is then introduced into animals for, again, basic pharmacology, basic dose response. Provided things go well, pharmacokinetics are studied. This cycle continues until the chemical is tested in man in a preliminary clinical study in a subpopulation for drug safety (toxicokinetics) and pharmacokinetics. Then, once again, scale up to a larger population. The Build and Test

Paradigm has served the pharmaceutical industry well for many decades. But the cost of clinical trials of INDs/NDAs is rising (Peck etal, 1994). And so is the failure rate of INDs/NDAs, based mainly on poor efficacy and/or poor pharmacokinetics in humans (Borchardt etal, 1996; Lesko LJ etal, 2000; Kennedy, 1997; Peck CC etal, 1994). Based solely on these two facts, the pharmaceutical industry is already hearing death knells for the Old Paradigm and it is looking at more cost efficient methods of modeling to ease the pressure (Holford etal, 1999). The pharmaceutical industry is pushed by economic forces within to develop new means for determining pharmacokinetics.

The toxicology community has used modeling for quite some time and its favored method, the physiologically based pharmacokinetic model, is highly mechanistic. Until recently however, simulations were done in mean animals, that is, virtual animals simulated with only means of each parameter. Values for state variables (most of which are considered covariates) such as organ volume may now be measured in live animals by ultrasound (Chang FM etal, 1997; Riccabona etal, 1997; Patlas etal, 2002) while until recently, values reported in the literature have been typically used. And finally, statistical validation of the complex high dimensional models is not commonly done, despite existing methods of doing so. The toxicology community is pushed by the need to catch up to its pharmaceutical cousins.

Advances in computing and modeling have been implemented at a much more rapid rate in other fields, taking products from concept and design, through scale up and production, and to a final marketed product with little need for physical testing. The seminal example is the Boeing 777, a product designed and tested virtually and only then was the first model built, only to validate the virtual model (Boeing, 1999). Because physiological systems are much more complex than physical systems, produced from parts manufactured with little interlot variability, like airplanes and spaceshuttles, and because we only now understand a mere fraction of human physiology, physiologically based pharmacokinetic modeling is a long way from the computer assisted design of drugs.

The need for reform in the Drug Development paradigm is not a new concept. The institution of the Prescription Drug User Free Act (PDUFA) in 1993, whose purpose was to decrease review time for "off label" uses of prescription drugs, was a first step in pharmaceutical reform. Carl Peck, founder of the Center for Drug Development Sciences (CDDS) as early as 1996 in his testimony to the House Subcommittee on Health and Environment, stated that drug development time can be dramatically shortened pendant on several reforms, one of which is improvements in the simulation of clinical trials based on the incorporation of new science and new technologies (Peck, 1996, 1999). Since pharmacokinetic modeling is a very large part of clinical trial simulation, and since poor pharmacokinetics in man is one of the major causes of failure of drug candidates, it stands to reason that improvements in pharmacokinetic modeling must be made. But, Dr. Peck's recommendations were based only on notions of ill-conceived or redundant trials and inefficient use of investor money; since the time of his recommendation a further monumental achievement in science has brought a new stressor to pharmaceutical development.

With the mapping of the human genome the number of potential pharmacological targets has increased dramatically. The concomitant increase in the number of drug candidates combined with the increasing cost of drug testing and the high failure rate of new drug applications (NDAs), the need for accurate screening of pharmacokinetic properties before costly animal and clinical testing has already begun to increase. If the current paradigm were used, the cost of even preclinical studies in a mere fraction of these chemicals would eclipse the possibility of long term profits, thereby introducing downward economic spiral into a heretofore stable pillar of global economy. Thus, the strategy for screening compounds identified by combinatorial chemists based on the mapped genome must change dramatically from one of testing a chemical in man to prescreening a combinatorial "hit" in a simulated man *before* the chemical is ever synthesized, in silico design meeting in silico testing. The pharmaceutical sciences are slowly entering a new era in which simulation of clinical trials becomes highly

mechanistic and therefore more predictive, and more applicable across different situations and occasions.

According to Lesko, etal (2000), pharmacokinetic modeling must come to incorporate human in vitro data (including metabolism, saturable distribution/excretion phenomena, and biopharmaceutics data), richer protein binding data, more whole-body physiologically based models, and more quantitative structure activity relationships in the PK/ADME model. Because the ultimate goal of pharmacokinetic/pharmacodynamic analysis is predictive, not descriptive, pharmacokinetic models should be more mechanism based (Derendorf etal, 2000); this trend is increasing but will need to do more so in the future to accommodate the input of the recent advances in proteomics and genomics. This level of simulation will likely be the thrust of large pharmaceutical industry as it attempts to reduce failure and cost by conducting virtual clinical trials on virtual drugs (Holford etal, 2000).

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CHAPTER 2

PHARMACOKINETICS OF 5-IODO-DIOXOLANYL-URACIL IN RATS[1]

[1]Boyd JL, FD Boudinot, CK Chu. To be submitted to *Antiviral Chemistry and Chemotherapy*.

ABSTRACT

The nucleoside analog (2S, 4R)-1-2-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (IOddU) has potent activity against Epstein-Barr virus. A reverse-phase HPLC analytical method using UV detection for its determination in biological matrices was developed and the pharmacokinetics of IOddU were characterized in rats. The retention time for IOddU was 4.9 min and 7.0 min for internal standard (AZdU). The extraction recovery of the compound was greater than 91%. The standard curve was linear from 0.25 mg/L to 200 mg/L and the limit of quantitation of IOddU in rat plasma was 0.25 mg/L. Inter- and intra-day relative standard deviations (rsd) were less than 15%. Mean (± rsd) AUC after intravenous administration (n=6) in DMSO and AUC after oral administration (n=5) in DMSO were 32.83 (0.374) and 32.06 (0.447); bioavailability was 97.7%. Mean (± rsd) plasma clearance after iv administration and renal clearance were 1.44 (0.588) and 0.69 (0.46) L/h/kg. Mean volume of distribution was 0.643 (0.619) L/kg. MRT (h), λ_z (min^{-1}), and fraction of dose excreted in urine were 0.510 (0.527), 1.08 (0.714), and 47.8%. Fraction excreted in bile was 0.9% (n=1).

INTRODUCTION

Epstein-Barr virus (EBV) is the member of the herpes virus family responsible for infectious mononucleosis and fatal acute infectious mononucleosis/X-linked lymphoproliferative syndrome and correlated with the appearance of Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, post transplantation and other lymphomae, and several other malignancies. The newly synthesized nucleoside analog (2S, 4R)-1-2-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (L-lOddU, lOddU) has been shown to have potent activity against the replication of EBV (Lin etal, 1999),

Various compounds have been shown to have activity against EBV replication and transformation including polypeptide fractions isolated from porcine liver and gastric mucosa (Ablashi et al, 1994a,b; Venanzoni et al, 1997), acridone alkaloids (Takemura et al, 1995), lantadene triterpenoids (Inada et al, 1995), NSAIDs (Kapadia etal, 2000), cyclic diarylheptanoids (Ishida etal, 2000), citrus flavanoids (Iwase etal, 2000), *Mellittia* isoflavanoids (Ito etal, 2000), ferulic acid derivatives (Murakami etal, 2000), and sequiterpenes from *Maytenus* cuzcoina (Gonzáles etal, 2000). Nucleoside analogs have also been shown to have antiviral activity (Wang etal, 1998), and several have been evaluated for activity against EBV (Lin et al, 1985; Yao et al, 1996; Chu et al, 1995). Several L-nucleoside analogs have been shown to be more resistant to p53 exonuclease dependant excision from the 3' end of DNA than the more natural D-nucleoside analogs (Kukhanova et al, 2000) and it is reasonable to assume the same property exists with lOddU. lOddU has lower EC_{50} and EC_{90} (mean (±sd): 0.033 (0.017), 0.016 (0.075) /M or 11.22 (5.78), 5.44 (25.5) mg/L) versus EBV DNA replication than several other nucleoside analogs while maintaining higher cytotoxic inhibitory doses ID_{50} (>1000 /M or 340 g/L) and a lower degree of mitochondrial DNA toxicity than the same compounds in the high-yield EBV-producing cell line H1 (Lin

etal, 1999; Bacon and Boyd, 1995). The purpose of this study was to characterize the preclinical pharmacokinetics of lOddU in rats.

MATERIALS AND METHODS

Chemicals and Reagents - lOddU was synthesized as previously described (Kim, et al, 1993). 3, Azido, 2',3'-dideoxyuridine (AZdU) used as the internal standard was synthesized as described previously (Chu etal, 1989). Acetonitrile, HPLC grade, and all other reagents, analytical grade were purchased from J. T. Baker (Phillipsburg, NJ).

Preparation of standards – Standard solutions of lOddU were prepared in 50% acetonitrile. Standard curve concentrations for lOddU covered a range of 0.05 g/L to 200 g/L. Internal standard solution of 50 g/L AZdU was prepared in water.

Sample handling - To 100 /L plasma samples were added 50 /L internal standard (AZdU 50 g/L). Tubes were vortex mixed for 30 sec,50 /L 2 M perchloric acid was added, tubes were vortex mixed again for 30 sec, and centrifuged at 5000 g for 10 min. The supernatant was withdrawn and 50 /L was injected onto the column. Urine was diluted 1:100 with filtered, deionized water. One hundred microliters (100 /L) diluted urine was withdrawn, 50 /L AZdU was added and 100 /L was injected onto the column. Bile was prepared in the same way as urine to 1:10 or 1:100 dilution.

For determination of lOddU excreted as glucuronide, the following procedure was used. To 1 mL rat urine was added 100 /L -glucuronidase (reconstituted to 10000 U/mL) then vortexed for 30 sec and incubated in a 37° C water bath for 12 h. The same procedure was followed on separate one mL aliquots of urine except that phosphate buffer was added instead of -glucuronidase solution, as a control. Urine was analyzed by HPLC for lOddU content by the method described above.

Chromatography – The HPLC system consisted of a Waters Model 510 solvent delivery system, Model Waters WISP 712 autosampler, Waters Lambda-Max Model 481

LC Spectrophotometer, and LDC Analytical D-2500 computing integrator. A mobile phase of 7% (v/v) Acetonitrile in 40 mM sodium acetate, pH 7.4. was used with a Hypersil (Alltech) ODS (C18) 5 /l/ column (150 x 4.6 mm). The flow rate was 1.5 mL/min. The UV wavelength used for detection was 280 nm.

Quantitation – Concentrations of lOddU in unknown samples were determined by the use of the slope of the standard curves of peak-area ratio (of drug/internal standard) versus standard drug concentration. Slopes were determined using linear regression analysis with a $1/x^2$ weighting factor. With this weighting factor, a normal distribution of the weighted residuals around the fitted standard curve was seen over the entire range of drug concentrations.

Assay specifications – The extraction recovery of lOddU and internal standard was determined at concentrations of 0.25, 0.5, and 50 g/l by comparing the peak area of the analyte from six extracted serum samples and six direct injections of the same amount of the drug in mobile phase. Percentage recovery was calculated from 100% x (peak area of the extract)/(mean peak area of nonextracted samples). Precision and accuracy were determined at the aforementioned concentrations by injecting seven replicates at each concentration in addition to the standard curve for each day of validation; precision in this case being the equivalent of the relative percent standard deviation subtracted from 100% and accuracy being observed concentration divided by intended concentration and multiplied by 100%.

pKa determination - The pKa was determined for lOddU by preparing a series of pH dilutions ranging from 2.5 to 12.5 (Toerell and Stenhagen, 1938). A two mL aliquot of each solution was dispensed into vials containing lOddU. The pKa was determined as the inflection point of the absorbance curve as measured by a Bausch & Lomb Spectronic 2000 spetrophotometer.

Animals – Twelve male Sprague-Dawley rats used for the pharmacokinetic studies were obtained from Harlan-Sprague-Dawley (Indianapolis, IN). Rats were

housed in a temperature controlled environment (22° C) on a 12 h light, 12 hr dark cycle and fed a diet of Lab Diet 5001 (PMI Feeds, St. Louis, MO) ad libitum. Rats were allowed a one week adjustment period before experiments began. The day before the study, jugular vein cannulas were surgically implanted under ketamine:acepromazine:xylazine (50:3.3:3.3 mg/mL per 100 g body weight) anesthesia. For the biliary excretion study, one rat was cannulated at the right external jugular vein and at the bile duct under the same anesthesia protocol as above.

Experimental Design – For the pharmacokinetic study, six rats were either infused with 50 mg/mL lOddU in DMSO (total volume did not exceed 0.25 mL) via the jugular cannula over a three minute period or dosed by intragastric gavage with similar solution of lOddU. Blood samples (0.3 mL) were collected via the jugular cannula prior to and at 0.08, 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8 h after drug administration and placed into heparinized tubes. Blood volume was replaced with normal saline. Heparinized blood was centrifuged and plasma was frozen at -20° C for later HPLC analysis.

For the biliary excretion study, bile was collected into previously weighed tubes over 30 minute intervals, weighed, and frozen at -20° C.

Pharmacokinetic analysis – Pharmacokinetic parameters were determined by area moment analysis. The area under the concentration versus time curve (AUC) and the first moment (AUMC) were calculated by Lagrange polynomial interpolation and integration from time zero to the last sample with extrapolation to infinity using the nonlinear least squares terminal slope. Systemic clearance (CL) was calculated from dose/AUC, mean residence time (MRT) from AUMC/AUC, and volume of distribution at steady state (V_{SS}) from MRT*CL. The Two Stage Method was used to first determine individual pharmacokinetic parameters and from these determining the mean and relative standard deviation (rsd).

Protein binding – To determine protein binding of the compound, a range of concentrations of IOddU in 1 mL aliquots of rat plasma was prepared (0.5, 0.75, 1.0, 1.5, 2.5, 1.0, 5, 10, 25, 50, 100 mg/L) in Centrifree YM-30 devices (molecular weight cut off, 30,000; Amicon Bioseparations, Millipore). The devices were accelerated over two minutes to a relative centrifugal force of 2,000 g and at 37 C in a Jouan 4.22 benchtop centrifuge for 30 minutes. Both filtered and unfiltered fractions were measured by HPLC using the method described previously for IOddU in plasma.

RESULTS & DISCUSSION

Chromatograms corresponding to extracts of blank rat plasma spiked with IOddU and AZdU (λabs = 280nm) and of blank rat plasma spiked with AZdU are illustrated in Figures 2.1. IOddU and AZdU each eluted with a single sharp peak and distinct baseline resolution at retention times of 4.7 and 6.9 min, respectively. Drug free plasma samples were consistently free of interference at the retention times corresponding to both drug and internal standard.

Calibration plots were linear over the range of 0.25 mg/L to 200 mg/L and the limit of quantitation for IOddU was 0.25 mg/L. Assay specifications, including extraction recovery, intra- and inter-day precision and accuracy, are presented in Table 2.1. The extraction recovery of IOddU was above 90%. The intra- and inter-day precision of the assay was satisfactory. The relative standard deviations were less than 15% at the low concentration and less than 10% at medium and high concentrations. The accuracy of the analytical method was above 85% at low and above 90% at medium and high concentrations. The pKa of IOddU was 9.6. The compound is negligibly water soluble.

Plasma concentrations as a function of time for IOddU after intravenous and oral administration are shown in Figures 2.2 and 2.3 and a summary of the pharmacokinetic parameters are given in Table 2.1. As would be expected from a highly lipophilic compound of low molecular weight, peak drug concentration was obtained rapidly

followed by a rapid distribution phase with maximum serum concentrations of 306 g/l (at 5 min) and 19.45 g/l (at 27 min) after intravenous and intragastric administration, respectively. The area under the plasma concentration time curve (AUC) following intravenous administration of lOddU to rats was 32.83 (0.374) mgh/L (mean (rsd)) and 32.06 (0.460) mgh/L following oral administration. The oral bioavailability of the compound was 97.7%. The MRT and λ_z after intravenous administration were 0.510 (0.527) h and 1.08 (0.714) h^{-1}, respectively. The compound's average total clearance from plasma was 1.44 (0.588) L/h/kg and 1.8 (0.375) L/h/kg after intravenous and gavage administrations, respectively. With a mean volume of distribution of 0.643 (0.619) l/kg for intravenous administration it is freely distributed throughout body water. The compound's half-life was 0.74 (0.712) h after intravenous administration and 1.54 (0.635) h after oral administration. Variability in the estimation of the volume of distribution and half-life may be a result of incomplete characterization of the terminal phase of the compound. Mean renal clearance was 0.69 (0.46) L/h/kg after intravenous administration, which is less than a renal blood flow value of 2.2 L/h/kg (Davies & Morris, 1993) but higher than glomerular filtration rate (0.27 l/kg/h), denoting some active secretion. The mean fraction of the drug bound to plasma proteins was 0.74 (0.236).

Excretion of the unchanged drug in urine accounted for 47.8% (0.173, n=6) and 35.2% (0.541, n=5) of the dose after iv and ig administration, respectively. Excretion of the unchanged drug into bile accounted for 0.9% (n=1) of the administered dose. Excretion of the drug as glucuronide conjugates accounted for 15.7 (0.406) % of the dose in urine.

Due to the compound's insolubility in either water, methanol, or ethanol, the aprotic solvent DMSO was used as the vehicle for both intravenous and intragastric administrations (Morrison & Boyd, 1987). DMSO is also a penetration enhancer whose penetration potential is suspected to be due to its membrane disruption properties (Osol

& Hoover, 1975). At least one animal showed toxicity in the form of transient hemolysis of blood (up till 2 h) and poor affect, an effect potentially attributable to DMSO; however, its effect on volume of distribution or clearance in this experiment is unknown. DMSO has been shown to alter pharmacokinetics in the past. It is a well known permeation enhancer (Li & Birt, 1996) due to its ability to alter membranes. DMSO has been shown to inhibit the metabolism of urethane (Waddell etal, 1989) which is suspected to be metabolized by aldehyde or alcohol dehydrogenase or an alcohol preferring cytochrome, acetaminophen (Jeffery etal, 1988), sulindac (Swanson), lindane (Chadwick etal, 1977), monocyclic aromatics (Stock & Fouts, 1971). The extent and specificity of hepatic metabolism of lOddU was not studied in this experiment; however, there is potential for interaction with DMSO.

Other uridine analogs have shown similar pharmacokinetics. A uridine analog with similar pharmacokinetics to lOddU is CDU (5-carboranyl-2'-deoxyuridine; Jarugula etal, 1994). Its mean volume of distribution of 0.70 (0.33) l/kg is very similar to the lOddU. Because CDU is explicitly cleared hepatically, it has a relatively CDU's low mean plasma clearance of 0.69 (0.29) l/kg/h; thus in terms of clearance the two compounds are not comparable. Additionally, it has a high mean fraction bound to plasma proteins, 0.95 (0.2); lOddU's mean fraction bound to plasma proteins is 0.74 (0.24) l/kg/h. The compound, AZdU (3'-azido-2', 3'-dideoxyuridine) was shown to have mean plasma clearance at 50 mg/kg of 1.27 l/kg/h and mean volume of distribution of 0.939 l/kg in mice (Doshi etal, 1989). AZddU, because of its substituents, is expected to be more water soluble than lOddU whose iodine group is only weakly electronegative and therefore weakly polar imparting low solubility in water (Morrison & Boyd, 1987, p.31-3), and also due to its polar azide group. AZdU's higher mean plasma clearance may be due to its higher solubility in water or less tubular reabsorption. A third uridine analog, LFMAU (1-(2-fluoro-5-methyl-β-L-arabinofuranosyl)-uracil), has a mean plasma clearance of 1.15 (0.24) L/h/kg in rats

(Chu etal, 1998), lower than lOddU's; as their renal clearances are similar (0.67 and 0.679 L/h/kg for LFMAU and lOddU, respectively) the difference in clearances is extrarenal and likely due to increased hepatic metabolism. Although it has a 5' methyl group which as stated earlier would give similar solubility properties to lOddU, LFMAU's mean volume of distribution, 1.12 (0.29) l/kg, is nearly twice that of lOddU perhaps owing to both the fluoro-substitution at the 2-position, and the 3-hydroxy group of the pentose moeity imparting greater ability ot bind to tissue proteins.

Concentrations were above the EC_{50} and EC_{90} for virus inhibition for just under one and two hours respectively. Concentrations never approached the ID_{50}, which was larger than 340 g/l, for cytotoxicity, and never approached concentrations which would cause mitochondrial toxicity (over 50 /_/M, or approximately 17 mg/mL).

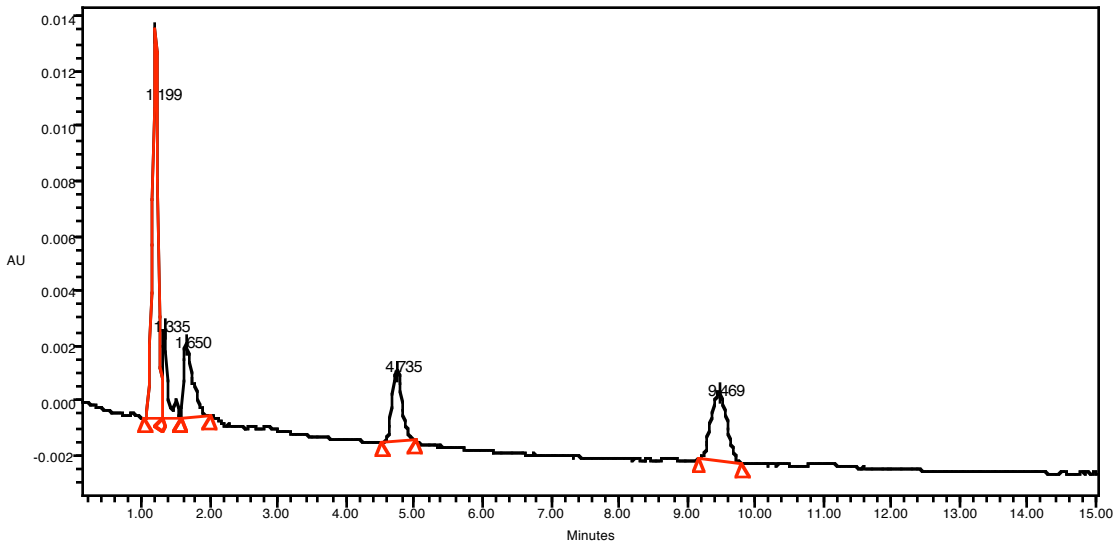
CONCLUSION

Pharmacokinetics of lOddU are similar to other nucleoside analogs. Its body weight adjusted volume of distribution at steady state (0.643 l/kg), corresponding to total body water, is low in comparison to other nucleoside analogs. Its total body weight adjusted clearance (1.4 L/h/kg) is median in comparison with other uracil analogs though it should be noted that compounds with similar clearances have been considered good candidates for further development (Chu etal, 1998). The concentration at this dose was above the EC_{50} for nearly two hours. Its pharmacology and low toxicity profiles in vitro in combination with favorable pharmacokinetics make it a good candidate for further studies. This study was supported in part by US Public Service Grants AI-25899.

Table 2.1: Mean pharmacokinetic parameters (rsd)

parameter	iv	oral
AUC, mgh/L	32.83 (0.37)	32.06 (0.45)
MRT, h	0.51 (0.53)	
λ_z, h-1	1.08 (0.71)	
half life, h	0.74 (0.7)	
Vss, l/kg	0.64 (0.62)	
Cl_t, L/h/kg	1.4 (0.6)	
Cl_r, L/h/kg	0.69 (0.46)	
Cl_{nr}, L/h/kg	0.73 (0.36)	
f_u, %	47.8% (0.17)	
f_{gr}, %	15.7 (0.41)	
f_b, %	0.9	
f_{bound}, %	74 (0.24)	
k_a, h^{-1}		0.65
F, %		97.7%

Figure 2.1: Blank rat plasma with AzdU (top), blank plasma with lOddU and AzdU (bottom)



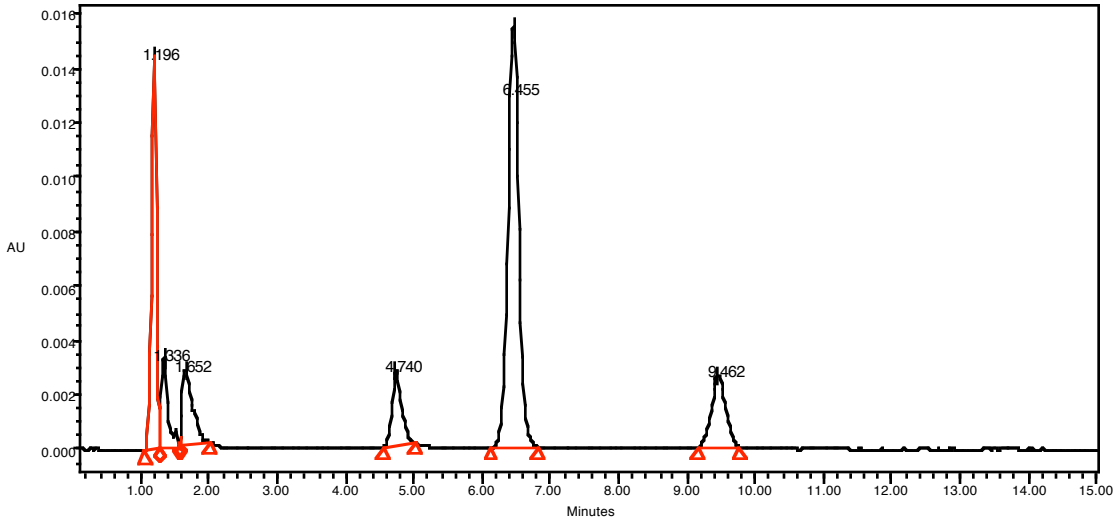


Figure 2.2: Average plasma concentration versus time curve in six rats after intravenous administration

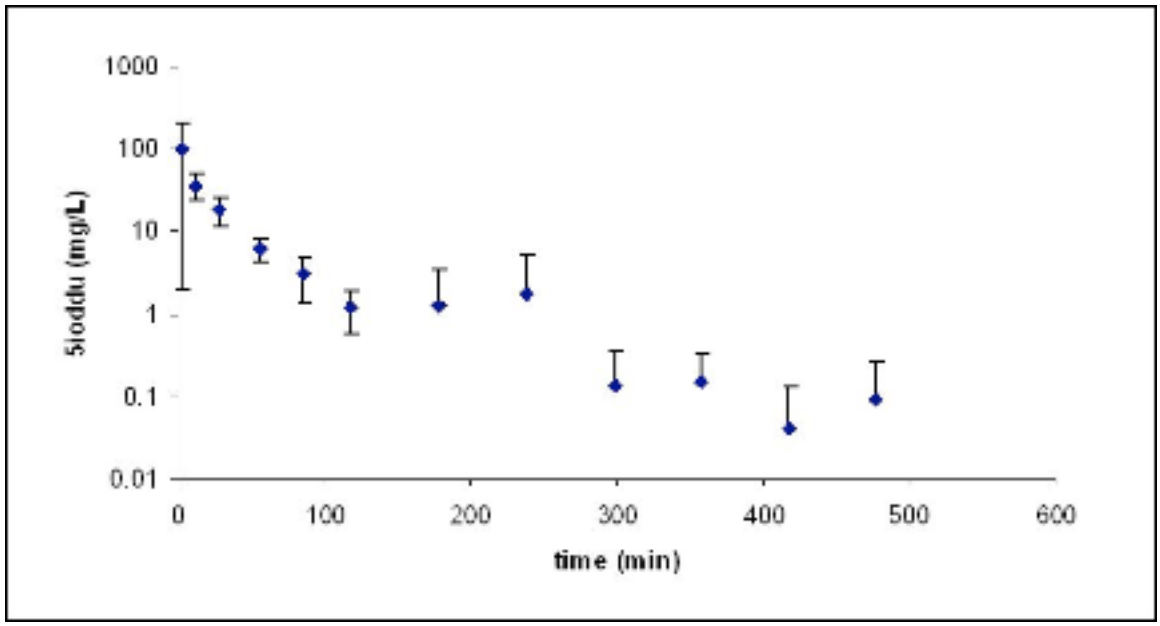
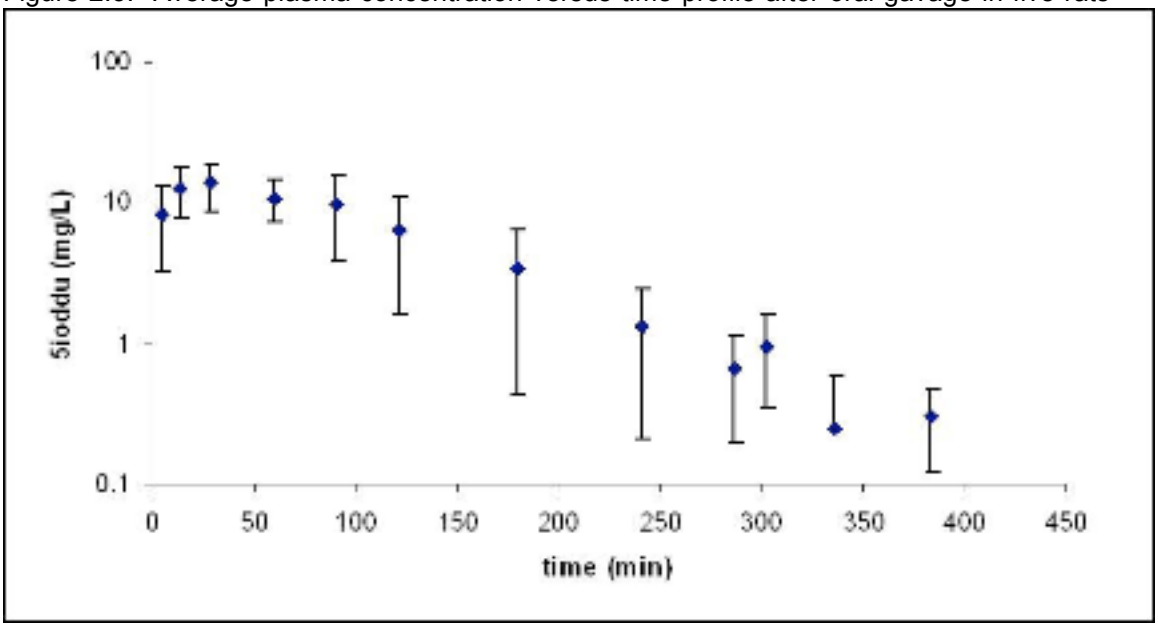


Figure 2.3: Average plasma concentration versus time profile after oral gavage in five rats



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CHAPTER 3

ANALYSIS OF IOHEXOL IN LEAN, FATTENED, AND OBESE DOGS[1]

[1]Boyd JL, FD Boudinot, DM Finco. To be submitted to *American Journal of Veterinary Research*.

ABSTRACT

Because it is desired to determine iohexol's suitability to be used in veterinary practice as a convenient means for determining glomerular filtration rate, its clearance, and therefore, glomerular filtration rate, in lean, fattened, and obese dogs was investigated and compared with urinary clearance of creatinine. Plasma clearance of iohexol was calculated by four methods: area moment, compartmental, terminal phase (using five points), and a three point (three points in the terminal phase) method. Area moment and compartmental clearances, both absolute and body weight adjusted, of iohexol underestimate creatinine clearance. Both methods using the terminal elimination phase resulted in clearance values higher than plasma iohexol clearance by the full time course methods; both however were similar, by ratio comparison, to urinary creatinine clearance indicating that either method may be appropriate for use in veterinary practice. Absolute clearance of iohexol increased with obesity whereas body weight adjusted clearances of iohexol did not. Body weight adjusted volume of distribution decreased with obesity, whereas absolute volume did not.

INTRODUCTION

The first choice for assessment of glomerular filtration rate (GFR) and renal function by clinicians is often creatinine clearance. Creatinine is endogenous and excreted in urine which makes it a convenient marker for GFR; however, because it is secreted by the renal tubules, it consistently overpredicts GFR. Glomerular filtration is most accurately described by compounds which are freely filtered at the glomerulus, and neither secreted nor reabsorbed by the renal tubules, are not metabolized, and are not protein bound. Many compounds have been used in human medicine for decades for this purpose. Iohexol is one of these compounds. It is a radio contrast agent which distributes to extracellular water and is neither secreted nor reabsorbed by the renal tubules, and whose elimination is fully renal. In clinical veterinary practice, GFR is most commonly assessed using the 24 hour timed urine collection method assaying for creatinine, a time and manpower consuming and messy practice. Use of iohexol for the determination of GFR would abolish messy timed urine collections, and by collection of plasma samples within the terminal elimination phase of an iv bolus dose, provide a rapid assessment of GFR in contemporary veterinary practice.

In support of the effort to establish a three point sampling method for using iohexol to determine glomerular filtration rate, various methods were used to analyze data sets of dog plasma concentrations. Ten dogs were dosed with iohexol in three different dosing periods. The first period, the dogs were lean. The dogs were fattened, and dosed with iohexol again. Finally, the third period of dosing involved the same dogs futher fattened to the point of obesity. A single bolus of iohexol was administered intravenously during each period. Blood samples were taken and analyzed for iohexol by two different methods. The concentration of creatinine in urine was determined for the sake of comparison.

The purpose of this study was to determine the effects of obesity on the glomerular filtration rate, and thus total clearance, of iohexol. Comparisons were made

between clearance of iohexol determined by a number of methods, and urinary creatinine clearance. A secondary aim of this experiment was to determine if obese dogs display different pharmacokinetics from lean dogs.

METHODS

Area moment analysis using the full 12 point data set was accomplished via Lagran. Winnonlin was used for compartmental modeling using the entire data set of 12 data collection points. Models fit were one, two, and three compartment models. Model fit was chosen by comparison of AIC, SC, and distribution of residuals. Clearance of iohexol was also determined using the terminal phase of the blood concentration versus time curve. Finally, clearance was determined using the slope and intercept of a regression line fit through three predetermined time points. In humans, iohexol usually displays a two or three compartment model, the third compartment being disposition to extravascular interstitial water. Differences in compartmentalization in humans may be due to physiological differences in the state of the vascular system between two different times or two individuals, or may be artefactually related to sampling times and duration. Reported means and rsd's are derived by the Two Stage Method.

Equations

Area moment derived GFR

$$GFR = Cl_R = Cl_{tot} = D/AUC, \qquad \text{Equation 3.1}$$

where AUC was determined by Lagrange interpolation of the concentration versus time data to the last time point and extrapolated to infinity based on the slope of the terminal phase, which was assumed to include the last five time points.

Compartmental derived GFR

$GFR = Cl_R = Cl_{tot} = D/(A/\!/ + B/\beta)$, Equation 3.2

where A is the y-intercept of the regression of the distribution phase line, $/\!/$ is its slope and the B, β terms are corresponding values for the regression of the elimination phase line. Weighting was 1/y(observed).

Elimination phase compartmental GFR

$GFR = Cl_R = Cl_{tot} = D/(B/\beta)$, Equation 3.3

where B and β are the y-intercept and slope of the terminal phase (final three or five points) regression line, and B/β being the equivalent of a partial AUC.

As the two terminal phase derived clearances are derived from only part of the data, their clearances will overestimate the true clearance of iohexol. Therefore, clearance derived in these methods were reported as is, and also after tranformation using the Brochner-Mortensen method (Brochner-Mortensen, 1972).

RESULTS & DISCUSSION

Table 3.1 lists GFR as determined by the methods described, as well as the urinary clearance of creatinine and volume of distribution parameters. Volumes of distribution derived from the full compartmental analysis are nearly identical to those derived by area moment analysis. Figure 3.1 gives ratio values of plasma clearance of iohexol versus urinary clearance of creatinine. When compared by ratio to creatinine clearance, the clearance of iohexol was consistently and across groups less than unity for both area moment derived and compartmentally derived methods. A ratio lower than one is expected in accordance with the fact that creatinine is secreted by the renal tubules. It is expected that the area moment derived and compartmentally derived clearances of

iohexol be the most accurate representations of glomerular filtration rate because of their utilization of the full concentration versus time data set.

Based on ratio comparison to urinary creatinine clearance, both the three and five point methods overestimate both the plasma clearance of iohexol and the urinary clearance of creatinine. The degree of overestimation appears to increase with obesity. The overestimation is due to a partially derived AUC (corresponding to only the terminal phase of the concentration versus time curve) which is smaller than the AUC of the full data set; in light of equation 3.3 can be seen that smaller values of AUC at a given dose lead to higher clearance values. The full time course derived clearance is the true value of plasma clearance of iohexol (and therefore GFR); conveniently for the practicing veterinarian, however, the ratio of iohexol plasma clearance to urinary creatinine clearance is close to one.

Comparison of pharmacokinetic parameters at different levels of obesity is also useful. Volume of distribution and clearance of iohexol, both absolute and body weight adjusted, as determined by the full compartmental method, were compared across the different levels of obesity. Body weight adjusted plasma clearance of iohexol, and therefore GFR, did not change significantly with obesity (p-value = 0.1846, Tables 3.2 & 3.3); absolute plasma clearance of iohexol increased significantly (p-value = 0.0013). Obesity has been shown to increase retention of sodium in humans resulting in higher blood pressures, and therefore renal blood pressure and GFR (Hall et al, 1993). Granger and Nakamura (1992) have indicated that blood pressure in obese dogs is insenstive to increases in sodium however. Renal plasma flow and GFR have been shown to increase in obese patients and dogs compared to lean subjects (Hall et al, 1993; Reisin et al, 1984) due to a combination of the increase in sodium, increase in sympathetic nervous system activity (Tuck, 1992; Hall, 1997), increases in plasma renin concentrations, thereby involving the renin-angiotensin system (Hall, 1994), leptin activity, or freely circulating fatty acids (Hall, 1997).

Absolute volume of distribution at steady state (V_{ss}) was found to increase, though not significantly (p-value = 0.2006), with obesity. This increase is intuitive as increase in body mass is accompanied by mariginal increase in water volume. Additional increases of extracellular water volume, beyond that which is required by increased body fat, are related to the nonlinear sodium retention caused by obesity (Hall et al, 1993) Body weight adjusted volume of distribution at steady state (V_{ss}/bw) was found to significantly (p-value = 0.0016) decrease across groups. In obesity, the fraction of body weight as water is smaller than the same fraction in lean patients; the result of decreasing body weight adjusted volume of distribution is not surprising since iohexol only distributes to extracellular body water and has a low octanol:water partition coefficient of –1.155 (Krause et al, 1994). Atracurium, bisoprolol, and nebivolol, all polar compounds with low LPCs, have shown similar decreases in body weight adjusted V_{ss}, yet with increases in absolute Vss with obesity (Varin F et al, 1990; Le Jeunne et al, 1991; Cheymol et al, 1997).

Use of either of the full time course methods (area-moment derived and compartmentally derived), the elimination phase (five point) method, or the three point method for determination of plasma clearance of iohexol may be appropriate for the determination of GFR while keeping in mind the differences between the methods. Use of the full time course methods are ideal and give an estimate of the free clearance of iohexol, which should be taken to be the true value of GFR (in comparison to the value arrived at by urinary clearance of creatinine). Use of either of the abbreviated time course profiles may be appropriate and provided there is predictable variability between urinary creatinine and plasma iohexol clearances between animals of varying disease states (both renal intactness, impairments of protein production, impairments of body fluid volumes).

With further investigation into the relationship between creatinine clearance and plasma iohexol clearance in differing disease states and physiological or anatomical

conditions (which impact upon iohexol clearance), iohexol may emerge as a readily available and easily interpretable marker for GFR in the modern veterinary practice.

Table 3.1: Pharmacokinetic parameters: Iohexol in various stages of obesity, analyzed by various methods

		mean phase I	rsd ph I	mean ph II	rsd ph II	mean ph III	rsd ph III
bw	kg	9.78	0.1195	13.80	0.1563	15.32	0.1967
dose	mg	2352.00	0.1178	2762.64	0.3928	3576.00	0.2037
dose/bw	mg/kg	240.54	0.0047	197.28	0.3497	233.15	0.0389
CCr(u)/bw	mL/min	4.19	0.1159	4.05	0.1230	3.85	0.0793
CCr(u)	mL/min/kg	41.19	0.1948	56.26	0.2322	58.79	0.1994
Vss/bw	L/kg	0.26	0.1619	0.21	0.1882	0.20	0.0887
Vss	L	2.53	0.2125	2.94	0.2570	3.02	0.1930
Cl/bw	mL/min/kg	3.52	0.0772	3.45	0.1555	3.20	0.1131
Cl	mL/min	34.62	0.1739	47.84	0.2333	48.24	0.1441
Wnl elim Cl/bw		4.4168	0.2047	3.6732	0.3907	4.0271	0.1850
3pt cl/bw		3.9908	0.2250	4.5273	0.1466	4.0791	0.0925
Lagran Cl/bw		3.5319	0.0734	3.4644	0.1552	3.2104	0.1167

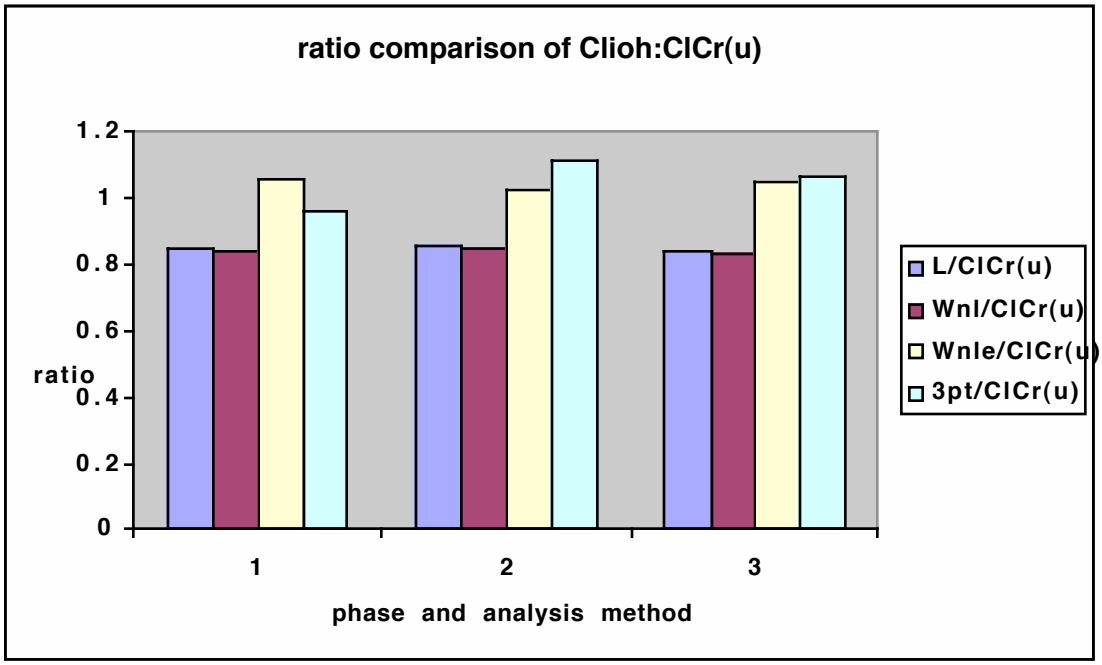
Table 3.2: Ratios of body weight adjusted plasma clearance of iohexol by various methods to body weight adjusted urinary clearance of creatinine

		L/ClCr(u)	wnl/ClCr(u)	wnle/ClCr(u)	3pt/ClCr(u)
ph i	avg	0.849	0.847	1.055	0.960
ph ii	avg	0.854	0.851	1.023	1.117
ph iii	avg	0.837	0.834	1.051	1.065

Table 3.3: P-values of clearances and volumes when compared by anova across levels of obesity

parameter	p-value
Cl	0.0013
Cl/bw	0.1846
Vss	0.2006
Vss/bw	0.0016

Figure 3.1: Bar chart of ratios of Iohexol clearance by different methods compared with urinary clearance of creatinine



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CHAPTER 4

AZT & 3TC IN CAT LYMPH TISSUE[1]

[1]Boyd JL, JK Mauldin, CW Schmiedt, CW Brockus, FD Boudinot, MA McCrackin-Stevenson. To be submitted to *American Journal of Veterinary Research*.

ABSTRACT

The tissue distribution of AZT and 3TC (zidovudine, (3'-azido-3'-deoxythymidine, AZT); and lamivudine, ((-)-2',3'-dideoxy-3'-thiacytidine) in cat lymph tissue was investigated. Five and six healthy, intact, barrier reared, 10 month old, domestic, short haired cats were infused to steady state with a solution of AZT and 3TC, respectively. Plasma samples taken and cats slaughtered; lymph tissues excised were tonsil, thymus, submandibular, bronchiolar, sternal, and mesenteric lymph nodes. Mean (±rsd) lymph tissue concentrations of AZT and 3TC in 8.13 (0.79) and 7.74 (0.66). Tissue to plasma concentration ratios were 0.36 (0.76) and 0.44 (0.51) for AZT and 3TC.

INTRODUCTION

Feline Immunodeficiency Virus (FIV), first isolated in 1987 (Pedersen etal, 1987), is a retrovirus of the family Retroviridae, subfamily Lentivirinae, to which also belong human and simian immunodeficiency viruses (HIV, SIV). Its geographic distribution is global (Olmsted etal, 1992; Sparger etal, 1989). Like both HIV and SIV, FIV has an initial stage of infection of and proliferation in the lymph nodes (Bach etal, 1994; Beebe etal, 1994; Hurtrel etal 1994; Matsamura etal, 1994; Parodi etal, 1994) in which most if not all nodes are infected by the fourth week after innoculation (Dean etal 1996); this initial stage is characterized by a flu-like illness and generalized lymphadenopathy (Yamamoto etal, 1988). Symptoms of the primary infection subside; however, viral activity continues as evidenced by the gradual decline of the CD4+/CD8+ T-lymphocyte ratio, presence of the virus in the blood, and production of viral variants (Beebe etal, 1994). The lymph node is of particular importance in lentivirus infection as the follicular dendritic cells (FDC) there serve as a continual reservoir for viral replication (Spiegel etal 1992; Pantaleo etal 1993; Beebe etal 1994) and production of viral variants (Haddad etal 2001); other tissues, such as the spleen (Bach etal, 1994), thymus (Dean & Pedersen, 1998; Heeney, 1995; Woo etal 1997, Norway etal 2001), and CNS (Pomerantz, 2002) have been implicated as well in the role of viral sanctuaries. Viral replication in lymphatic tissues is several orders of magnitude over that in blood cells (Burgisser etal, 1997). Lymph nodes in FIV infected cats are involved, in order of severity of lymphadenopathy, like so, hindlimbs, forelimbs, and head; whereas lymph nodes associated with the alimentary tract showed little evidence of enlargement (del Fiero etal, 1995).

As to the importance of the thymus in FIV replication Dean & Pedersen (1998) found the thymus had the highest level of FIV replication of four target sites investigated in the study (thymus, spleen, mesenteric lymph node, and cervical lymph node); other studies have proposed the thymus as a major site for FIV replication and CD4+ depletion

in the acute infection stage (Heeny, 1995; Norway etal, 2001; Haynes & Hale, 1998).

AZT (zidovudine, 3'-azido-3'-deoxythymidine) is the most commonly prescribed nucleoside analog anti-HIV drug. It has been found to have potent in vitro activity against FIV in feline thymocytes (Egberink etal, 1991) and in vivo by improving the CD4+/CD8+ ratio in FIV infected cats (Hartmann etal, 1992; Hartmann etal, 1995). However, AZT's toxicity makes for a controversial treatment (Gregory etal, 1997; Meers etal, 1993; Smyth etal, 1994a). The pharmacokinetics of AZT in cats have been determined (Zhang, 2001) with a mean (rsd) body weight adjusted plasma clearance of 0.41 (0.10) L/h/kg and body weight adjusted volume of distribution at steady state of 0.82 (0.15) l/kg both of which are less than the respective values reported for humans of 0.93 (0.36) and 9.38 (0.29). The disposition of AZT has been studied in female C57BL/6 mice (Chow H-H etal 1998; Chow H-H etal 1997); among the findings of these studies by Chow etal was the fact that the thymus, while presenting a lower AZT:AZT-MP (AZT monophosphate) ratio than many other investigated tissues, showed a much higher AZT-TP:AZT (AZT triphosphate, the active metabolite) denoting either a lack of affinity for AZT-MP or low efficiency in converting AZT-MP to AZT-DP (low density or efficiency of thymidine kinase) and/or high efficiency in converting AZT-DP to AZT-TP and AZT-MP to AZT-DP.

3TC (lamivudine, (-)-2',3'-dideoxy-3'-thiacytidine) is a nucleoside analog commonly used in combination therapy with AZT against HIV and singly against Hepatitis B virus (Soudeyns etal, 1991; Coates etal 1992a,b; Chang etal, 1992). Smyth etal (1994b) found that 3TC is active against FIV with a lower IC_{50} and IC_{90} for FIV-E77 and FIV-8 production in primary feline lymphocyte cell line and a sub-line of Crandell-Reese feline kidney cells than AZT as well as higher CT_{50}'s (50% toxic concentration) for lymphocytes and CRFK cells than AZT. Similar findings of FIV's susceptibility to 3TC were corroborated by McCrackin-Stevenson and McBroom (2001). The pharmacokinetics of 3TC in cats have been investigated (Zhang, 2002) and were found to be similar to its pharmacokinetics in humans with a volume of distribution of

0.6 (0.30) l/kg, clearance of 0.22 (0.41) L/h/kg comparable to yet both still slightly less than those reported for humans (1.3 l/kg and 0.32 L/h/kg; Hussey etal, 1994). In a comparison study by Smyth etal (1994b) 3TC was found to be the most effective at inhibiting FIV in primary feline lyphocyte cell lines out of a group of eighteen nucleoside analogs; of these eighteen it was also one of the least toxic.

Because of the endemic presence of FIV, the lack of safe and effective treatments for FIV, the proven track record of AZT and 3TC as antiviral agents in treating HIV infection, their activities against FIV, the importance of the thymus and lymph nodes in the life cycle of FIV, and the popularity of domestic cats as pets, the nature of AZT and 3TC's disposition into lymphoid tissue needs to be determined. Therefore, the objective of this study was to determine if AZT and 3TC penetrate cat lymph and thymus tissues and if so to what degree, and to determine any patterns in the disposition among lymph nodes within each cat.

MATERIALS AND METHODS

Materials

AZT, 3TC, AZdU, d4T, ketamine, diazepam, xylazine were purchased from GlaxoWellcome Inc., (Retrovir), Research Triangel Park, NC 27709, GlaxoWellcome Inc., Research Triangel, azdu, d4t), Fort Dodge Animal Health (Ketaset), IA, 50501, Bayer Corp. (Rompum), Shawnee Mission, KS, 66201.

Animal handling

Six healthy, intact, 10-month old, barrier reared domestic short haired cats weighing 3.21 (0.22) kg were used in the AZT study and 3.30 (0.18) kg for the 3TC study. Cats were housed at the University of Georgia Veterinary Medical Animal Care Facility. Animal studies were approved by the University of Georgia Animal Care and Use Committee and were conducted in accordance with guidelines established by the National

Institutes of Health Guide for the Care and Use of Laboratory Animals. Complete blood counts (CBC), serum chemistry, and urinalyses were performed on all cats within three days of their arrival to ensure health.

Cats were canulated at the jugular vein for sample collection with an 18.5 gauge catheter (I-Cath, Charter Medical, Ltd., Lakewood, NJ, 08701) under ketamine (12.5-15.0 mg/kg) and xylazine (1.0 mg/kg) anesthsia given by intramuscular injection. A 22 gauge Intracath (Sovereign, Sherwood Medical Industries, Tullamore, Ireland) catheter was placed into the medial saphenous vein of a rear leg with the insertion point of the catheter being the caudal vena cava for drug administration. Jugular catheters and Intracath catheters were flushed with one and 0.5 mL 0.9% saline containing 5 u/mL sodium heparin.

Dosing

Food was withheld for 24 hours before and after drug administration. Water was withheld for 12 to 16 hours prior to drug adminsitration but was freely available after drug was given. AZT solution (10 mg/mL) was diluted to 6.67 mg/mL with 5% dextrose in water as recommended by the manufacturer and injected into the jugualr catheter as bolus (7.0 mg/kg AZT) over one to three minutes as loading dose. The same dilution of AZT was given for three hours at a rate of 14.5 mg/kg/hr. 3TC (7.0 mg/kg) was diluted to 10.0 mg/mL with 0.9% saline and injected into the jugular catheter as bolus over one to three minutes. Infusion of 3TC at 5.5 mg/kg/hr at a concentration of 5.0 mg/mL 3TC in 0.9% saline was accomplished through the jugular catheter.

Sample collection

Blood samples were collected via jugular catheter immediately before drug administration and one, two, and three hours thereafter. A 0.3 mL aliquot sample was collected into a sterile tuberculin syringe and transferred into a heparinized polypropylene microcentrifuge tube from a larger (2.5 mL) sample collected via the

jugular catheter with a 3 mL syringe; the remaining blood sample was returned through the jugular catheter. Catheter was flushed with one mL heparinized saline. Heparinized blood samples were immediately centrifuged at 3000 g and the harvested plasma stored at -20° C until analysis. Cats were sedated with diazepam and ketamine (0.12-0.2 and 2.5-4.0 mg/kg, respectively) IV and anesthetized with ketamine and xylazine (12.5-15.0 mg/kg and 1.0 mg/kg respectively) IM at the end of the infusion. Lymph tissue was harvested within 10-15 minutes of dosing and stored at -80° C.

Chemical analysis

AZT method

Chromatographic conditions were as follows: mobile phase was 15% (v/v) acetonitrile 40 mM KH_2PO_4 pH 7.0, flow at 2.0 mL/min, column Alltech Hypersil ODS 5 /_/ 150 mm x 4.6 mm with a Waters Guard-Pak Nova-Pak C18 guard column. Internal standard was CS85. Respective retention times for AZT and CS85 under these conditinos were 3.1 and 5.5 minutes.

Lymph tissue samples from five cats dosed with AZT were prepared by thawing (from -80° C to room temperature), cut into smaller pieces, and homogenized with a volume (mL) of phosphate buffered saline (pH 7) equal to the weight (mg) of the tissue sample. One hundred microliters of homogenate were dispensed into microcentrifuge vials, 10 /_/L of internal standard (40 /_/g/mL) and 50 /_/L 2 M perchloric acid were added, vortexed for 30 sec, centrifuged for ten minutes at 9000 g, and 50 /_/L of supernatant injected onto the column. Absorbance was read at 260 nm. Standard curve was made in cat plasma (r^2 = 0.9977).

3TC method

Chromatographic conditions for analysis of 3TC in cat lymph were as follows: mobile phase was 4% (v/v) acetonitrile 40 mM KH_2PO_4 (pH 7), 2 mM triethylamine, flow at 1.5 mL/min, Alltech ODS 5/ 150 mm x 4.6 mm column with a Waters Guard-Pak Nova-Pak C18 guard column. Internal standard was d4T. Respective retention times for 3TC and d4T under these conditions were 5.8 and 9.2 minutes.

Lymph tissue samples from three cats dosed with 3TC were prepared in the same manner as described for AZT with the exception that 10 /L of 40 /g/mL d4T was added to each sample prior to vortexing as internal standard. Standard curve was prepared in cat plasma (r^2 = 0.9953).

RESULTS

AZT was found in all lymph nodes of all cats except the tonsil of one cat due to paucity of samplable tonsil tissue. The average (rsd) concentration of AZT in all lymph nodes from all five cats was 9.77 (0.80) mg/L. Table 4.1 represents the average concentrations of AZT present per lymph node type/location as a bar chart. Mean concentrations of AZT in all cats was similar except in one cat which displayed lower concentrations in all lymph nodes. Average concentrations were similar across lymph nodes with highest concentrations appearing in sternal lymph node and following in decreasing magnitude tonsil, bronchiolar lymph node, thymus, mesenteric lymph node, and submandibular lymph node; the highest concentration lymph node had just over two times the concentration of the lowest. Ratio comparison of lymph node concentrations to plasma concentrations results in a disposition pattern of declining concentration of sternal lymph node, tonsil, bronchiolar lymph node, thymus, mesenteric lymph node, and submandibular lymph node (Figure 4.1).

When compared by one way anova with tissues as treatment factor and cats as blocking factors, mean concentration per tissue across animals were found to be not significantly different from the overall mean ($p=0.5602$); whereas cats were found to be significant ($p=0.0388$). That cats would be significant may be explained by the fact that one cat showed concentrations that were an order of magnitude lower than concentrations in other cats; why this cat showed differences may potentially be explained by intraindividual pharmacokinetic differences, either the cat did not absorb AZT well, metabolized or excreted it rapidly, or potientially through differences in handling of extracted tissues.

Similarly, 3TC was found in all samples. The average concentration of 3TC in all lymph nodes from all six cats dosed with 3TC was 7.74 (0.66) mg/L. Table 4.1 lists the average concentrations of 3TC present per tissue. Concentrations were similar across cats, although concentrations in all lymph nodes of two cats were nearly an order of magnitude above those of other cats. Concentrations of 3TC across lymph nodes within individual cats were usually very similar except in two cats which displayed a larger range in concentrations; one of these cats was also one of the cats which displayed higher concentrations than the others. The order of lymph nodes from highest to lowest concentration was mesenteric lymph node, tonsil, thymus, bronchiolar lymph node, sternal lymph node, and submandibular lymph node. When concentrations in lymph nodes were compared by ratio (Figure 4.1) to concentrations in plasma a resulting pattern of disposition, starting with highest ratio and in order of descending ratio, as follows, mesenteric lymph node, tonsil, thymus, bronchiolar lymph node, sternal lymph node, and submandibular lymph node.

DISCUSSION

That drugs are retained into different lymph nodes preferentially seems counterintuitive in that lymph nodes have largely the same composition regardless of

location. However, a combination of factors can contribute to differential loading including differences in both amount and rate of blood flow, degree of vascularization or association with true vasculature, and rate of lymph flow.

Concentrations of AZT in all lymph nodes of all cats were above the EC_{50} reported for AZT against FIV antigen production in feline thymocytes of 0.013 mg/L (0.4) while at the same time being below the CC_{50} (concentration at which the lymphocytes were depleted by cytotoxicity by 50%) of 26 (0.07) mg/L in all but one tissue, that being the submandibular lymph node (33 mg/L) of a single cat (Vahlenkamp etal 1995). Tissues were sampled within 10-15 minutes post dose; concentrations were similar to those observed after IV and PO administration at ten and 15 minutes reported elsewhere (Zhang, 2002). Concentrations of AZT in plasma measured 26.3 (0.16) mg/L resulting in an overall lymph:plasma concentration ratio of 0.36 (0.76) mg/L. Assuming concentration ratios between lymphatic tissues and blood remain the same throught the drug's residence in the cat's body (ie, assuming steady state conditions) then a thrice daily oral dosing with 25 mg/kg AZT should be sufficient to inhibit FIV replication in lymphatic tissues by 50% compared with replication in untreated cats.

Concentrations of 3TC were above the IC_{50}, the concentration at which FIV antigen presentation in infected feline lymphocytes was reduced by 50% from control (nontreated) lymphocytes (0.04 (0.25) mg/L, Smyth etal 1994b) in all tissues. Likewise, concentrations of 3TC were above the IC_{90}, 0.16 (0.125) mg/L (Smyth etal 1994b) for inhibition of FIV replication in all samples. Likewise, concentrations of 3TC were well below the CT_{50} (the toxic concentration, or the concentration at which triatiated thymidine was taken up by cells was inhibited by 50%) of 250 (0.16) mg/L by nearly two orders of magnitude (the highest concentration in assayed tissues was 16.8 mg/L in the tonsil of one cat). At an oral schedule of 25 mg/kg twice daily, concentrations in lymph nodes should be above the IC_{90} for the inhibition of FIV replication in lymphoocytes.

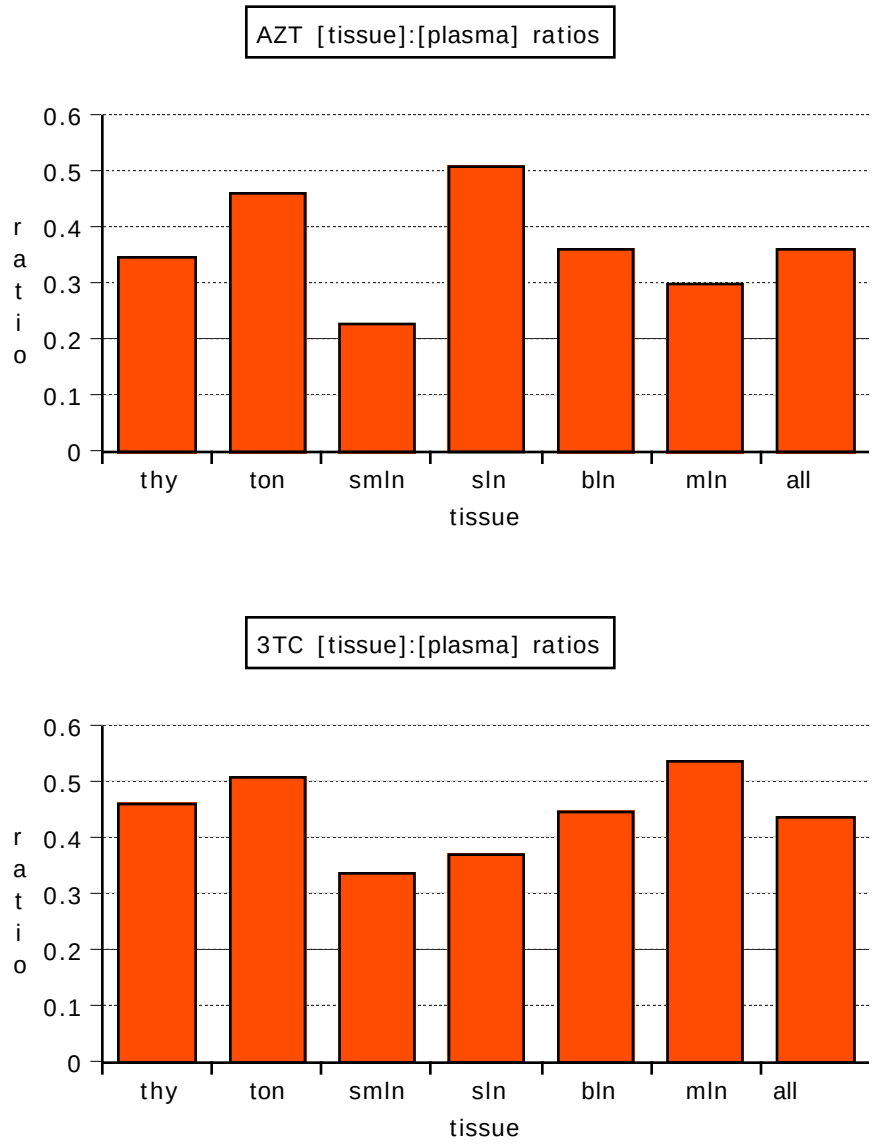
In a study by Dean & Pedersen (1998) comparing FIV replication in four lymphatic tissues (thymus, spleen, mesenteric and cervical lymph nodes) FIV replication was found to be "highest in thymus, followed by spleen, mesenteric lymph nodes, and cervical lymph nodes." Mean concentration ratios between thymus and plasma for AZT and 3TC were 0.35 (0.89) and 0.46 (0.70) and when compared to plasma concentration versus time profiles at 25 mg/kg orally should provide at least 8 (6) and more than 12 (more than 12) hours of concentrations above the IC_{50} (IC_{90}) in the thymus. Concentrations in the mesenteric lymph nodes of cats for AZT were below but similar to concentrations in thymus. Viral replication in the mesenteric lymph node was less than in the thymus as reported by Dean & Pedersen (1998) and thus concentrations which are above the IC_{50} & IC_{90} in the thymus should be protective in the mesenteric lymph node as well. Concentrations of 3TC in the mesenteric lymph node were above those of the thymus and therefore replication of FIV in infected lymphocytes in the mesenteric lymph node should be inhibited at this schedule. Pattern or rate of replication of FIV in other lymph nodes has not been studied thus the level of protection afforded to these tissues is unknown.

Differences in the regional distribution of AZT have been reported previously for mice (Manouilov etal, 1995). This study reports higher concentrations of AZT in axillary lymph nodes in comparison with neck and mesenteric lymph nodes; whereas, our study reports AZT concentrations in head/neck lymph nodes both above and below those in mesenteric lymph nodes. Distribution of 3TC was different as well, with the mesenteric lymph node accumulating the highest concentrations of 3TC. The conclusion that lymphatic distribution of nucleoside analogs must therefore be both animal and compound specific is simply reiterative of general pharmacokinetic principles.

Table 4.1: Concentrations (mg/L) of drug per node (avg=average, rsd=relative standard deviation, ln=lymph node)

	AZT avg	AZT rsd	3TC avg	3TC rsd
thymus	8.82	0.87	8.34	0.73
tonsil	13.2	0.68	8.87	0.62
submandibular ln	6.02	0.93	5.78	0.56
sternal ln	14.2	0.84	6.34	0.59
bronchiolar ln	9.52	0.51	7.57	0.75
mesenteric ln	7.52	0.94	9.50	0.72
overall	9.97	0.80	7.74	0.66

Figure 4.1: Ratios of concentration of drug in lymph node to concentration in plasma (thy=thymus, ton=tonsil, smln=submandibular lymph node, sln=salivary lymph node, bln=bronchiolar lymph node, mln=mesenteric lymph node)



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CHAPTER 5

DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR BROMODICHLOROMETHANE IN THE JAPANESE MEDAKA (*ORYZIAS LATIPES*) FISH[1]

[1]Boyd JL, FD Boudinot, MW Toussaint, JW Fisher.

ABSTRACT

A physiologically based pharmacokinetic model with associated pharmacodynamic effects was developed for bromodichloromethane (BDCM), a suspected human carcinogen, in the Japanese medaka fish (*Oryzias latipes*). Concentrations of BDCM were quantitated and modeled in blood, gill, liver, and muscle after a four hour exposure in the first part of the study. The second part of the study involved a bioassay in which the animals were exposed for 180 days at the end of which histopathology was performed in various tissues. The effect of hepatocellular adenoma in naive fish (uninitiated with DNA alkylating agent) was chosen as the pharmacodynamic endpoint based on the fact that it is the major carcinogenic effect in the medaka and because it is a pathological finding present in rodents treated with BDCM. The model was determined to provide a good fit based on its ability to simulate concentrations in all assayed tissues within the range of one standard deviation for two exposure concentrations. Some cancerous effects in the gill were also highly correlated to gill exposure levels.

KEY WORDS: Bromodichloromethane, Medaka, PBPK, “Physiologically based pharmacokinetic modeling”

INTRODUCTION

Bromodichloromethane (BDCM) is a byproduct of drinking water chlorination, one of the most commonly used methods to disinfect water throughout the world. It is a suspected human carcinogen (Cantor et al, 1978; Morris et al, 1992; Bull et al, 1995; King & Marrett, 1996) and a known rat carcinogen (Brennen et al, 2001). Because of its ubiquitous presence and potential deleterious effects, the need exists to determine exposure levels which are protective of human health. Although many dose response models are developed by relating administered dose to effect, dose response relationships are developed best with pharmacokinetic models. Pharmacokinetic models describe the absorption, distribution, metabolism, and excretion (ADME) of drugs and chemicals. Several types of pharmacokinetic models exist, including classical pharmacokinetics, population based pharmacokinetics, and physiologically based pharmacokinetics. The first two methods typically employ structural models which describe ADME on the basis of statistically derived compartments; compartments are abstract representations of generalized drug disposition, not necessarily corresponding to an anatomical unit. The last method, physiologically based pharmacokinetic modeling, simulates concentrations in compartments which are anatomically based, such that each compartment represents an actual organ or tissue (or group thereof). Among other things, PBPK models derive parameters related to exposure including the concentration in a tissue compartment and the area under the curve (AUC) which is the integral of the concentration versus time function; in cases of carcinogenic effects due to chronic exposure to chemicals, the AUC is the preferred metric with which to relate exposure.

A physiologically based pharmacokinetic (PBPK) model describing BDCM's pharmacokinetics in various tissues, including the liver, in the rat exists (Lilly et al, 1998). The experimental design used in developing this model employed dosing rats with BDCM in corn oil and aqueous media. While appropriate for toxicology testing, such a model describes disposition due to intermittent dosing. In order to remove

pharmacokinetic variability in the dose-response model, a model employing continuous exposure is needed. Additionally, the rat model (Lilly et al, 1998) does not include pharmacodynamic effects. Therefore, a model utilizing continuous aqueous exposure and pharmacodynamic data was developed in the Japanese medaka fish (*Oryzias latipes*).

Physiologically based pharmacokinetic models have been developed in a number of large fish such as rainbow trout (Nichols et al, 1996), catfish (Nichols et al, 1996), sea bass (Poher, et al, 1997), and others. These models are developed by sampling chemical concentrations in various organs at a variety of predetermined time points. Sampling of chemical concentrations is accomplished by a variety of chemical analysis methods, typically assaying concentrations in extracts of tissue homogenates. The principal limitation placed on PBPKs in small fish, or any small animal, is the limited volume of tissue available for sampling. Yet the trend in toxicology and biomedical sciences in general is to develop models in smaller animals thereby reducing expenditure, related to food and housing costs, reducing time of testing, due to shorter lifespans, and increasing statistical power. Pharmacokinetic models exist in several small fish species, including goldfish (Podowski et al, 1991), guppies (Layiwola et al, 1983), and fathead minnows (Lien & McKim, 1993); for the most part, these models are classical pharmacokinetic models or chemical uptake studies.

The Japanese medaka is a small teleost native to Japan with low background incidence in cancers. Cultivation methods for the medaka are well known, handling of the fish is easy, and an established cancer bioassay exists in it. The medaka has been used for a single published pharmacokinetic study to date (Lien & McKim, 1993).

The purpose of this study was to develop a physiologically based pharmacokinetic model describing the absorption, distribution, metabolism, and excretion of bromodichloromethane (BDCM), including the pharmacodynamic effect of liver cancer so that the medaka fish may serve as a continuously exposed surrogate for humans.

PHARMACOKINETIC STUDY DESIGN

All animal research was conducted in accordance with the Animal Welfare Act, other federal guidelines relating to animal experimentation, and the Guide for the Care and Use of Laboratory Animals (1996) and in facilities that are fully accredited by the Association of the Assessment and Accreditation of Laboratory Animal Care, International. Full conditions of the in-house breeding of Japanese medaka have been previously reported (Toussaint, 2001). Sexually mature medaka fish of at least six months of age were exposed to three different treatment levels, 0, 1.5, and 15 mg/L BDCM for a duration of four hours. Fish were randomly assigned to each of the three treatment levels, with three tanks per gender and per treatment level such that at 120 fish per tank and nine tanks, there were 1080 fish total. Because of the difficulty of assaying concentrations of BDCM in such small tissues, three fish served as an experimental unit of one. Upon end of exposure, fish were removed from the tanks, euthanized with MS-222, and tissues were collected. Tissues collected were blood, liver, muscle, and gill. All experimental work was done at the U.S. Army Center for Environmental Health Research, Fort Detrick, MD.

PHARMACODYNAMIC STUDY DESIGN & HISTOPATHOLOGY

The pharmacodynamic effects reported in this study are from a companion experiment which investigated histopathology of the fish exposed to three levels of BDCM (below detection limit of 0.001, 1.82 (0.12), and 17.14 (0.15) mg/L; Toussaint et al, 2001). Briefly, fish were exposed at the three levels of BDCM for 180 days and then euthanized with MS-222, tricaine methanesulfonate. Necropsy, fixation, section, staining, and histopathology were performed.

CHEMICAL ANALYSIS

At four hours fish were euthanized and tissues harvested. Fish blood, liver, gill, muscle were collected and sealed in 10 mL glass headspace vials, and stored at 0° C for analysis. Thawed samples were individually denatured in 5 mL of 2% (w/v) sodium dodecyl sulfate at 60° C for 2 hours. Samples were then immediately analyzed by flame ionization detector interfaced to a model 6890 capillary gas chromatograph with model 7694 automatic static headspace sampler and fused silica capillary column (30 m x 0.25 mm ID) coated with cross-linked 1% methylsilicon gum phase 0.3 /_/m film thickness (Hewlett-Packard, Wilmington, DE). This method is described elsewhere (Toussaint, 2001). The detection limit for this method was 0.001 mg/L and percent recovery was 95%.

PHARMACOKINETIC MODEL

Concentrations of BDCM were modeled in various tissues, including blood, liver, muscle, and gill. Remaining tissue was lumped together and represented as 'other' compartment. Metabolic parameters, Vmax and Km, for metabolism in the liver were taken from the rat (Lilly et al., 1998); initial estimate of Vmax was scaled from the reference value in the rat using the relationship of reference value (Vmaxc) raised to the 0.75 power and multiplied by body weight of the fish. Cardiac output was taken from a reference value in a one kilogram rainbow trout (Nichols et al., 1996) raised to the 0.75 power and multiplied by body weight. Blood flow through tissues was determined by multiplying the relative volume of the organ by the body weight scaled cardiac output; thus, blood flow through the various tissues was also scaled. Adjustments to the model were made in order to provide better fits of model generated concentrations of BDCM as compared to assayed concentrations.

Partition coefficients were estimated by calculating the concentration ratio of tissue/blood for the muscle, gill, and liver in the high dose group (15 mg/L BDCM). The blood/water ratio was determined using measured water concentrations.

The differential equations that constituted the structural model to simulate the pharmacokinetics of BDCM, and the structural model itself, are shown in Figures 5.1 and 5.2. A full list of all the parameters used in the model may be found in Table 5.1. A good fit was defined as model predicted BDCM tissue concentrations falling within one standard deviation of the measured tissue concentration. We required an acceptable model as one robust enough to simulate tissue concentrations within one standard deviation of observed concentrations for both the 1.5 and 15 mg/L BDCM treatment groups.

SYSTEM DESCRIPTION

The program ACSLTox, version 11.5.2 (MGA Software, 1997) was used for modeling. The environment in which ACSLTox ran was Microsoft Windows 98 version 4.10.2222 A (Microsoft, 1998). The machine was a Dell Genuine Intel x86 Family 6 Model 8 Staging 3 128 MB RAM.

RESULTS & DISCUSSION

In our model, we determined that the ‘other’ compartment represents a lipophilic compartment. BDCM has moderate lipophilicity (Lilly et al., 1998). In the medaka fish, no fat tissue was removed for analysis; however those remaining tissues are fatty in nature. Our modeling efforts suggest that a fat depot probably exists for BDCM in the medaka fish. We were unable to simulate the kinetic behavior of BDCM in the medaka fish without this assumption and we thus assigned the tissue/blood partition coefficient of the 'other' compartment to 18 in accordance with that of the fat

compartment in Lilly et al (1998). Methods to measure partition coefficients for small tissues and organs from the medaka failed.

The model simulated concentrations of BDCM in medaka fish tissues approached observed concentrations only after critical adjustments to the PBPK model were attempted. A term, GEE (gill extraction efficiency), was added to the PBPK model in the gill compartment to account for diffusional limitations after initial model runs produced highly exaggerated tissue concentrations in the initial medaka model. The value for GEE was based on a reported extraction efficiency of 7% for butanol in trout gills (McKim et al., 1995). This finding is significant because it suggests that only a very small fraction of the BDCM dissolved in water is actually inspired into systemic circulation. It is important to note that this attempt to describe the gill/water exchange is taken from approaches used for inhaled gases that have a high water solubility and modest lipid solubility (Fisher et al., 2000). Other fish PBPK models for much larger fish describe the gill/water exchange by more rigorous methods (McKim et al, 1995; Erickson & McKim, 1990).

Initial iterations completed with the maximum (prescaled) rate of metabolism, Vmaxc, set at the value reported in rats (Lilly et al, 1998), and multiplied by body weight scaled to the 0.75 power of the fish failed to simulate the observed BDCM concentration profiles measured in the medaka fish. It is important to note that rates of metabolism are generally not scaleable between different species; scaling of the maximum rate of metabolism was intended to serve as a starting point for fitting. Scaling the metabolic rate from rats to the medaka fish resulted in extremely rapid clearance of BDCM from systemic circulation. We then set the maximum rate of metabolism, Vmaxc, to zero and increased its value, in an iterative fashion, until suitable fits were seen between observed and simulated tissue concentrations. Adjustment of this Vmaxc was done only to improve fits of observed concentrations; however, no samples were taken at later time points. The behavior of the concentration of BDCM in the liver at later time points is likely artifactual, resulting from a high value of intrinsic clearance

(Vmax/Km), and that in order to accurately predict the concentration of BDCM in the liver, later sampling must be done. To determine true metabolic constants, Vmax and Km, for oxidation of BDCM in medaka liver, additional experimentation is required. Metabolism of BDCM is thought to be predominantly dependant on *cyp*2E1 in the rat (Allis et al, 2001). There is conflicting evidence as to this iosform's presence in fish, one report suggests that this isoform is not expressed in the medaka fish (Lipscomb et al, 1997). Yet the presence of *cyp*2E1 in fish has been confirmed in strains of Poecilius fish of Arizona (Kaplan et al, 2001). Other isoforms of the cytochrome p450 system as well as other enzymes may metabolize BDCM in the medaka. Plots of simulations of BDCM in the Japanese medaka at the two exposure concentration levels in an acceptable model are shown in Figures 5.3 & 5.4.

Teleost fish are known to have a secondary circulatory system (Satchel, 1991). Due to lack of information in this area, no attempt was made to model BDCM's behavior in relation to this secondary circulatory system. Information as to the relation between chemical disposition and this secondary circulatory system may be of benefit to future physiologically based pharmacokinetic studies in fish.

Sensitivity analysis was performed to determine the parameters to which the model was most sensitive. By varying input parameters by ten percent, their affects on the output parameters, AUC in blood and the liver, were observed. These two endpoints were chosen because the liver is the previously determined target organ of carcinogenesis, and because all tissues equilibrate with the blood and it is the blood concentration from which most classical pharmacokinetic parameters are derived. AUC in venous blood was most sensitive to PGW such that an change in PGW of ± 10% provided a change in venous blood AUC of -7%/-17%. AUC in the liver was sensitive to PGW in a similar fashion; changes (± 10%) in PGW produced AUC values in the liver of +18%/-17%. Venous blood AUC and AUC in the liver were also sensitive to PB, GEE, and QPC (in that order) though to a lesser extent than PGW. This indicates that further work concentrate initially on partitioning in the gill and kinetics there.

Hepatocellular adenoma incidence rates in the medaka fish were chosen as the pharmacodynamic endpoint because of reported findings of BDCM induced liver neoplasms in rats (NTP, 1987; Dunnick et al, 1987). One of the important information gaps in the use of medaka fish as an alternative model for rodents is understanding the differences in routes of exposure. The model derived dosimetry parameter, the weekly averaged area-under-the-concentration-versus-time-curve for liver (AUCL) was selected for regression analysis with the percent incidence of hepatocellular adenomae found in the medaka fish study. This parameter is simply the total AUCL divided by the number of weeks in the 180 day study, or 25.7 weeks. This metric was chosen because the liver is the organ of selected carcinogenic effect and was averaged in order to provide a meaningful metric which can be applied to short and long term human exposures. There was very high linear correspondence of percent liver incidence with AUCL (r^2=1.0; Table 5.2). The bioassay study for the medaka was carried out over six months, while the pharmacokinetic study was a four hour study. The PBPK model was developed based on the four hour study and used to extrapolate to longer term exposures that were used in the medaka fish bioassay study. All regression parameters relating percent incidence to the exposure metric of AUCL are presented in Table 5.2. More pharmacokinetic data is needed to describe the pharmacodynamic responses with a Hill equation (which would describe the data in a physiological manner) or Weibull cumulative density function (which would describe the data but not physiologically), which would provide more a more accurate description of the dose-toxic-effect relationship of BDCM (Gibaldi & Perrier, 1982; Newman, 1995).

Interestingly, effects in the gill were also highly correlated with model simulated exposures. Gill hyperproliferation had a very high correlation (r^2 = 0.9988) with weekly averaged AUCG, the area under the curve in the gill. Though this effect was not initially selected for pharmacodynamic modeling, nor is it immediately translatable to rat or human effects, this finding may be important. These results are also listed in Table 5.2.

SUMMARY

This preliminary PBPK model for BDCM in the medaka fish represents the first PBPK model for a small fish that distinguishes between different tissue groups and incorporates a pharmacodynamic endpoint into the model. Data sets to support either compartmental or PBPK models for small fish are based on ‘lumped’ kinetic data, which is usually the entire fish. We have developed and partially validated a true PBPK model for the medaka fish. This modeling effort provided significant insights into important species extrapolation issues related to exposure and dosimetry of BDCM. With further research improvements in the PBPK-PD model can be accomplished in several areas. In future pharmacokinetic studies, a richer time course data set could be used to obtain optimized model parameters. The physiological parameters we used for the medaka fish were 'scaled down' from large fish. Further research on blood flows and lamellar ventillation rates in the medaka fish would improve the PBPK model robustness. Separate metabolic studies are required to quantify the metabolic capability of the medaka fish. Finally, research to quantify the diffusional limitations associated with gill mucous and gill diffusion would allow for more accurate estimates of chemical intake.

Figure 5.1: Differential equations used to model the concentration of bromodichloromethane in the Japanese medaka (*Oryzias latipes*), see Figure 5.2 for definitions

dAG/dt = QC(CT - CA) + QP*GEE(CI*PGW – *CG/PGW)

dAM/dt = QM(CA – AM/(VM*PM))

dAL/dt = QL(CA – (AL/(VL*PL)) – RAMX)

RAMX = dAMX = VMAX*(AL/(VL*PL))/(KM + (AL/(VL*PL))

dAO/dt = QO(CA – (AO/VO*PO))

CA = AG/VG*PB

CV = (QM*(AM/VM*PM) + QL*(AL/VL*PL) + QO*(AO/VO*PO))/QC

Figure 5.2: Structural model of the physiologically based pharmacokinetic model for bromodichloromethane in the medaka fish. Terms beginning with C are concentrations, Q are flow rates, V are volumes, P are partition coefficients, ending with I refer to intake, X refer to expiration, G refer to gill, GW refer to gill:water, GB refer to gill:blood, L refer to liver, M refer to muscle, O refer to the lumped "other" compartment, A refers to arterial, V or V(O/M/L) refer to venous concentrations, C refers to cardiac, and P refers to ventillation.

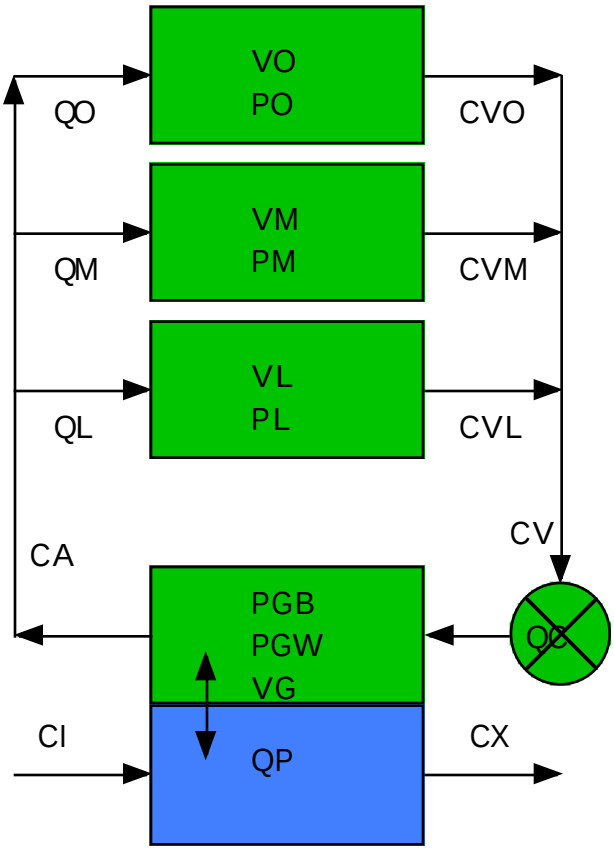

Figure 5.3: High dose group, observed (points) and simulated (lines) concentrations

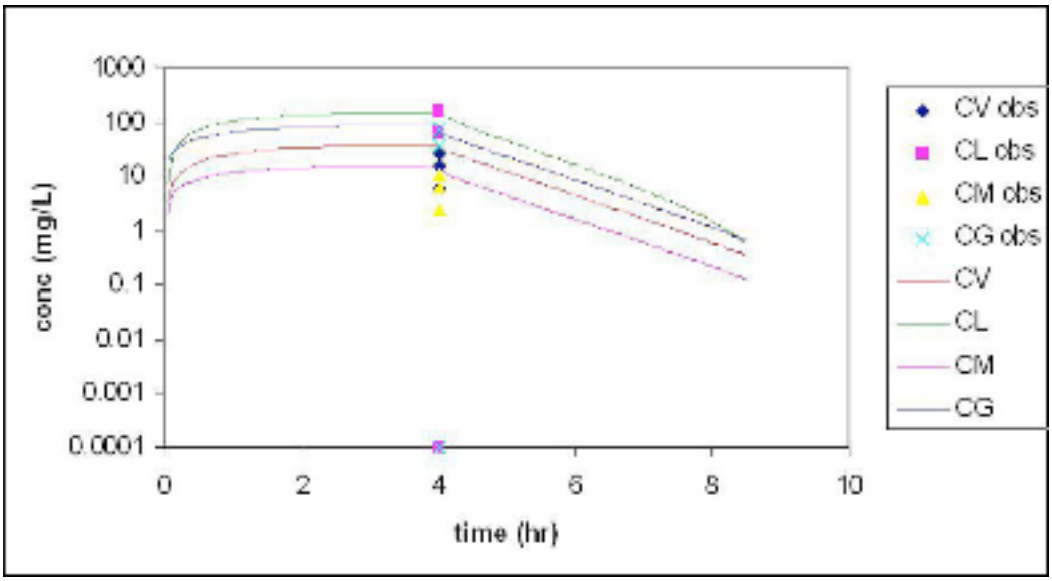

Figure 5.4: Low dose group, observed (points) and simulated (lines) concentrations

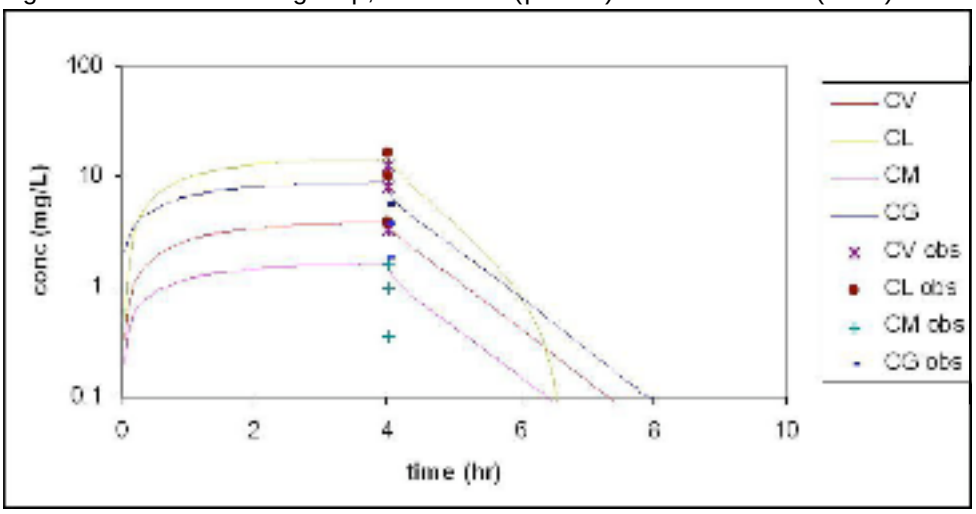

Table 5.1: Model parameters and description for the physiologically based pharmacokinetic-pharmacodynamic model of bromodichloromethane in the Japanese medaka (*Oryzias latipes*)

abbreviation	term	value
QCC (L/kg/h)	cardiac output	2.07
QPC (L/kg/h)	rate of uptake at gill	7.06
Fractional blood flows (%CO)		
QLC	fractional blood flow to liver	0.03
QMC	fractional blood flow to muscle	0.6
QGC	fractional blood flow to gill	1
Relative volumes (%BW)		
VGC	volume of gill	0.1
VLC	volume fo liver	0.02
VMC	volume of muscle	0.78
Partition coefficients		
PGW	gill/water	2.45
PB	gill/blood	0.46
PL	liver/blood	1.25
PM	muscle/blood	0.12
PO	“other”/blood	18
Metabolism		
VMAXC (mg/L/h)	scaleable Vmax	0.01
KM (mg/L)	Michelis-Menten metabolic constant	0.5

Table 5.2: Regression parameters for modeled pharmacodynamic effects

effect	dose metric	slope	intercept	correlation
hepatocellular adenoma	weekly AUCL	4×10^{-5}	1.47	1.0
gill hyperproliferation	weekly AUCG	2×10^{-5}	0.55	0.9988

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CHAPTER 6

CONCLUSIONS

Pharmacokinetics is a tool used to determine or describe physiological features of xenobiotics. It can be used in the context of determining straightforward baseline, chemical specific, physiological values of volumes and clearances, tissue distributions, or describe some physiological process. On the other hand, physioanatomy itself may be simulated in relation to kinetics of xenobiotics in effort to understand physiological response. From the standpoint of basic drug discovery, area moment, or noncompartmental, methods have a great deal of utility, as a means of measuring baseline phenomena (clearances, volumes, etc) while at the same time being physiologically relevant. Compartmental models give similarly physiologically relevant pharmacokinetic parameters as noncompartmental while at the same time being predictive as well, thereby extending the use of pharmacokinetics into drug safety and clinical trials. Yet the predictive nature of compartmental models comes with the price of increased assumption making. Physiologically based models provide the ability to simulate both pharmacokinetics as well as physioanatomy. Physiologically based pharmacokinetic models allow for a great deal of manipulation in a virtual man; yet with detailed elaboration in the model comes an increase in number of assumptions.

Pharmacokinetics of 5-iodo-dioxolanyl-uracil in rats

Pharmacokinetics of (2S, 4R)-1-2-[2-(hydroxymethyl)-1,3-dioxolan-4-yl]-5-iodouracil (IOddU) were determined in rats after intravenous and oral administration. Its total (mean ± rsd) plasma, urinary clearances, and volume of distribution, after intravenous bolus injection were 1.44 (0.588), 0.69 (0.46) L/h/kg, and 0.643 (0.619) L/kg. MRT (h), λ_z (min^{-1}), and fraction of dose excreted in urine were 0.510 (0.527), 1.08 (0.714), and 47.8%. Bioavailability after oral gavage administration was nearly complete.

Analysis of iohexol in lean, fattened, and obese dogs

The effect of increasing levels of obesity on glomerular filtration rate were deteremined in dogs. Absolute plasma clearance of iohexol increased with obesity as expected; body weight adjusted plasma clearance of iohexol did not increase significantly. A three point method sampling in the terminal phase overpredicted urinary creatinine clearance slightly; yet gives values for GFR similar to urinary creatinine clearance providing continuity of interpretablity to practicing veterinarians.

Lymphatic distribution of AZT and 3TC in cats

3'-azido-3'-deoxythymidine and (-)-2',3'-dideoxy-3'-thiacytidine (AZT & 3TC) concentration ratios between lymph tissues and blood were determined at steady state in the domestic house cat. Lymph tissues included were tonsil, thymus, submandibular, bronchiolar, sternal, and mesenteric lymph nodes. Mean lymph tissue concentrations of AZT and 3TC were 8.13 (0.79) and 7.74 (0.66). Tissue to plasma concentration ratios were 0.36 (0.76) and 0.44 (0.51) for AZT and 3TC.

Development of a physiologically based pharmacokinetic model for bromodichloromethane in the Japanese Medaka fish (Orynchias latipes)

A physiologically based pharmacokinetic model was developed to relate concentrations in plasma and tissues of the Japanese Medaka fish (Orynchias latipes) in which an established cancer bioassay exists. Incidence of hepatocellular adenoma in male medaka exposed at three levels (0, 1.5, and 15 mg/L) correlated very well (r^2=1.00) with weekly averaged simulated area under the concentration versus time curve

Summary

A variety of pharmacokinetic methodologies were used to determine and describe/simulate the pharmacokinetics of several compounds. The results of the lOddU study can be immediately used by medicinal chemists in identifying issues in

antineoplastic therapy synthesis. Veterinary practicioners may immediately benefit from both the iohexol and AZT/3TC studies. With further study, the BDCM study may be used as an inexpensive liver cancer model of halogenated aliphatics as a surrogate for the same cancers in humans.