Pharmaceutical Research, Vol. 21, No. 8, August 2004 (© 2004)

Commentary

The Role of Metabolites in Bioequivalence

Kamal K. Midha,[1,2,3] Maureen J. Rawson,[3] and John W. Hubbard[2,3,4]

Received January 21, 2004; accepted April 5, 2004

The role of metabolites in bioequivalence studies has been a contentious issue for many years. Many papers have published recommendations for the use of metabolite data based on anecdotal evidence from the results of bioequivalence studies. Such anecdotal evidence has validity, but the arguments lack weight because the "correct" answers are always unknown. A more promising area of exploration is recommendations based on simulated bioequivalence studies for which the "correct" answers *are* known, given the assumptions. A review of the literature, however, reveals scant evidence of attempts to apply to real data the pharmacokinetic principles on which the recommendations from simulated studies relied. We therefore applied those principles (based on estimates of intrinsic clearance after oral administration of the parent drug) to four bioequivalence studies from our archives, in which the parent drug and at least one metabolite were monitored. In each case, the outcome is discussed in the context of the complexity of the metabolic processes that impact on the parent drug and the metabolite(s) during the first passage from the intestinal lumen to the systemic circulation. Our observation is that no simple generalization can be made such that each drug/metabolite combination must be examined individually. Our recommendation, however, is that in the interests of safety, bioequivalence decision-making should be based on the parent drug whenever possible.

KEY WORDS: average bioequivalence; bioequivalence studies; intrinsic clearance; metabolites; simulations; within-subject variability.

INTRODUCTION

This commentary will focus on orally administered drugs that reach the circulation and are active systemically. Bioequivalence (BE) based on test/reference comparisons of pharmacokinetic measures serves two purposes (1). The first is to act as a surrogate for therapeutic equivalence. The second is to provide *in vivo* evidence of pharmaceutical quality. The overall objective of BE is to ensure that generic formulations have similar efficacy and safety characteristics to the corresponding brand formulations. For the most part, traditional BE studies have been carried out on the basis of measurement of only the parent drug in body fluids such as plasma or serum. The role of metabolites in bioequivalence (BE) studies, however, has been a controversial issue for decades. A number of reasons for use of metabolite data have been put forward, such as (i) the parent is an inactive prodrug, (ii) plasma concentrations of the parent drug are too low to monitor because of inadequate assay sensitivity, (iii) the parent drug is metabolized rapidly to an active metabolite, and (iv) the parent drug and a metabolite both have therapeutic activities but the metabolite is present in higher concentrations.

Literature on this subject is divided into two broad groups. In the first group, recommendations on the use of parent and/or metabolite data in bioequivalence studies are based on anecdotal evidence from actual bioequivalence studies. Such recommendations lack weight, however, because the "correct" answer is always unknown simply because there is no way of knowing whether or not the two formulations under review are truly bioequivalent. In the second group, recommendations based on simulated bioequivalence studies appear more promising because the "correct" answer is known, given the assumptions on which the model is constructed. There are five key publications based on simulations in which a parent drug gives rise to a single metabolite: (i) Chen and Jackson, 1991 (2); (ii) Chen and Jackson, 1995 (3); (iii) Tucker and co-workers, 1993 (4); (iv) Rosenbaum and Lam, 1997 (5); and (v) Jackson, 2000 (6) A review of the literature revealed there has been little or no attempt to apply the pharmacokinetic principles on which the simulated studies were based to actual bioequivalence data. For the purposes of this commentary, therefore, we applied the pharmacokinetic principles used in simulations to four bioequivalence studies from our archives in which a parent drug and at least one metabolite were monitored.

THE PHARMACOKINETIC BASIS OF BIOEQUIVALENCE

Bioequivalence is concerned with the comparison of the bioavailabilities of a drug (or a metabolite) from two formu-

[1] College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 0W8, Canada.
[2] College of Pharmacy & Nutrition, University of Saskatchewan, Saskatoon, SK S7N 5C9, Canada.
[3] Pharmalytics Research Institute, University of Saskatchewan, Saskatoon, SK S7N 3R2, Canada.
[4] To whom correspondence should be addressed. (e-mail: hubbard@pharmalytics.ca)

0724-8741/04/0800-1331/0

lations of that drug and is usually assessed by measures obtained from the respective plasma concentration vs. time curves. The United States statutory definition of "bioavailability" reads as follows: "Bioavailability is measured by assessing the rate and extent to which an active drug or active moiety is absorbed from the drug product and becomes available at the site of action (7)." This definition is theoretically elegant but is not applicable in practice. It did, however, have the effect of focusing attention on rate and extent of absorption of drug from the site of administration into the systemic circulation. It has long been recognized that the area under the (usually plasma or serum) concentration vs. time curve (AUC) is a robust measure of extent of absorption. The fundamental relationship (Eq. 1) relates clearance (CL) to the dose of drug, AUC, and the fraction of the dose absorbed from the gastrointestinal tract into the systemic circulation (F), which is also known as the "bioavailability." The subscript T in Eq. 1 refers to the test formulation, whereas subscript R refers to the reference formulation.

$$CL_T = \frac{F_T \times Dose_T}{AUC_T};\ CL_R = \frac{F_R \times Dose_R}{AUC_R} \quad (1)$$

Simple manipulation of Eq. 1 gives the ratio of bioavailabilities (F_T/F_R) as shown in Eq. 2.

$$BE \approx \frac{F_T}{F_R} = \frac{CL_T \times AUC_T}{dose_T} \times \frac{dose_R}{CL_R \times AUC_R} = \frac{AUC_T}{AUC_R} \quad (2)$$

Normally, one would expect clearance after administration of the test product (CL_T) to be the same as that after administration of the reference formulation (CL_R) and the dose of drug to be the same in each formulation, in which case Eq. 2 may be simplified to a comparison of AUC values. Thus, the ratio of AUCs is a valid means of comparison of the extents of bioavailability of the drug after administration of the two formulations. If a nonmedical ingredient in the test product (say an unfortunate choice of excipient) did cause a change in clearance after administration of the test product, the bioavailability (F_T) and AUC_T would be affected accordingly, and the bioequivalence study would fail.

For many years, C_{max} was considered as an important measure of rate of absorption of drug from the gastrointestinal tract into the systemic circulation. More recently, however, a simulated bioequivalence study demonstrated that C_{max} of the parent drug was insensitive to a 25% difference in the absorption rate constants (k_a) of drug from the test and reference formulations when intrinsic clearance was less than liver blood flow (6). This clearly indicates that C_{max} is a not a reliable measure of rate of absorption. Various other direct and indirect measures of rate of absorption have been proposed, but all of them have disadvantages that limit their practical value in bioequivalence studies (8). It is now recognized, however, that k_a is a complex, hybrid rate constant that embraces many competing processes and is of limited clinical importance in the context of bioequivalence. On the other hand, C_{max} is influenced both by volume of distribution (V_d) and extent of distribution, both important pharmacokinetic measures that do indeed have clinical relevance.

In view of the foregoing difficulties, in recent years focus has shifted away from consideration of rate and extent of absorption to the *exposure* concept of bioequivalence. Here, C_{max} and AUC are considered to be clinically relevant measures representing peak exposure and total exposure, respectively. In addition, a relatively new measure termed "early exposure" has been introduced for situations in which it is envisaged that careful monitoring at early time points is considered important. "Early exposure" has been defined as area under the plasma concentration vs. time curve truncated at the median t_{max} of the reference formulation. At present, however, no drug regulatory agency has proposed bioequivalence limits for the new measure. For more details on exposure concepts in bioequivalence, the reader is referred to a good recent review on the subject (8).

THE STATISTICAL BASIS OF BIOEQUIVALENCE

Estimation of bioequivalence is based on the "two one-sided test" (9) in which the (1-2α) confidence interval around the geometric mean ratio (GMR) of the test and reference values of an appropriate measure (such as C_{max} or AUC) is required to fall within preset bioequivalence limits. The latter are based on a consensus among physicians that a difference of 20% in dose between two formulations would have no clinical significance for many drugs. Measures derived from plasma concentrations are log-normally distributed, however, so 0.8–1.2 on the raw scale becomes ±0.223 on the natural log scale (often written as 0.8–1.25, or 80–125%). A (1-2α) confidence interval is used to allow for the fact that a test to reference ratio may either be less than unity or greater than unity. In other words, the "two one-sided test" allows for either situation with the set value of α in either tail. The "two one-sided test" is unique in that the null hypothesis is one of nonequivalence, such that enough evidence must be found to reject the null hypothesis in order to accept the alternative hypothesis of bioequivalence. The type-I error (a declaration of bioequivalence for two products that are truly not bioequivalent) is referred to as the "consumer risk" and is typically set at ($\alpha = 0.05$); that is, 5%. Similarly, the type-II error (a declaration of nonequivalence for two products that are truly bioequivalent) is known as the "producer risk" and is determined by the experimental conditions.

One of the important functions of bioequivalence is to provide assurance that two formulations of a given drug are interchangeable in any individual subject. For this reason, the "two one-sided test" is based on the within-subject variability (WSV), which is commonly estimated from the residual mean square (S_W^2) in analysis of variance (ANOVA) in which the fixed effects are typically Formulation, Period, Sequence and Subject (Sequence). The WSV is often expressed as the "ANOVA-CV," which is estimated from the residual mean square by Eq. 3 in which EXP is the exponent.

$$ANOVA - CV = \sqrt{EXPs_w^2 - 1} \times 100\% \quad (3)$$

The width of the 90% confidence interval depends on the magnitude of the WSV and the number of subjects in the bioequivalence study. Some drugs have a low ANOVA-CV (5–15%), whereas others are considered highly variable with an ANOVA-CV of ≥30%. Thus, the higher the ANOVA-CV, the greater the number of subjects required to give adequate statistical power.

DRUG REGULATORY VIEW: METABOLITES IN BIOEQUIVALENCE

The U.S. statutory definition quoted above (see the section, "Pharmacokinetic Basis of Bioequivalence") makes ref-

erence to "an active drug or active moiety," which permits consideration of an active metabolite in addition to, or instead of, the parent drug. Guidelines issued in various jurisdictions by drug regulatory agencies, however, tend to be rather equivocal in their reference to the use of metabolite data. For example, the Draft Guidance for Industry issued by the U.S. Food and Drug Administration (FDA) (10) states "for BE studies, measurement of only the parent drug released from the dosage form is generally recommended. The rationale for this recommendation is that the concentration-time profile of the parent drug is more sensitive to changes in formulation performance than a metabolite, which is more reflective of metabolite formation, distribution, and elimination." The Draft Guidance then goes on to cite instances when metabolite data may be preferred, such as "when parent drug levels are too low to allow reliable analytical measurement in blood, plasma or serum for an adequate length of time. The metabolite data obtained from these studies should be subject to a confidence interval approach for BE demonstration."

The Draft Guidance then refers to the formation of a metabolite as a result of "gut wall or other presystemic metabolism." The formation of a major, active metabolite during the first pass from the lumen of the gastrointestinal tract to the systemic circulation can be an important issue in bioequivalence because formulation factors usually have the most impact during this period. The Draft Guidance continues: "If the metabolite contributes meaningfully to safety and/or efficacy, the metabolite and the parent drug should be measured." In many cases, however, knowledge of the activity of metabolites is based on receptor binding studies on animal tissues or other pharmacological tests in animals. The extent to which the results of such studies extrapolates to safety and/or efficacy issues in human patients is often unknown.

The foregoing recommendations imply, but do not state that the same exposure measures used for the parent drug would also be applied to metabolite data, that is, peak exposure, total exposure, and early exposure, and that confidence intervals and preset bioequivalence limits would be applied similar to those recommended for a parent drug. Certainly in our experience, the FDA recommends submission of data on the same exposure measures for metabolites as those required for the parent drug, notably C_{max}, AUC_{last}, and AUC_{∞}.

IMPACT OF MULTIPLE ANALYTES IN BIOEQUIVALENCE STUDIES

The section on metabolites in the FDA Draft Guidance of 2002 (10) concludes with the statement that "metabolite data can be used to provide supportive evidence of comparable therapeutic outcome." This statement appears to suggest the potential use of multiple analytes in bioequivalence decision-making. As outlined above, however, a traditional bioequivalence study is based on a single analyte, usually the parent drug, and the consumer risk is set at 5%. In the present day, it has become common for drug regulatory agencies to request data on one or more (active) analytes simply because it is possible to monitor them. Before a drug regulatory agency receives such data, however, a decision should be made *prospectively* as to which single analyte will be used for the BE decision. The consumer risk then remains at 5% whether the analyte selected is a parent drug or a metabolite. If an agency selects one of several analytes *retrospectively,* then the consumer risk could be reduced and the producer risk increased.

The consumer risk was set at 5% as a reasonably conservative level of uncertainty when a generic formulation is allowed to compete with the brand product in the market place. An increase in producer risk makes it more likely that a truly BE generic formulation will be excluded from the marketplace. It is therefore important to be clear about the potential consequences if metabolite data are used inappropriately as "supporting evidence" in BE evaluations. This problem becomes all the more acute when there are multiple active metabolites that contribute to overall therapeutic efficacy of the drug treatment.

"SENSITIVITY" IN BIOEQUIVALENCE STUDIES

The U.S. Draft Guidance for Industry (10) states that the "concentration-time profile of the parent drug is more sensitive to changes in formulation performance than a metabolite, which is more reflective of metabolite formation, distribution, and elimination." Here the word "sensitive" may be interpreted as "sensitive to error" and is commonly used as such in bioequivalence jargon. A generic manufacturer may, for example, use an inappropriate nonmedical ingredient (such as an excipient) in attempting to copy the brand product, which may lead to greater variability or "error" in the performance of the generic product. Sensitivity to "error" then is regarded as a useful characteristic in bioequivalence. In the context of the above quotation from the FDA Draft Guidance, greater sensitivity of the parent drug compared with a metabolite means that the 90% confidence interval for the parent will be wider than that of the metabolite, such that a greater number of subjects is required to achieve adequate statistical power than would be required if bioequivalence were to be based on the metabolite. Other authors imply that whichever analyte (parent drug or metabolite) displays the wider confidence interval is the more "sensitive to error" or the more "discriminating" for bioequivalence. As we shall demonstrate, however, this simple approach may be greatly compromised by the presence of "outliers." In our view, the most promising theoretical approach to this question lies in the application of the fundamental pharmacokinetic principles based on the "well-stirred" hepatic model, which is outlined below.

BRIEF REVIEW: METABOLITES IN BIOEQUIVALENCE STUDIES

One of the most commonly cited reasons for consideration of metabolite data is the case of the inactive prodrug where it is commonly accepted that the active metabolite is the appropriate analyte for the assessment of BE. An example of this concept is the bioequivalence of two formulations of the antipsychotic prodrug fluphenazine decanoate, which is eventually completely metabolized to the active fluphenazine metabolite (11). The title of the foregoing manuscript erroneously refers to the "steady state pharmacokinetic profile of fluphenazine decanoate" (11), whereas the analyte actually monitored was fluphenazine, not the prodrug. The results showed a statistically significant higher mean C_{max} of fluphenazine and a non-significant trend toward a shorter t_{max} after intramuscular administration of the test formula-

tion compared with the reference product, although these differences were considered to be unimportant clinically.

A second reason for the application of metabolite data in BE studies arises when plasma concentrations of the parent drug are too low to permit analysis, thus necessitating the use of a quantifiable metabolite. Chloral hydrate is an example of a drug for which concentrations of the parent drug in plasma are very low. Therefore, a BE study (12) was carried out in which the active metabolite trichloroethanol (elimination half-life of about 10 h) was used as the discriminating analyte.

The situation becomes more complex in cases where plasma concentrations of the parent drug are too low to monitor but there are more than one active metabolites to consider. An important example of this phenomenon is the case of the antiparkinsonian drug selegiline which has a very short half-life variously reported to be only about 9 min (13) or 1.9 ± 1.0 h (14). Unpublished data from our labs supports the latter value. In view of low plasma levels and the short half-life of the parent drug, a BE study on two formulations of selegiline was therefore based on three active metabolites, desmethylselegiline, l-methamphetamine and l-amphetamine (13). Similarly, the parent antiarrhythmic drug nitroglycerin was reported as "virtually" undetectable after oral administrations of a solution, sublingual tablets, or a sustained-release tablet (15). When the delivery system avoided first-pass metabolism, however, the parent drug and its two active metabolites, 1,2-dinitroglycerin and 1,3-dinitroglycerin, were all quantifiable in a comparative pharmacokinetic and bioavailability study (16) on three different transdermal formulations. Similar observations were reported for isosorbide and two active metabolites, isosorbide 5-mononitrate, and isosorbide 2-mononitrate (17). In this study, after buccal administration in which the drug is absorbed directly into the systemic circulation through the buccal mucosae, the bioavailability of the parent drug was more than double than that found when a tablet formulation was swallowed. In cases such as the foregoing, there may be justification for the use of metabolite data when parent drug concentrations are too low to be monitored. Questions still remain, however, especially when there are multiple active metabolites.

A third situation for which the use of metabolite data has been advocated is for the bioequivalence of formulations of highly variable drugs. The latter have been defined as drugs with a within-subject variability (WSV) in terms of the ANOVA-CV on the maximum plasma concentration (C_{max}) and/or area under the plasma concentration vs. time curve (AUC) of equal to or greater than 30% (18). Very large numbers of subjects are required in BE studies to give adequate statistical power when the WSV is high. Ezan and co-workers (19) stated that "in situations of low absorption, high first pass metabolism and intrasubject variability, metabolites may reflect absorption more adequately than the parent drug, and their determination may help decision-making in bioequivalence issues." Based on a study with α-dihydroergocryptine and metabolites (19), they concluded that their study "describes a particular case where only measurements on the basis of the metabolites can justify the assumption of bioequivalence." This conclusion, however, was based on the measurement of the parent drug and at least four metabolites cross-reacting in an enzyme-linked immunoassay for which the 90% confidence intervals around the geometric mean ratio of AUC happened to fit within preset BE limits of 80–125%, whereas those for C_{max} did not. Parent α-dihydroergocryptine concentrations were very low as measured by a radioimmunoassay method, and the 90% confidence intervals for both C_{max} and AUC failed to meet preset BE limits of 80–125%. Therefore, closer scrutiny of these data suggest that there was in fact no firm basis for the recommendation that bioequivalence should be declared based on the simplistic measurement of an uncharacterized mixture of metabolites.

The use of metabolite data in bioequivalence studies involving highly variable drugs is appealing because metabolites are often less variable than the parent drug such that smaller numbers of subjects are required to achieve statistical power. For example, in a single-dose study on the antipsychotic drug loxapine and two active metabolites (20), the ANOVA-CVs of C_{max} and AUC of the parent drug were greater than those of either metabolite, and the 90% confidence intervals of the metabolite were therefore narrower than those of the parent drug. The ANOVA-CVs are generally lower ("dampened") at steady state compared with single oral doses. In a steady-state study on two oral formulations of spironolactone, the parent drug and two metabolites, canrenone and 7-α-thiomethylspironolactone were considered (21). Plasma concentrations of the metabolites were higher than those of the parent drug and the authors recommended that metabolite data should be included in bioequivalence studies on spironolactone formulations. Manuscripts such as the foregoing are examples of hundreds of such documents in the scientific literature that provide valid evidence of the potential value of metabolite data in bioequivalence studies, but by virtue of their anecdotal nature, cannot provide a coherent framework from which a regulatory decision-making process involving metabolites can evolve.

INTRINSIC CLEARANCE AND THE "WELL-STIRRED" LIVER

Three seminal manuscripts in the field of pharmacokinetics appeared in the 1970s. The first of these papers by Gibaldi and co-workers (22) described a simple method to estimate the fraction of the parent drug absorbed from the gastrointestinal tract into the systemic circulation (F) (Eq. 4) where Q_H is liver blood flow, set at 90 L/hr,

$$F = \frac{Q_H}{Q_H + (dose/AUC_{po})} = \frac{Q_H}{Q_H + CL_o} \quad (4)$$

AUC_{po} is the area under the plasma concentration vs. time curve after oral dosing of the parent drug, and CL_o is apparent oral clearance. It is assumed that the kinetics are linear, the drug is metabolized only in liver.

The second and third seminal manuscripts, published by Perrier and Gibaldi (23) and by Wilkinson and Shand (24), introduced the concepts of intrinsic metabolic clearance and the "well-stirred" model of hepatic function. In this, hepatic extraction (E_H) is a function of the intrinsic clearance (CL_{int}) and the fraction of the parent drug unbound (f_u) as shown in Eq. 5. The product of CL_{int} and f_u estimates apparent oral clearance (CL_o) when the drug is metabolized entirely in liver.

$$E_H = 1 - F = \frac{CL_{int} \times f_u}{Q_H + (CL_{int} \times f_u)} = \frac{CL_o}{Q_H + CL_o} \quad (5)$$

Intrinsic clearance is given by Eq. 6, in which the product of E_H and Q_H is hepatic clearance. The latter approximates to intravenous systemic clearance when the drug is metabolized

entirely in liver, under which conditions the quotient of hepatic clearance and the fraction absorbed (F) estimates apparent oral clearance. The important

$$CL_{int} = \frac{E_H \times Q_H}{F \times f_u} = \frac{CL_o}{f_u} \quad (6)$$

point as far as bioequivalence studies are concerned is that intrinsic clearance can be estimated from the estimate of apparent oral clearance from the study data and from values of the fraction unbound that, for many drugs, can be obtained from the literature.

Subsequently, more sophisticated pharmacokinetic models have been developed to take into account metabolism in the intestinal wall as well as in the liver (25,26), but they tend to be of limited practical application to oral data. Moreover, the simple model appears to work reasonably well in situations where there is more than one metabolite, where there is sequential phase I and/or phase II metabolism, and even when there is some metabolism occurring in the intestinal wall, provided pharmacokinetics of the parent drug are linear.

KEY SIMULATED STUDIES ON THE ROLE OF METABOLITES IN BIOEQUIVALENCE

Simulated studies have the advantages that all the parameters are specified, and the correct answers are known given the assumptions. The disadvantage is that biological complexity may be oversimplified. The published papers about simulations on the use of metabolites are divided into two broad categories: (i) simulations seeking to detect the analyte most discriminant for bioequivalence decision-making based on the relative widths of the confidence intervals, and (ii) simulations based on intrinsic clearance and the well-stirred model. In this commentary, we shall review five important publications based on simulations of oral administration of a parent drug from which a single metabolite is produced: (i) Chen and Jackson, 1991 (2); (ii) Chen and Jackson, 1995 (3); (iii) Tucker and co-workers, 1993 (4); (iv) Rosenbaum and Lam, 1997 (5); and (v) Jackson, 2000 (6).

Simulations Seeking the Most Discriminant Analyte Based on Relative Widths of the Confidence Intervals

Simulations on C_{max}: Chen and Jackson, 1991

Chen and Jackson (2) used a simple pharmacokinetic model (Fig. 1) to generate plasma concentration time profiles for the parent drug and a single metabolite, with the elimination of the metabolite being limited either by its formation in the body (model I) or by its excretion from the body (model II). The title of this manuscript with its reference to drugs with "no first pass effect" is misleading because the authors are actually referring to low hepatic extraction drugs with no *extensive* first-pass effect. The assumptions were that the drug was absorbed faster after administration of the test product than after administration of the reference formulation and that absorption of the drug from the gastrointestinal tract was complete after either product. To simulate bioequivalence studies, bivariate distributions of the absorption rate constant (k_a) were generated and k_a was varied to represent the test and reference formulations. The values for the formation rate constant for the metabolite (k_f) and the elimination rate constant for the metabolite (k_e) were assumed to be constant for each subject. Normally distributed errors (high CV at 49% and low CV at 20%) were added to k_a to account for potential variability in assay, formulation, and subject.

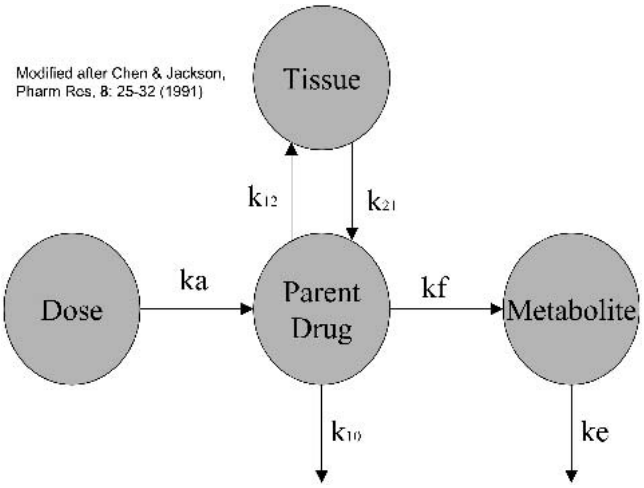

Fig. 1. Pharmacokinetic model employed by Chen and Jackson, 1991 (2).

The result of the simulations of this simple, uncomplicated situation was that the WSV associated with C_{max} was invariably greater for the parent drug than for the metabolite, regardless of the metabolite model or how random errors were applied. This meant that the 90% confidence intervals for the parent drug were invariably wider than those of the metabolite. The authors (2) quoted four bioequivalence studies (Table I) to support the findings of their simulations. In each case, the 90% confidence interval for the parent drug was wider than that of the metabolites. Two of the four drugs selected (acetohexamide and procainamide) appear to meet the simple criteria of linear kinetics and low hepatic extraction. Allopurinol and sulindac, however, are not appropriate examples of such drugs.

Allopurinol is rapidly absorbed from the gastrointestinal tract and is rapidly cleared from plasma with an elimination half-life of 1–2 h. About 20% of the dose is to be found in the feces, probably due to incomplete absorption from the gas-

Table I. Examples of Actual Bioequivalence Studies on Drugs with Linear Kinetics Without First-Pass Effect in Terms of C_{max}: Chen and Jackson, 1991 (2)

Drug/metabolite	WSV%[a]	90%CI[b]	Width CI[c]
Acetohexamide	23.7	81–105	24
Hydroxyhexamide	13.1	95–108	13
Allopurinol	31.9	91–126	35
Oxypurinol	6.8	95–102	7
Procainamide	13.3	98–111	13
N-Acetylprocainamide	9.0	96–105	9
Sulindac	37.5	86–120	34
Sulindac sulfide	27.1	84–108	24

[a] Within-subject variability expressed as the square root of the residual variance in ANOVA of untransformed data divided by the reference mean.
[b] 90% confidence interval.
[c] Width of the 90% CI.

trointestinal tract. The drug is extensively metabolized during the first pass into its active metabolite, oxypurinol, which has a much longer elimination half-life than the parent compound (27). Thus, allopurinol appears to be an intermediate hepatic extraction drug rather than low extraction drug. Consequently, C_{max} and AUC values of allopurinol were very much lower than those of the oxypurinol (2), and the parent drug was highly variable (ANOVA-CV 31.9%), whereas the metabolite was much less variable (6.8%). Chen and Jackson (2) implied that BE should be decided on the basis of the parent drug, which had wider confidence intervals than the metabolite. A worrying problem with this simple approach is that it is very sensitive to the influence of outliers.

The choice of sulindac (an inactive sulfoxide) and its active sulfide metabolite was also an inappropriate example of a simple case involving linear pharmacokinetics without extensive first-pass metabolism (2). Sulindac (Fig. 2) is essentially a prodrug with complex pharmacokinetics involving reversible metabolism to and from the active sulfide metabolite and irreversible metabolism to the inactive sulfone. In the BE study cited by Chen and Jackson (2), the parent drug was also highly variable (ANOVA-CV 37.5%), whereas the metabolite was somewhat less variable (27.1%) and thereby had narrower 90% confidence intervals than the parent drug. Again, a case could be made for basing the BE decision on the active sulfoxide metabolite, despite the fact that the metabolite had narrower 90% confidence intervals than the parent drug.

Simulations on C_{max}: Chen and Jackson, 1995

In a second paper in the series, Chen and Jackson (3) used a more sophisticated model (Fig. 3) in which they divided the central compartment into two components in order to accommodate the absorption rate constant of the parent drug (k_a) and the formation rate constant of the metabolite (k_s) during the presystemic absorption of the parent drug. This model also allowed provision for a formation rate constant (k_f) of the metabolite during subsequent recirculation through the liver, which corresponds to the rate constant one would find after intravenous administration of the drug. The model also made provision for the parent drug to partition into and out of a tissue compartment (Fig. 3). Plasma concentration vs. time curves were generated for the parent drug and metabolite by means of the relevant rate constants obtained from a bivariate normal distribution and designated random errors.

The key finding in this study was that the model was able

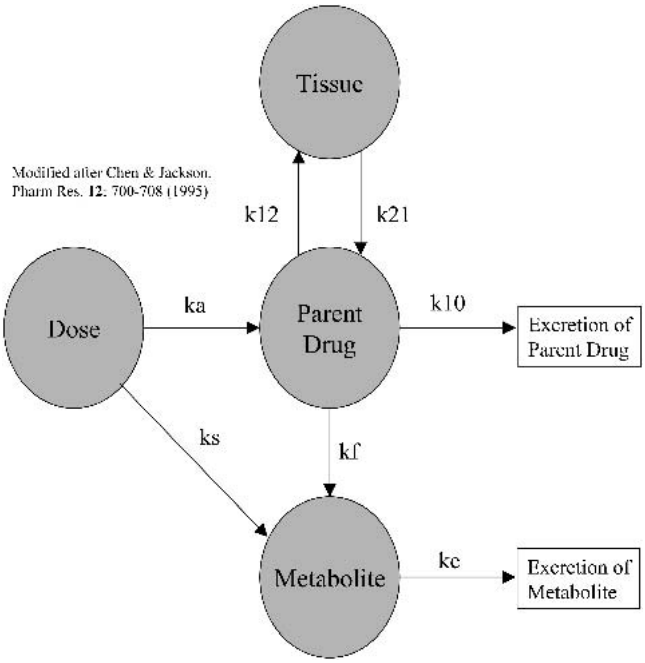

Fig. 3. Pharmacokinetic model employed by Chen and Jackson, 1995 (3).

to distinguish between the discriminating power of C_{max} of the parent drug and that of the metabolite, depending on the relative WSV of the absorption rate constant (WSV-k_a) of the parent drug, and the first-pass formation rate constant of the metabolite (WSV-k_s). In cases where WSV-k_a > WSV-k_s, the width of the confidence interval of the parent drug was invariably greater than that of the metabolite in terms of C_{max}. On the other hand, when there was higher WSV associated with first-pass metabolism than with the presystemic absorption of the drug (i.e. WSV-k_s > WSV-k_a), then the metabolite had wider confidence intervals in terms of C_{max} than the parent drug. Discrepancies were observed when the WSV associated with elimination was greater than that associated with presystemic absorption, but otherwise the study produced a rationale for use of drug or metabolite C_{max} data, if one accepts that a wider confidence interval is reason enough to prefer one analyte over another.

Again, these authors quote from real data from the FDA archives to support their conclusions; five drug-metabolite combinations for which the WSVs associated with C_{max} were

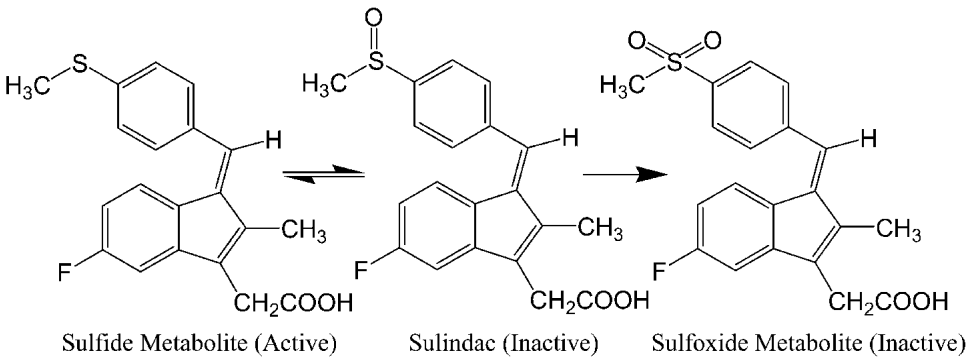

Fig. 2. Sulindac and metabolites.

greater for the parent than for the metabolite with corresponding wider confidence intervals for the parent drug and two drug-metabolite combinations for which the reverse was true (Table II). A weakness of this approach is that C_{max} is largely influenced by extent of absorption and is not very sensitive to the absorption rate constant (k_a), whereas the ratio C_{max}/AUC is much more sensitive to k_a but is not sensitive to extent of absorption (28,29). Thus, the ANOVA-CV of the C_{max}/AUC ratio of the parent drug gives a better estimate of the within-subject variability of k_a than can be obtained from C_{max}. Correspondingly, the ANOVA-CV of the C_{max}/AUC ratio of the metabolite gives an estimate of the WSV associated with the formation rate constant (k_s) of the metabolite during the presystemic absorption of the parent drug.

Simulations Based on Intrinsic Clearance and a "Well-Stirred" Liver

Simulations on AUC: Tucker and Co-workers, 1992

Tucker and co-workers (4) carried out simulations based on a more sophisticated pharmacokinetic model than those used by Chen and Jackson (2,3) in which the drug is converted into a single metabolite on first passage and on subsequent recirculation through the liver, followed by renal clearance of both drug and metabolite (4). The basic assumptions were linear, time invariant disposition pharmacokinetics and a "well-stirred" liver. The relative bioavailability based on drug and metabolite kinetics was derived in terms of the fundamental kinetic variables intrinsic clearance, renal clearance (CL_r) of drug and metabolite and hepatic blood flow. Plasma protein binding was subsumed into intrinsic clearance. Tucker and co-workers argued that in practice, the ratio of test/reference AUC values (drug or metabolite) that will be the more sensitive to differences in the fraction of the dose absorbed from the gastrointestinal tract is dependent on the relative within-subject variabilities in relevant metabolic and renal clearances.

The results (Table III) (4) indicated that for *extent* of relative bioavailability (test/reference AUC ratios), use of parent drug *or* metabolite data (active or inactive) may be appropriate for BE testing, provided the choice is made prospectively. Tucker and co-workers also pointed out that if the kinetics of the drug are well understood and there is some appreciation of the WSV, it should be possible to determine *a priori* whether drug or metabolite data are more discriminant of BE. In situations where $CL_{int} < Q_H$ (low extraction ratio), data on the parent drug are preferred except in cases where CL_r is low, in which case metabolite data are preferred. Estimation of CL_r entails collecting total urine for between four and five elimination half-lives, which is a tedious imposition on volunteers in a BE study, but data on renal clearance are available in the literature for some drugs. Metabolite data are also preferred when $CL_{int} \geq Q_H$.

Simulations on C_{max} and AUC: Rosenbaum and Lam, 1997

Rosenbaum and Lam (5) carried out a similar study to the foregoing, based on the fundamental pharmacokinetic parameters of intrinsic and hepatic clearances in which a parent drug and a single first-pass metabolite were considered. Their study focused on the relative sensitivities of the parent drug and metabolite to variabilities imposed on the key pharmacokinetic measures in the model. The results indicated that the parent drug displayed greater sensitivity to all forms of error, such that with a given number of subjects, the 90% confidence intervals around the geometric mean test/reference ratios of C_{max} and AUC were invariably wider for the parent drug than those of the metabolite. These results (5) give support to the widely held view based on empirical evidence that the parent drug is the more discriminating for BE because it is more sensitive than a metabolite to the key within-subject variabilities operative on the complex process of presystemic absorption.

Simulations on C_{max} and AUC: Jackson, 2000

In a third paper in the series, Jackson (6) examined the role of metabolites in BE assessment of highly variable drugs under conditions of pharmacokinetic linearity and extensive first-pass metabolism. Highly variable drugs were defined at Bio-International '92 as drugs for which the ANOVA-CV from studies based on the traditional 2-treatment, 2-period, 2-sequence design was equal to or exceeded 30% (18). The pharmacokinetic model used (Fig. 4) was based on a general model of drug and metabolite kinetics proposed by Weiss

Table II. Examples of Actual Bioequivalence Studies on Drugs with Linear Kinetics and First-Pass Effect in Terms of C_{max}: Chen and Jackson, 1995 (3)

Drug/metabolite	WSV%[a]	90%CI[b]	Width CI[c]
Examples of WSV-parent > WSV-metabolite			
Triamterene	27.9	99–127	28
OH-triamterene sulfate	17.1	90–107	17
Doxepin	28.4	79–102	23
N-Desmethyl	16.2	86–99	13
Isosorbide dinitrate	64.8	79–150	71
2-mononitrate	21.0	103–126	23
5-mononitrate	19.3	99–120	21
Metoprolol	17.4	100–114	14
OH-metoprolol	9.3	103–110	7
Amitriptyline	18.5	94–112	18
Nortriptyline	12.0	94–106	12
Examples of WSV-metabolite > WSV-parent			
Imipramine	14.3	95–110	15
Desipramine	19.2	89–110	21
Nortriptyline	11.7	91–102	11
OH-nortriptyline	19.3	89–107	18

[a] Within-subject variability expressed as the square root of the residual variance in ANOVA of natural log transformed data.
[b] 90% confidence interval.
[c] Width of the 90% CI.

Table III. Results of Simulations on AUC: Tucker *et al.,* 1993 (4)

Condition	Preferred analyte
$CL_{int} < Q_H$	Parent drug preferred
	Except where CL_r is low: Metabolite preferred
$CL_{int} \geq Q_H$	Metabolite preferred

CL_{int}: intrinsic clearance, estimated as the quotient of apparent oral clearance and the fraction of the parent drug unbound, where the kinetics are linear and the drug is metabolized primarily in the liver. AUC, area under curve.

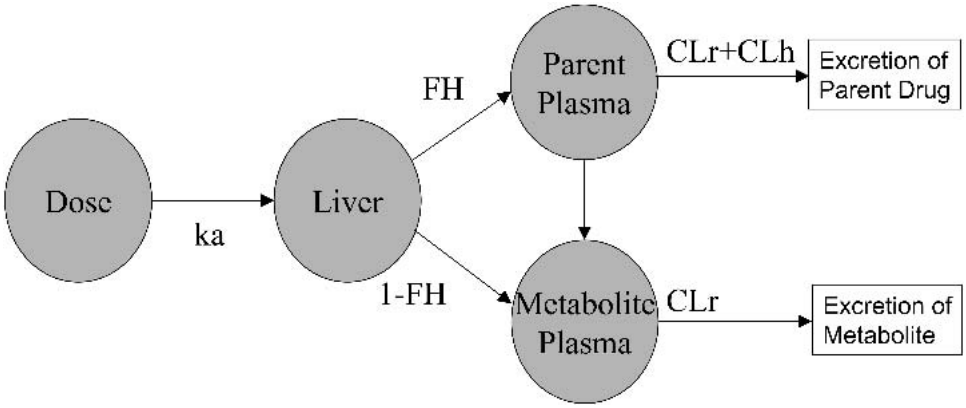

Fig. 4. Pharmacokinetic model employed by Jackson, 2000 (6).

(30). The model depended on a well-stirred liver (24), with the fraction of the drug unbound (f_u) subsumed into intrinsic clearance. Simulations were carried out with WSV values ranging from 20% to 60% applied to the absorption rate constant (k_a), the formation rate constant of a single metabolite (f_m), volume of distribution, liver blood flow, intrinsic clearance, and renal clearance. The test to reference ratios of the absorption rate constant (k_{a_T}/k_{a_R}) and the ratios of the fraction of drug absorbed into the systemic circulation (F_T/F_R) were each studied at ratios 1.0 and 1.25.

The results showed that C_{max} was insensitive to a 25% difference in k_a when intrinsic clearance was less than liver blood flow. Thus, the probability of concluding bioequivalence was the same for parent drug and metabolite when intrinsic clearance was less than liver blood flow. When intrinsic clearance approached or exceeded liver blood flow, however, the parent drug responded to a 25% difference in k_a, whereas the metabolite remained insensitive, thus allowing a much greater chance of a conclusion of bioequivalence for the metabolite than for the parent drug. In this situation, however, a 25% difference in the absorption rate constant of the parent drug after administration of the test and reference products would result in a difference in the time to maximum concentration (t_{max}) of the parent drug, which in itself should cause concern for BE assessment. For example, in a study (31) in which the pharmacokinetics of an immediate release formulation of methylphenidate were compared with a slow release formulation, there was a 30% difference in t_{max} between the formulations in the fasting state ($p = 0.0001$, Wilcoxon signed rank test) and a 46% difference after a high fat breakfast ($p = 0.0001$). Similarly, the ratio C_{max}/AUC (sensitive to rate but not extent of absorption) was also highly significant ($p = 0.0001$) in the F-test from ANOVA, reflecting the expected difference in absorption rate after administration of the immediate and sustained release formulations. In practical terms therefore, one should never ignore the failure of the 90% confidence interval of the parent drug to fall within preset bioequivalence limits, together with a significant difference in t_{max}, and declare bioequivalence based on a metabolite.

In simulations in which the test/reference ratios of both k_a and F were set at unity (perfect bioequivalence) and intrinsic clearance exceeded liver blood flow, there was only about a 70% probability of concluding bioequivalence in terms of C_{max} of the parent drug, whereas the metabolite registered a 100% probability. In other words, there was roughly a 30% chance of committing a type II error and declaring the test product not to be bioequivalent with the reference product in terms of the C_{max} of the parent drug. For the metabolite, however, depending on the WSV set for renal clearance, there was a 90–100% probability of declaring bioequivalence based on the metabolite in terms of C_{max}. The results of the simulations on AUC were similar to those for C_{max} in that the metabolite was the better predictor of true bioequivalence when intrinsic clearance exceeded liver blood flow. This was consistent with the findings of Tucker and co-workers (Table III).

Application of the Simulation Methods to Actual Bioequivalence Studies

The simulated studies provide two different methods that can be applied to actual BE data. The first of these methods compared within-subject variability associated with the hybrid absorption rate constant (k_a) with that of the hybrid formation rate constant (k_s) of the metabolite. Here, the ANOVA-CVs of C_{max} normalized to AUC (C_{max}/AUC) of the parent drug and of the metabolite(s) give more appropriate estimates of WSV-k_a and WSV-k_s than those associated with C_{max}. In this conception, the process with the greater WSV (WSV-k_a or WSV-k_s) will be used to select the analyte (parent drug or metabolite) with the greater sensitivity to error and therefore the greater discriminatory power for bioequivalence-decision-making in the actual BE studies to be examined below.

The second method is based on the intrinsic clearance of the parent drug in relationship to liver blood flow. Generally speaking results of simulations showed the parent drug is preferred when $CL_{int} < Q_H$, whereas a major active metabolite is preferred when $CL_{int} \geq Q_H$. We shall also apply this method to the examples of actual BE studies in which intrinsic clearance will be estimated as the quotient of apparent oral clearance and the fraction unbound (Eq. 6).

APPLICATIONS TO ACTUAL BIOEQUIVALENCE DATA

Doxepin and *N*-Desmethyldoxepin

Doxepin is an antidepressant that is marketed as an irrational mixture of geometric isomers, 15% of the more active

cis isomer and 85% of the less active trans isomer. Doxepin is biotransformed to a variety of phase I and phase II metabolites (32–34) of which the major *N*-desmethyl metabolite is active and appears to contribute to therapeutic activity (35,36). Despite the apparent complexity of its metabolism, however, estimation of the fraction absorbed (F) by the method of Gibaldi and co-workers (22) was exactly the same (F = 0.29 for each isomer) as was found when doxepin was administered intravenously and orally to a group of healthy volunteers in a pharmacokinetic study based on a crossover design (37). Doxepin therefore behaves as a highly extracted drug (E_H = 0.71). Apparent oral clearance for (*Z*)-doxepin was 238 L/h and that for (*E*)-doxepin was 262 L/h, and with the unbound fraction reported as 0.18, the corresponding values for intrinsic clearance were (*Z*) 1322 L/h and (*E*) 1456 L/h. Thus for both isomers, intrinsic clearance was well in excess of liver blood flow, which would suggest that the metabolite data would be an appropriate choice for both C_{max} and AUC in a bioequivalence study.

Bioequivalence studies on doxepin have been problematical from the analytical point of view because plasma concentrations of (*Z*)-doxepin are very low as a consequence of its presence as only 15% of the total doxepin in the formulation. For example, a bioequivalence study on two formulations of doxepin in 30 healthy volunteers (38) used a validated stereoselective analytical procedure, but it was possible to monitor (*Z*)-doxepin plasma concentrations in only 3/30 subjects. Consequently, only total doxepin plasma concentrations were available for bioequivalence decision-making based on the parent drug, although plasma concentrations of both *N*-desmethyldoxepin isomers were high enough for separate monitoring. For purposes of comparison with Chen and Jackson (3), however, we shall consider total doxepin levels for both parent drug and metabolite.

The results of the study which were based on a 2-formulation, 2-treatment, 2-period crossover design are summarized in Table IV. Immediately noticeable is the fact that the parent drug was highly variable in terms of C_{max} (ANOVA-CV ≥ 30%), whereas the metabolite was not highly variable in any measure. The ANOVA-CVs for both C_{max} and AUC_{last} of the parent drug were higher than those of the metabolite and therefore the 90% confidence intervals were wider for the parent drug than for the metabolite. These results are consistent with a different bioequivalence study on total doxepin quoted by Chen and Jackson, 1995 (3) such that 90% confidence interval around the geometric mean ratio of C_{max} failed to fall within present bioequivalence limits of 80–125% in both studies. The parent drug ANOVA-CV of the ratio C_{max}/AUC (21.0%) was considerably less than that of C_{max} (34.1%) suggesting that large portion of the variability on C_{max} was not due to k_a. A comparison of C_{max}/AUC for the parent drug and metabolite, however, showed that the greater variability was associated with the parent drug (k_a) rather than the metabolite (k_s), consistent with Chen and Jackson, 1995 (3). The argument that the measure with the higher WSV and therefore the wider confidence interval is the most discriminating for bioequivalence decision-making is not convincing, however. In this case, the intrinsic clearance of the parent drug was greatly in excess of liver blood flow, which suggests the metabolite is the preferred analyte for both C_{max} (6) and AUC (4,6) particularly as a Wilcoxen signed rank test showed no significant difference between test and reference t_{max} values of either parent drug or metabolite. This interpretation seems particularly reasonable in a case such as doxepin (38) where plasma concentrations of active (*Z*)-doxepin were below the lower limit of quantification in 27/30 subjects such that measurement of total doxepin in reality was a reflection of the much less active (*E*)-isomer. Thus, it is also reasonable from both pharmacokinetic and clinical standpoints to base the bioequivalence decision on the active metabolite (*Z*)-*N*-desmethyldoxepin.

Table IV. Results of a Bioequivalence Study on Two Formulations of Doxepin in 30 Healthy Volunteers

Measure[a]	WSV%[b]	90%CI[c]	Width of CI
Doxepin[d]			
C_{max}	34.1	102–138	36
AUC_{last}	23.2	96–119	23
C_{max}/AUC_{last}	21.0	101–122	21
N-desmethyldoxepin[e]			
C_{max}	13.9	99–113	14
AUC_{last}	11.6	93–104	11
C_{max}/AUC_{last}	14.8	100–115	15

WSV, within-subject variability; CI, confidence interval.

[a] All measures calculated from natural log transformed data (SAS).

[b] Within-subject variability estimated from ANOVA.

[c] 90% confidence interval.

[d] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.1887 (Wilcoxon).

[e] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.3724 (Wilcoxon).

Nortriptyline and 10-Hydroxynortriptyline

Chen and Jackson, 1995 (3) included a study from FDA archives on nortriptyline and 10-hydroxynortriptyline as an example of the situation where there is a greater WSV associated with the formation rate constant of the metabolite (k_s) than with the absorption rate constant (k_a) of the parent drug (Table II). The complexity of the practical issues involved in studies of this kind can be illustrated by closer examination of nortriptyline. This antidepressant drug is a substrate for cytochrome P450 2D6 (CYP2D6), which hydroxylates the molecule at the 10-position in a highly stereospecific manner, thereby creating both geometric and optical isomerism. (–)-(*E*)-10-hydroxynortiptyline is a major metabolite that is pharmacologically active and may be a better antidepressant than the parent drug. For a brief review, see Nordin and Bertilsson (39). (–)-(*E*)-10-hydroxy metabolite was produced in human liver and intestinal microsomal preparations in higher concentrations than the (+)-(*E*)-10-hydroxy antipode. The metabolic conversion of nortriptyline into (–)-(*E*)-10-hydroxynortriptyline was inhibited by quinidine to a much higher degree than the formation of the (+)-(*E*)-10-hydroxymetabolite in human liver homogenates, suggesting that the former process is mediated by CYP2D6. In most patients, plasma concentrations of (–)-(*E*)-10-hydroxynortriptyline are higher than those of nortriptyline, although poor metabolizers with low CYP2D6 produce very little of the hydroxymetabolites. Moreover, glucuronidation is a major pathway in the disposition of the (*E*)-10-hydroxynortriptyline metabolites (40,41). It is evident that nortriptyline metabolism is more complicated than the assumptions of the simulations (3) allow.

A study on the bioequivalence of two formulations of nortriptyline from our laboratories (42) gave results very similar to those quoted by Chen and Jackson, 1995 (3). The WSVs for both C_{max} and C_{max}/AUC of the (±)-10-hydroxy metabolite were higher than those of the parent drug (Table V). Thus, in terms of the pharmacokinetic model used by Chen and Jackson, 1995 (Fig. 3), the WSV for the formation rate of the metabolite (WSV-k_s = 16.2%) was greater than that of the absorption rate of the parent drug (WSV-k_a = 12.8%). The confidence intervals for C_{max}/AUC of the metabolite were therefore wider than those of the parent drug (Table V) from which one might conclude the metabolite was the better discriminant of bioequivalence. The ratio C_{max}/AUC is not recognized by any drug regulatory authority, however, leaving only a similar, but weaker argument based on C_{max}, which is only marginally sensitive to absorption rate of the parent drug or formation rate of the metabolite.

The more fundamental approach to the problem taken by Tucker and co-workers (4) and Jackson (6) does, however, provide a more challenging dilemma to drug regulatory authorities. The fraction of parent drug absorbed into the systemic circulation (22) estimated from the nortriptyline bioequivalence study data (42) (F = 0.65) was similar to the value obtained in a two-period study in which nortriptyline was dosed intramuscularly and orally in humans (F = 0.66) (43). The hepatic extraction was in the low end of the intermediate range (E_H = 0.35) which suggests that nortriptyline is not subject to extensive first-pass metabolism. Apparent oral clearance was 48 L/h, but taking into account the fraction unbound (f_u = 0.08), intrinsic clearance was estimated as 600 L/h, which is greater than liver blood flow. Therefore, one would conclude that bioequivalence decisions on C_{max} and AUC should be based on the (active) metabolite, rather than on the parent drug.

The selection of the metabolite as the analyte of choice when intrinsic clearance exceeds liver blood flow makes sense intuitively, as one would anticipate that plasma concentrations of the metabolite would be greater than those of the parent drug. As outlined earlier, however, the metabolism of nortriptyline is more complicated than the simple assumptions of the simulations allow. Close scrutiny of the data in this case reveals that although intrinsic clearance appears to be greater than liver blood flow, hepatic extraction is relatively low (0.35). Moreover, examination of the plasma concentration vs. time curves of the parent drug and metabolites reveals they are both flat curves, at similar concentrations with mean t_{max} values at 8.7 h (test) and 7.6 h (ref) for the parent drug, and 7.7 h (test) and 7.4 h (ref) for the metabolite. These data are difficult to reconcile and cast doubt on the wisdom of choosing to make the bioequivalence decision on the basis of the metabolite data.

Table V. Results of a Bioequivalence Study on Two Formulations of Nortriptyline in 23 Healthy Volunteers

Measure[a]	WSV%[b]	90%CI[c]	Width of CI
Nortriptyline[d]			
C_{max}	10.2	95–106	11
AUC_{last}	9.4	95–106	11
C_{max}/AUC_{last}	12.8	93–107	14
(±) (*E*)-10-OH-nortriptyline[e]			
C_{max}	18.2	93–113	20
AUC_{last}	11.1	92–104	12
C_{max}/AUC_{last}	16.2	96–114	18

WSV, within-subject variability; CI, confidence interval.
[a] All measures calculated from natural log transformed data (SAS).
[b] Within-subject variability estimated from ANOVA.
[c] 90% confidence interval.
[d] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.1094 (Wilcoxon).
[e] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.6475 (Wilcoxon).

Amoxapine, 7-Hydroxyamoxapine, and 8-Hydroxyamoxapine

Amoxapine is a dibenzoxapine-type tricyclic antidepressant with antipsychotic properties that blocks the reuptake of norepinephrine into presynaptic terminals and weakly blocks the reuptake of serotonin (44). 8-Hydroxyamoxapine is a major metabolite that blocks uptake of norepinephrine and is more potent than the parent drug in blocking the reuptake of serotonin. 7-Hydroxyamoxapine, on the other hand, is a minor metabolite that has roughly equipotent activities in blocking the reuptake of norepinephrine and serotonin but has similar activity to the structurally related antipsychotic drug loxapine in binding to dopamine receptors and dopamine sensitive adenylate cyclase (44–46). In a bioequivalence study on two formulations of amoxapine (47), the parent drug and the two metabolites were monitored in our laboratories. Arguments (3) based on the relative WSVs of the absorption rate constant (k_a) of the parent drug and of the formation rate constant (k_s) would suggest that 7-hydroxyamoxapine should be most discriminant analyte. The WSV of C_{max} of the 7-hydroxy metabolite (21.7%) was higher than that of the parent drug (18.8%) and the width of the 90% CI of the metabolite (20%) was therefore wider than that of the metabolite (18%) (Table VI). These data might be taken to suggest that these

Table VI. Results of a Bioequivalence Study on Two Formulations of Amoxapine in 27 Healthy Volunteers

Measure[a]	WSV%[b]	90%CI[c]	Width of CI
Amoxapine[d]			
C_{max}	18.7	86–104	18
AUC_{last}	17.8	83–99	16
C_{max}/AUC_{last}	19.1	95–115	20
7-OH-amoxapine[e]			
C_{max}	21.4	90–111	21
AUC_{last}	20.3	87–106	19
C_{max}/AUC_{last}	18.5	96–115	19
8-OH-amoxapine[f]			
C_{max}	13.8	93–107	14
AUC_{last}	7.6	92–99	7
C_{max}/AUC_{last}	13.3	98–112	14

WSV, within-subject variability; CI, confidence interval.
[a] All measures calculated from natural log transformed data (SAS).
[b] Within-subject variability estimated from ANOVA.
[c] 90% confidence interval.
[d] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.9521 (Wilcoxon).
[e] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.9338 (Wilcoxon).
[f] t_{max} (test) vs. t_{max} (ref) no significant difference p = 0.0698 (Wilcoxon).

metabolite data are more discriminatory than the parent drug or the 8-hydroxy metabolite and therefore should be used in the bioequivalence decision-making process. A clue that there may be a problem with this interpretation lies in the fact the ANOVA-CV of AUC_{last} for 7-hydroxyamoxapine is 20.3%, whereas that for the extrapolated area (AUC) is only 11.5%. (47) A re-examination of the raw data showed that subject no. 6 (evidently an outlier) had a very low AUC_{last} value. It was impossible to estimate an elimination rate constant for this subject because of undulations in the terminal slope so that the area could not be extrapolated. Removal of data for subject no. 6 reduced the ANOVA-CV of AUC_{last} to 13.9% and that for C_{max} to 15.0%. Obviously, one cannot discard inconvenient data from a bioequivalence study, but this experience does illustrate potential problems in basing the decision-making process on the analyte with the greatest WSV and the widest confidence interval. Moreover, it is evident that the low plasma concentrations of 7-hydroxyamoxapine in subject no. 6 after administration of the test and reference products could certainly not be attributed to "formulation failure."

A more rigorous consideration of the pharmacokinetic properties associated with the presystemic absorption process, however, suggests amoxapine is an intermediate hepatic extraction drug ($E_H = 0.65$) with an apparent oral clearance of 167 L/h and intrinsic clearance of 1670 L/h ($f_u = 0.1$). These data would suggest that a metabolite would be the most discriminating for bioequivalence assessment based on the simulations on C_{max} (6) and AUC (4,6), as intrinsic clearance is greater than liver blood flow. Moreover, the lack of any significant differences in t_{max} (or C_{max}/AUC) suggests the absorption rates of the parent drug were similar after administration of the test and reference formulations, which would favor the choice of a metabolite, but which one? AUC_{last} of 7-hydroxyamoxapine was about 9-fold lower than that of the parent drug, whereas AUC_{last} of 8-hydroxyamoxapine was 3-fold greater than that of the parent drug. One could argue therefore that bioequivalence should be assessed on the basis of the 8-hydroxy metabolite.

Loxapine, 7-Hydroxyloxapine, and 8-Hydroxyloxapine

Though amoxapine is marketed as an antidepressant, it is also an active metabolite of the antipsychotic drug loxapine. The latter is extensively biotransformed in animals and humans to a variety of metabolites (45,46), some of which are pharmacologically active. Inclusion of a bioequivalence study on loxapine and its 7- and 8-hydroxy metabolites (20) is relevant to the current discussion because the parent drug is highly variable in terms of C_{max} (Table VII), which shows that the ANOVA-CVs of both C_{max} and AUC of the parent drug were higher than corresponding values for either metabolite. The fraction of the parent drug absorbed into the systemic circulation (F) was estimated as 0.29 (22), which means the drug is highly extracted ($E_H = 0.71$). Apparent oral clearance was estimated as 220 L/h and intrinsic clearance as 7333 L/h, greatly in excess of liver blood flow. There were no significant differences between t_{max} or C_{max}/AUC values after administration of test and reference formulations, which suggests the absorption rates of the parent drug were similar after administration of the two formulations. As in the case of amoxapine, these pharmacokinetic data suggest that a metabolite would be the appropriate choice for assessment of bioequivalence. Again, the selection of metabolite is confounded by the presence of at least two therapeutic categories (antipsychotic and antidepressant).

Table VII. Results of a Bioequivalence Study on Two Formulations of Loxapine in 30 Healthy Volunteers

Measure[a]	WSV%[b]	90%CI[c]	Width of CI
Loxapine[d]			
C_{max}	29.7	88–115	27
AUC_{last}	23.8	92–115	23
C_{max}/AUC_{last}	15.9	90–105	15
7-OH-loxapine[e]			
C_{max}	17.9	85–101	16
AUC_{last}	15.2	91–105	14
C_{max}/AUC_{last}	15.8	88–102	14
8-OH-loxapine[f]			
C_{max}	16.4	87–102	15
AUC_{last}	9.7	93–102	9
C_{max}/AUC_{last}	13.1	91–103	12

WSV, within-subject variability; CI, confidence interval.
[a] All measures calculated from natural log transformed data (SAS).
[b] Within-subject variability estimated from ANOVA.
[c] 90% confidence interval.
[d] t_{max} (test) vs. t_{max} (ref) no significant difference $p = 0.1191$ (Wilcoxon).
[e] t_{max} (test) vs. t_{max} (ref) no significant difference $p = 0.8184$ (Wilcoxon).
[f] t_{max} (test) vs. t_{max} (ref) no significant difference $p = 0.6109$ (Wilcoxon).

The key question remains, however, which metabolite should be chosen? One could make a case for the selection of either 7- or 8-hydroxyamoxapine based on potential contributions to therapeutic activity, although the presence of two important therapeutic actions (antidepressant and antipsychotic) confounds the issue in the case of both loxapine and amoxapine. One could argue that the metabolite with the larger ANOVA-CV would be the more discriminating for bioequivalence decision-making because it gives the widest confidence intervals, but as the case of 7-hydroxyamoxapine in the bioequivalence study cited (47) illustrates, an ANOVA-CV can easily be inflated by spurious data from a single subject (1/27 subjects) in a manner that does not betray formulation failure. It could also be argued from a pharmacokinetic point of view that the most abundant metabolite (active or inactive) would be the most appropriate determinant for bioequivalence, although clinicians would undoubtedly balk at this suggestion. The most abundant active metabolite in this case is 8-hydroxyloxapine for which AUC_{last} is 3.6-fold greater than that of the parent drug, whereas AUC_{last} for the 7-hydroxy metabolite is a little more than half that of the parent drug. One could therefore conclude that bioequivalence should be assessed on the basis of the 8-hydroxymetabolite.

DISCUSSION

Simulations on the role of metabolites in bioequivalence have all been based on the case of a parent drug biotransformed into a single metabolite that is not subject to subsequent phase I or phase II metabolism. In the simplest of cases (4,6) where hepatic extraction is low and intrinsic clearance is

less than liver blood flow, plasma concentrations of the parent drug almost invariably exceed those of the metabolite, which suggests bioequivalence decisions should be based on the parent drug. When first-pass hepatic extraction reaches the medium to high range and intrinsic clearance exceeds liver blood flow, the situation becomes more complex. Plasma concentrations of a major, active metabolite may equal or exceed those of the parent drug, which makes pharmacologically active metabolites appear much more important for consideration. In the most extreme cases, where hepatic extraction is high (>0.7) and intrinsic clearance is very much higher than liver blood flow (⩾90 L/h), the parent drug is likely to be highly variable (ANOVA-CV ≥ 30%). Here, a major, active metabolite (therapeutic or toxic) can look tempting as a potential candidate for bioequivalence assessment, especially as it is likely to be less variable than the parent drug. The results of simulations give support to the latter argument in that the metabolite was the better predictor of bioequivalence than the parent drug when intrinsic clearance was greater than liver blood flow, both in terms of C_{max} (6) and AUC (4,6).

Difficulties arise because, in practice, it is unusual to find situations that conform to the simple conditions used in simulated studies based on a single metabolite, eliminated solely by renal excretion in a system where there is no extrahepatic biotransformation and no sequential phase I and/or phase II metabolism. In a practical situation where none of these constraints apply, it becomes much harder to justify reliance on metabolite data as the basis for bioequivalence decision-making. In a few of the more complex practical situations, a case can be made for consideration of metabolite data when, for example, concentrations of the (active) parent drug are so low they cannot be measured sufficiently well to be useful. As described above, intrinsic clearance of (*Z*)-doxepin was greatly in excess of liver blood flow, which suggests that the active metabolite, (*Z*)-*N*-desmethyldoxepin, is arguably the most appropriate analyte for assessing the bioequivalence of two formulations of doxepin because the active (*Z*)-isomer of the parent drug was present in plasma in concentrations so low it could be measured in only 3/27 subjects. From a pharmacodynamic viewpoint, this is a more appealing solution to the problem than relying on the low activity (*E*)-doxepin, which accounts for the overwhelming majority of "total" doxepin. Similarly, both clinical and pharmacokinetic arguments can be advanced to justify base bioequivalence assessment of formulations of allopurinol on the metabolite, oxypurinol (27).

Nortriptyline is an intermediate extraction drug (E_H = 0.35). Intrinsic clearance was in excess of liver blood flow (CL_{int} = 600 L/h) suggesting that the therapeutically active metabolite, (*E*)-10-hydroxynortriptyline, would be the most appropriate analyte for the assessment of the bioequivalence of two formulations of the drug. Closer examination of the data, however, raises concern about reliance on the metabolite data for assessment of bioequivalence in this instance, especially in view of the fact that plasma levels of the active metabolite were not greatly in excess of those of the parent drug.

Amoxapine and loxapine represent intermediate and high hepatic extraction drugs respectively, both of which have very high intrinsic clearances (much greater than liver blood flow). Both drugs have at least two pharmacodynamically active phase I metabolites each of which is subject to sequential phase II metabolism. The two phase I metabolites possess different pharmacodynamic activities, which therefore make distinctly different contributions to the overall therapeutic activities of amoxapine or loxapine and complicates the question of appropriateness of analyte for bioequivalence assessment even further. At first sight, the simulation studies (4,6) appear to suggest metabolite data would be appropriate for assessment of bioequivalence of either drug given the high intrinsic clearances, although the presence of multiple metabolites, each subject to sequential phase II metabolism was outside the scope of the simple model used in the simulations. Moreover, when considering the phase I metabolite, how would one distinguish between them when their contributions to overall therapeutic activity are different, their plasma concentrations are different, and their binding affinities to their appropriate receptors are different? The simplest solution would be to assess bioequivalence based on an active metabolite that is present in plasma at concentrations markedly higher than those of the parent drug. Such is the case with the 8-hydroxy metabolites of both amoxapine and loxapine. Detractors of this view might point out that both amoxapine and loxapine are more complex in their pharmacokinetics than the simple model used in the simulations in view of the multiple metabolites, sequential phase II metabolism during the first pass, and the fact that renal clearance was not the only method of elimination.

CONCLUSIONS

It is impossible to implement simple rules about the potential use of metabolite data in bioequivalence studies that can apply to any but the simplest of situations. Each drug and metabolite combination must be given prospective, individual examination of the fundamental pharmacokinetic properties and applicable within subject variabilities. A metabolite would be the analyte of choice where the parent drug is inactive (i.e., a prodrug) or where the parent drug is present in plasma in concentrations so low as to be unquantifiable. Otherwise, given the two distinct purposes of bioequivalence testing (i) as a surrogate for therapeutic bioequivalence and (ii) as an indicator of *in vivo* pharmaceutical quality of the two formulations under investigation, it is safest to rely on the parent drug in situations where there are multiple metabolites (active, toxic, or inactive) and where sequential phase I and/or phase II biotransformations are involved.

Any decision to use metabolite data in a given bioequivalence study must be made *a priori* by a drug regulatory agency and should be communicated to the sponsor in the design stage of the study. This expedient approach permits a sponsor to plan the serial blood sampling time scheme to include sufficient samples adequately to define t_{max} and C_{max} of the metabolite. It will also avoid submission of data on multiple analytes for *a posteriori* evaluation, which may lead to unacceptable changes in the consumer and producer risks. It is important that physicians and pharmacists feel able to assure patients that all drug products for oral administration, brand or generic, are high-quality formulations.

ACKNOWLEDGMENTS

The authors thank Dr. Jane Alcorn and Mr. Mark Fidelak for helpful criticisms and comments on the first draft of this manuscript.

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