
Isotope Effects: Definitions and Consequences for Pharmacologic Studies

Agnes Van Langenhove, PhD

The use of stable isotope-labeled compounds for pharmacologic studies requires careful consideration of the nature of the stable isotope label (^2H , ^{13}C , ^{15}N , ^{18}O) and its position of incorporation in the molecule. When deuterium is used, improper positioning can lead to significant primary isotope effects. Primary isotope effects occur when the breaking of the bond to the heavy isotope is the rate-limiting step in a reaction (or metabolic transformation). A reaction will proceed slower for the molecule with the heavy isotope label because of the mass difference between the light and the heavy isotope. In addition to these primary isotope effects, smaller but nevertheless important secondary isotope effects, physicochemical isotope effects, active hydrogen/deuterium exchange, or isotope effects associated with either the enzyme-catalyzed biotransformation or the mass spectrometric ionization and fragmentation can be operative. In mechanistic studies, isotope effects are used to their advantage; however, in pharmacokinetic studies, the occurrence of isotope effects can lead to grossly misleading biologic and analytic results: the metabolism of the drug will differ when 'in vivo' isotope effects are operative, and isotope effects occurring during the analysis procedure will obscure the true metabolic profile of the drug.

Stable isotope-labeled drugs can be designed to be convenient tracers for *in vivo* pharmacokinetic studies and for *in vivo* and *in vitro* drug and enzyme mechanistic studies.^{1,2} The labeled form of the drug can easily be distinguished from the unlabeled form by mass spectrometry, because the incorporation of one or more stable isotopes has increased its molecular weight.

Use of stable isotope-labeled compounds in pharmacokinetic studies implies that the metabolism of the stable isotope-labeled compound is not significantly different from that of the unlabeled analogue, i.e., that there is no *in vivo* isotope effect, and also, that the stable isotope-label is not incorporated at a position where it can be lost during the *in vivo* metabolism or during the subsequent analysis. If these conditions are not met, the use of stable isotope-labeled compounds can lead to grossly misleading biologic and analytic results. For example, substitution of deuterium for the *N*-methylhydrogens of morphine (Figure 1) resulted in a significant reduction in the potency and lethality of morphine in mice regardless of the route of administration.^{3,4} *N*-demethylation of deuterated morphine by rat liver

microsomal enzymes was characterized by a smaller rate constant, a higher energy of activation, and a larger Michaelis constant (*K_m*), and thus weaker enzyme binding as compared with unlabeled morphine. Replacement of the *N*-methylhydrogens of methsuximide (Figure 1) by deuterium did not only result in decreased rate of formation of the *N*-desmethylmetabolite, but in addition, the metabolism was switched to a different pathway, namely to *para*-hydroxylation of the phenyl ring of methsuximide.⁵

On the other hand, when stable isotope-labeled compounds are used for mechanistic studies, isotope effects can be used to their advantage: they provide detailed information about the nature of the enzymatic or drug action mechanism. Although this information can then be used to modify a drug's action or its toxicity,⁶⁻⁸ it is also extremely useful when selecting stable isotope-labeled compounds for pharmacokinetic studies.^{9,10}

KINETIC ISOTOPE EFFECTS

Background

Any bond involving a heavy isotope and another atom will be stronger than the same bond between the corresponding light isotope and that atom because of the mass difference between the heavy and the light isotope.¹¹ In any reaction in which the breaking of this bond is the rate-limiting step, the

From the Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts. Address for reprints: Agnes Van Langenhove, PhD, Department of Nutrition, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115.

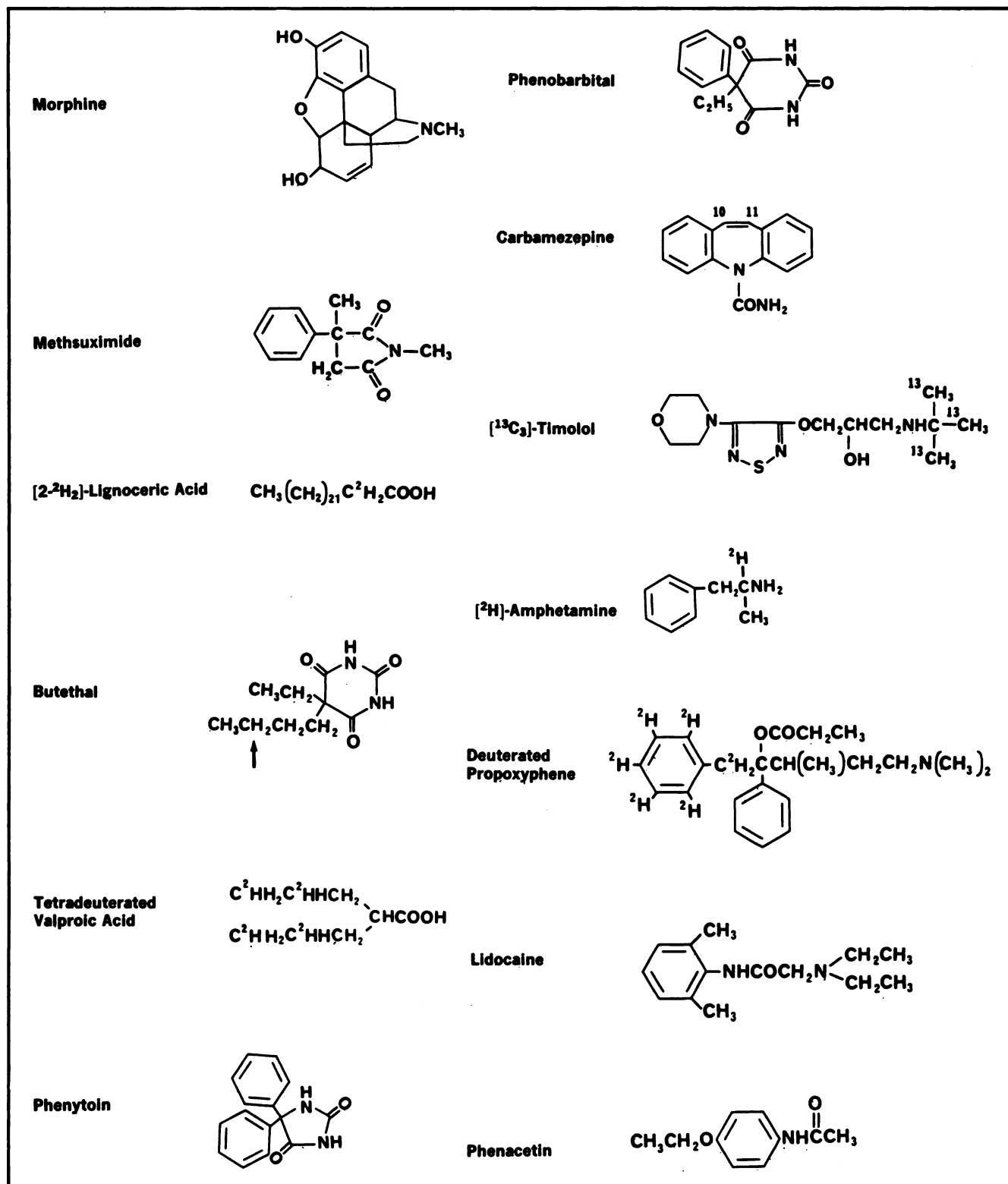


Figure 1. List of structural formulas. The arrow in the butethal structure indicates the position of 3'-deuterium atoms.

TABLE

Estimated Maximum Possible Rate Constant Ratios at 25°C for Various Stable and Radioactive Isotopes

Natural Abundance Isotope	Heavy Isotope	Rate Ratio k_1/k_2^*
^1H	^2H	18.00
^1H	^3H	60.00
^{12}C	^{13}C	1.25
^{12}C	^{14}C	1.50
^{14}N	^{15}N	1.14
^{16}O	^{18}O	1.19
^{32}S	^{34}S	1.05

*Rate constant for the light isotope is the numerator.

reaction will therefore proceed slower for the molecule with the heavy isotope. This primary kinetic isotope effect is usually expressed in terms of the ratio of the specific rate constants ($K_{\text{light}}/K_{\text{heavy}}$), and thus requires measurement of the concentration of reactants and products for the different isotopic variants. These measurements are usually done by conventional mass spectrometry with a precision well within 1% (within 5% if isolation of the analytes from body fluids and gas chromatography-mass spectrometry analysis is required). For in vivo biotransformations in particular, measurement of the rate of product disappearance by itself does not reflect the true kinetic isotope effect because different metabolic pathways, giving rise to different end products, can be followed. The kinetic isotope effect is largest for deuterium: Hydrogen and deuterium differ in mass by a factor of two. Substitution of ^{13}C for ^{12}C , ^{15}N for ^{14}N , or ^{18}O for ^{16}O , does not produce such large isotope effects, because the mass difference between the light and heavy isotope is much smaller.

Bigeisen¹² estimated the maximum possible ratios in specific rate constants for a number of stable and radioactive isotopes (Table). The magnitude of the isotope effect correlated with the use of ^{13}C , ^{15}N , and ^{18}O is within the error limits of normal rate measurements, and the effect is therefore usually insignificant. These isotope effects can be determined of course by much more elaborate methods, such as isolation and purification of the drug or metabolites, combustion to CO_2 and N_2 , and use of an isotope ratio mass spectrometer. It is worth mentioning that the coefficients of variation for multiple determinations of the same pharmacokinetic parameters have been reported to be 9% to 60% within subjects and 4% to 55% between subjects.¹³

Primary kinetic isotope effects are operative when cleavage of the bond to the heavy isotope is the rate-limiting step in the reaction; secondary kinetic isotope effects can occur if

cleavage of the bond to the heavy isotope (again, deuterium in particular) is not the rate-limiting step in the reaction. Incorporation of deuterium at sites one or more atoms removed from the bond being broken can also result in secondary kinetic isotope effects. Secondary isotope effects are much smaller than primary isotope effects: for deuterium, they usually have limiting values of 1.02–1.40 (J. F. Kirch, in Cleland and colleagues¹⁴). Rate measurements will again barely detect secondary isotope effects.

Theoretic Considerations for Deuterium Isotope Effects

The effect of the difference in mass on the stretching frequency (ν)—and thus on the zero-point energy (ZPE), ($\frac{1}{2}h\nu$)—of a bond to deuterium and a bond to hydrogen, is the major factor responsible for the generally lower reactivity of a bond to deuterium as compared with the corresponding bond to hydrogen (h = Planck's constant).^{15,16} The ZPE of a bond undergoing a chemical reaction is the lowest energy level of the bond and corresponds to the vibrational energy of the bond at absolute zero. The stretching frequency is defined by Hooke's Law (equation 1).

$$\nu = \frac{1}{2\pi c} \sqrt{\frac{\kappa}{\mu}} \quad [1]$$

in which μ is the reduced mass and κ is the force constant for the stretching vibration. The reduced mass is approximately 1 and 2 for most hydrogen- and deuterium-containing bonds, respectively. The force constant is governed by the shape of the bottom of the potential energy curve (Figure 2). As can be seen on Figure 2, the potential energy curves for a bond to hydrogen and the corresponding bond to deuterium are almost identical. Hooke's Law therefore shows that the mass factor is the important parameter that defines the magnitude of the stretching frequencies.

The difference in ZPE of a bond to deuterium and a bond to hydrogen has consequences on the dissociation energy of a compound and on rate processes. The effect on the dissociation energy is illustrated in Figure 2. Since the deuterated compound will have the lower ZPE, it will be more stable than the hydrogen analogue. Similarly, in a rate process, if one considers the curve relating the the potential energy of the system with the distance along the reaction coordinate (Figure 3), the effect of the ZPE on the rate again becomes apparent. The difference in ZPE in the reactants will result in a difference in height of the potential energy barrier for the reaction and will require a higher energy of activation for the deuterium-containing compound.

Whereas a significant primary kinetic isotope effect is generally attributed to a rate-determining transition state in which the bond to the isotope is "being more or less" broken, such a simple qualitative rationalization for secondary kinetic isotope effects is lacking. The manifestation of the latter in model reactions implies only that the force constant of the bond to the isotope has changed in proceed-

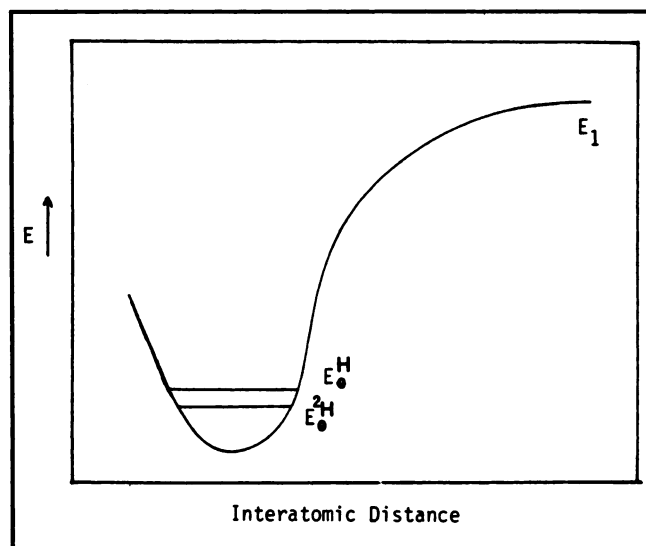


Figure 2. Morse curves relating potential energy and interatomic distance. E_D^0 = zero-point energy of a carbon-deuterium bond; E_H^0 = zero-point energy of a carbon-hydrogen bond.

ing from the ground state to the transition state of the reaction.

PHYSICOCHEMICAL DEUTERIUM ISOTOPE EFFECTS

Physicochemical differences between labeled and unlabeled forms of a compound such as differences in pKa, lipid solubility, and plasma protein binding can be responsible for in vivo isotope effects. An example is the previously increased basicity of *N*-methyl deuterated morphine, which resulted in decreased enzyme binding. Different physicochemical properties are equally important during sample processing. The increased basicity of deuterated morphine would again be an example. More widely documented isotope effects of physicochemical nature that can occur during the analysis are the differences in chromatographic mobility and differences in adsorption for deuterium labeled and unlabeled forms of a compound.^{1,17}

ACTIVE HYDROGEN-DEUTERIUM EXCHANGE

Active hydrogen-deuterium exchange can occur during sample processing.^{18,19} For example, an aromatic hydroxyl group renders hydrogens in the *ortho* position to the hydroxyl group subject to acid-catalyzed exchange (scheme 1, Figure 4). Deuterium in these positions, while retained at physiologic pH, may be lost when such a compound is treated with strong acid during isolation and workup of the sample, e.g., during hydrolysis of glucuronides. Another example is the scrambling of hydrogens (scheme 2, for toluene; Figure 5) that can occur during pyrolysis of com-

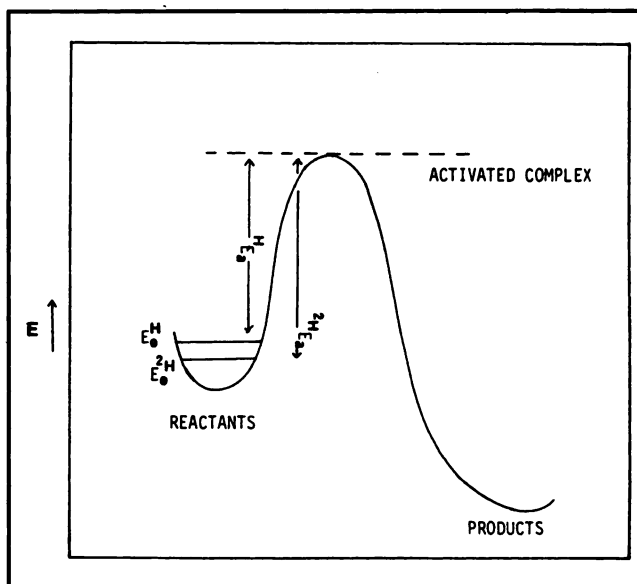


Figure 3. Potential energy profile. $E_D^‡$ = energy of activation for deuterium-containing compound; $E_H^‡$ = energy of activation for hydrogen-containing compound.

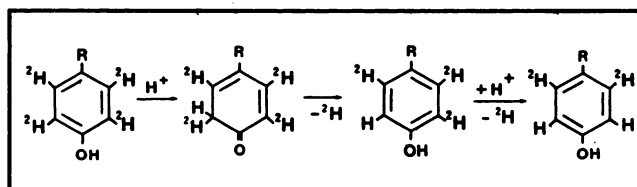


Figure 4. Scheme 1: Acid-catalyzed exchange of deuterium atoms ortho to the hydroxyl group.

pounds or during mass spectrometric analysis.^{20–22} Scrambling during mass spectrometric analysis implies that the hydrogen-deuterium exchange process occurs more rapidly than mass spectrometric fission.

ISOTOPE EFFECTS ASSOCIATED WITH MASS SPECTROMETRIC IONIZATION AND FRAGMENTATION

It is worth mentioning that there can be a small, usually nonsignificant, isotope effect associated with ionization and with the subsequent fragmentation or fission of labeled molecules during mass spectrometric analysis.^{17,23} McCloskey and co-workers²³ showed that the most striking effect of deuterium substitution is the increased stability of the molecular ion. The smaller the proportion of deuterium, the smaller these isotope effects will be, and with only one or two atoms in a molecule containing many other hydrogens, the effect may be extremely small. For multiply deuterated compounds, it is important that the mass spectra of labeled

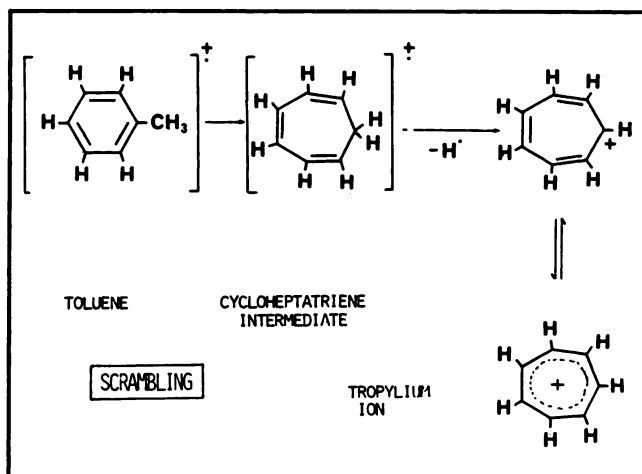


Figure 5. Scheme 2: Scrambling of hydrogens during mass spectrometric analysis of toluene.

and unlabeled analogues are critically compared and that appropriate steps are taken in assay procedures based on isotope dilution techniques.

ENZYME-CATALYZED BIOTRANSFORMATIONS

Whereas for nonenzymatic reactions, measured rates of reaction provide a full expression of the isotope effect, this is often not the case for enzymatic processes. Enzymatic reactions usually have more steps than their nonenzymatic counterparts and rarely provide full expression of the intrinsic isotope effect, i.e., the effect imposed by the heavy isotope on the isotopically sensitive step.^{14,25,26} Kinetic iso-

tope effects can be modified by physicochemical isotope effects such as substrate binding and conformational changes: A very broad range of values that are highly dependent on the particular experimental design employed to measure the isotope effect can therefore be displayed. Heavy isotope effects on enzymatic reaction rates are generally observed as effects on $U_{\max}/K_m$ and provide information about steps up to and including the first irreversible step in the reaction. A measured deuterium isotope effect on $U_{\max}/K_m$ is, in most cases, different from the estimated intrinsic isotope effect for the same reaction and different from the observed isotope effect as determined by, for example, measuring the rate of product disappearance.^{27,28} A critical attitude is therefore necessary in evaluating literature data in this field, with the purpose of designing a stable isotope-labeled compound to be used in pharmacokinetic studies.

USE OF MECHANISTIC DATA IN PHARMACOLOGIC STUDIES

The importance of mechanistic data for the design of a stable isotope-labeled compound to be used for specific pharmacologic purposes can best be illustrated by a discussion of mixed function oxidase reactions. The metabolism of a wide variety of drugs is catalyzed by these enzymes.

In oxidations at a saturated carbon (scheme 3, Figure 6) the carbon is first activated by abstraction of a proton, hydride, or hydrogen to form a readily oxidized carbonium ion, carbanion, or radical, or in a second mechanism, oxygen is inserted directly into the substrate.²⁹ In both of these reactions, the cleavage of the C-H (C-D) bond is the rate-limiting step in the reaction. The large isotope effect ($k_H/k_D = 5$) found for the α -hydroxylation of [2-²H₂]-lignoceric acid (Figure 1) proves the consistency of the mechanism, and fatty acids labeled with deuterium at the α -carbon should not be used in pharmacokinetic studies.³⁰ Similarly, Tanabe reports an in vivo isotope effect in the elimination half-lives for 5-ethyl-5-(3'-dideuteriobutyl) barbituric acid (butethal) (Figure 1): The half-life of 3'-dideuteriobutethal was approximately 2.5 times longer than that of the unlabeled analogue. The isotope effect measured for the in vitro hydroxylation at the 3' position of 3'-dideuteriobutethal (k_H/k_D) was 1.59. The pharmacologic properties also changed with deuteration: A more than twofold increase in sleeping time ($k_H/k_D = 2.28$) was observed for 3'-dideuteriobutethal; t_H/t_D for 4'-dideuteriobutethal was 1.47.³¹ No isotope effect was measured in the metabolism of tetradeuterated valproic acid (Figure 1).³² In this case, deuterium was not substituted at the major site of metabolism (the major metabolic pathway of valproic acid is β -oxidation and glucuronidation).

In oxidations at an aromatic carbon, an alternative pathway for the formation of hydroxylated metabolites can be followed, namely addition of oxygen through an arene

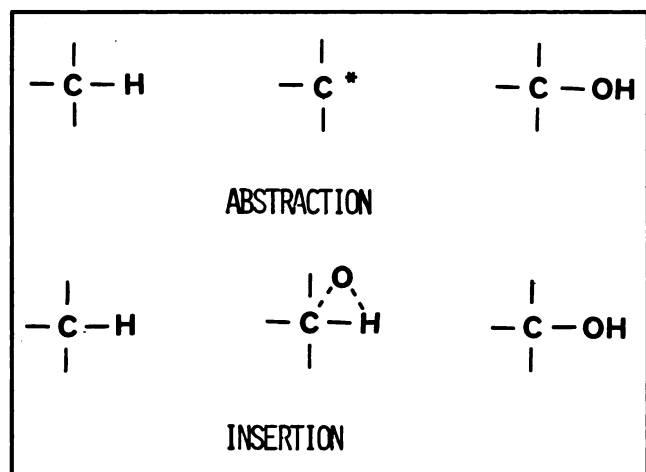


Figure 6. Scheme 3: Oxidation at a saturated carbon.

oxide mechanism.^{33,34} Here, cleavage of the C-H (C-D) bond is not the rate-limiting step. The arene oxide pathway is usually accompanied by the rearrangement of a hydrogen (NIH shift, scheme 4; Figure 7). This hydrogen rearrangement is responsible for small secondary kinetic isotope effects. Using the information that oxidation through the arene oxide mechanism does not result in a significant isotope effect on reaction rates, Tomaszewski and colleagues²⁹ showed that *ortho* and *para* hydroxylations of phenyl rings proceed via the arene oxide pathway: He found large isotope effects only for *meta* hydroxylations. The absence of isotope effects for *para* hydroxylations has been observed by other groups: phenytoin (Figure 1) labeled with deuterium in one ($[^2\text{H}_5]$ -phenytoin) or both ($[^2\text{H}_{10}]$ -phenytoin) phenyl rings,³⁵ phenobarbital (Figure 1) labeled with deuterium in the *para* position of the phenyl ring,³⁶ and carbamazepine (Figure 1) labeled with two deuterium atoms in each of the phenyl rings³⁷ did not show isotope effects.

As expected, experimental evidence shows that ^{15}N or ^{13}C labeling of compounds that undergo hydroxylations does not result in measurable kinetic isotope effects. In a well-documented study on the *in vivo* equivalence of unlabeled and $[^{13}\text{C}, ^{15}\text{N}_2]$ -hydantoin-labeled phenytoin, Browne and associates³⁸ did not find any significant isotope effects associated with the metabolism of the stable isotope-labeled drug. The pharmacokinetic parameters for labeled and unlabeled phenytoin were not significantly different and the plasma concentrations of labeled and unlabeled phenytoin and its *para* hydroxylated metabolite and the urinary excretion of labeled and unlabeled *para* hydroxylated metabolite remained the same over the collection period. A subsequent study also did not show any isotope effect in the metabolism of $[^{13}\text{C}, ^{15}\text{N}_2]$ -phenobarbital.² In a study with $[^{13}\text{C}_3]$ -timolol (Figure 1), evidence was not found for metabolic kinetic isotope effects although timolol is

metabolized by oxidation of the *t*-butyl group (i.e., the position where ^{13}C was incorporated).³⁹

N-dealkylation of primary amines proceeds through an unstable carbinolamine intermediate: a carbon-nitrogen bond is broken and a carbon-oxygen bond is formed. A second, alternative mechanism is the formation of an *N*-hydroxy derivative. The details of these mechanisms have been thoroughly described by Parll and co-workers.^{40,41} The studies justify the isotope effects (measured for the formation of oxime and ketone intermediates) in the oxidative deamination of $[^2\text{H}]$ -amphetamine (Figure 1), as well as the large negative isotope effect (indicative of switching) in the formation of *N*-hydroxyamphetamine from the deuterated form of the drug.⁴¹ Deuterated propoxyphene with deuterium located far enough away from the position of metabolic activity (Figure 1) did not show an isotope effect.^{42,43}

Therefore, the isotope effects in the metabolism of lidocaine (Figure 1) labeled specifically with deuterium in the α -methylene groups of the *N*-alkyl constituent, $[^2\text{H}_4]$ -lidocaine ($k_{\text{H}}/k_{\text{D}} = 1.49$) and in the terminal methyl groups, $[^2\text{H}_6]$ -lidocaine ($k_{\text{H}}/k_{\text{D}} = 1.52$) are not unexpected.⁴⁴ *O*-dealkylation also proceeds through hemiacetal intermediates.¹⁷ The *O*-deethylation of two differently deuterium-labeled phenacetin analogues (Figure 1) to *para* hydroxyacetanilide was also associated with isotope effects: A primary isotope effect ($k_{\text{H}}/k_{\text{D}} = 1.61$) was observed for $[1,1\text{-}^2\text{H}_2]$ -phenacetin, a secondary isotope effect ($k_{\text{H}}/k_{\text{D}} = 1.03$) for $[2,2,2\text{-}^2\text{H}_3]$ -phenacetin.⁴⁵

CONCLUSIONS

The consequences of isotope effects for pharmacologic studies are twofold: They are tolerated for mechanistic studies in which they can be used to their advantage, but for pharmacokinetic studies, they have to be absent (or nonsignificant, i.e., within the error limits of the measurement of the stable isotope label). Use of ^{13}C or ^{15}N or another heavy isotope differing from the light one by less than 10% in mass does not result in significant isotope effects. In regard to deuterium, the following conditions have to be met if a significant isotope effect is to be excluded: (1) Deuterium should not be incorporated at the position where cleavage of the bond to deuterium is the rate-limiting step in the biotransformation of the compound; substitution of deuterium in this position will result in primary kinetic isotope effects, i.e., the metabolism of the drug will be changed significantly; (2) deuterium should not be incorporated at positions where active hydrogen-deuterium exchange can occur; this could result in loss of the stable isotope label and invalidate the analysis procedure; (3) if multiply deuterated compounds are used, they could have different physicochemical properties. In this case, special attention to the analytic procedure and enzyme binding kinetics may be required.

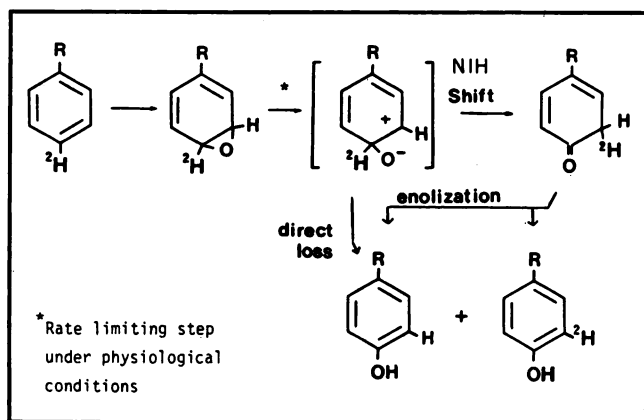


Figure 7. Scheme 4: Microsomal oxidation of aromatic substrates: The arene oxide pathway.

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