

The Application of Stable Isotopes in Biomedical Research

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Many of the ways in which stable isotopes are used in biomedical research are reviewed. The use of stable isotopes in stable isotope dilution assays, for metabolite identification and in pharmacokinetic studies, is discussed and relevant examples are given to illustrate the various points made. Isotope effects and their implications for future drug design are considered. Some of the toxicity problems associated with the use of stable isotopes are also considered. Finally, a brief subjective view of possible future advances is made.

INTRODUCTION

The increasing use of stable isotopes in the fields of biochemistry and medicine has resulted in a number of symposia recently dealing with this topic. Of these symposia the most important are those held at Argonne in 1973,¹ 1975² and 1978,³ and the British Pharmacological Society symposium in 1977.⁴ Additionally a number of meetings dealing with mass spectrometry have also discussed stable isotopes in depth, including the five meetings organized by Frigerio in Italy⁵⁻⁹ and the recent meetings on quantitative mass spectrometry in Ghent.^{10,11}

A number of review articles dealing with the application of mass spectrometry to biomedical research have appeared in the last few years, including reviews by Lawson¹² and Fenselau.¹³ The more specialized aspects of selected ion monitoring have been reviewed by Holmstedt and Palmer,¹⁴ and a list of references up to 1975 has been compiled by Palmer and Holmstedt,¹⁵ following a list of references up to 1973 published by Costa and Holmstedt.¹⁶ Undoubtedly selected ion monitoring represents the major use of stable isotopes to date, but other aspects of the use of stable isotopes have also been reviewed, especially with regard to their possible toxicity (Blake *et al.*,¹⁷ Greeg¹⁸). Clinical applications have also been discussed at the various symposia on stable isotopes.

This review will cover the application of stable isotopes in the following areas: (1) stable isotope dilution assays; (2) metabolite identification (stable isotope signature); (3) absolute bioavailability by co-administration; (4) determining pharmacokinetics during chronic administration studies; (5) bioequivalence measurements; (6) metabolic 'switching' and stable isotope effects; (7) toxic effects of stable isotopes. The review will concentrate on recent work, although some historical perspective will be given. Representative examples from the published literature will be given, rather than attempting a full review of all papers relating to stable isotopes.

Abbreviation: NAPA = acetylprocaineamide.

Terminology

Due to the rapidity with which advances have been made, a number of synonyms have arisen for various aspects of stable isotope terminology. A brief definition for terms the author will use in this present review, with synonyms to be found in literature, is presented below.

STABLE ISOTOPE. Generally an isotope of an element which is non-radioactive (i.e. does not decay by emission of α , β , or γ radiation). However, in this context the most common isotopes (that is ^1H (protium), ^{12}C , ^{16}O , ^{14}N , ^{32}S) of hydrogen, carbon, nitrogen, oxygen and sulphur are excluded, although they are stable isotopes. The most commonly used are as follows:

Isotope	Natural abundance (%)
^2H (deuterium)	0.0148
^{13}C	1.11
^{15}N	0.365
^{17}O	0.037
^{18}O	0.204
^{33}S	0.760
^{34}S	4.22

These are also referred to as **HEAVY ISOTOPES**.

ENRICHMENT FACTOR. The degree of enrichment of the stable isotope over its natural abundance.

SELECTED ION MONITORING. A method whereby the mass spectrometer is made to monitor a small number of selected ions. On magnetic instruments this is done by switching the accelerating voltage between preset values, or by changing the magnet flux. On quadrupole instruments this is done by rapid switching of the rod voltage in the mass filter. Synonymous terms are **MASS FRAGMENTOGRAPHY**, **MULTIPLE ION DETECTION**, **SELECTED ION RECORDING** and **MASS SELECTED ION DETECTION**. **MASS CHROMATOGRAPHY** refers to a separate technique whereby the intensity of selected ions are monitored from a file of full scan spectra using a computer.

EI (ELECTRON IMPACT IONIZATION). The normal method of forming ions in the mass spectrometer

by bombarding the vaporized sample with an electron beam.

CI (CHEMICAL IONIZATION). A method of forming ions by gas phase chemistry from a suitable ionized reagent gas.

FI (FIELD IONIZATION). A method of forming ions by electron extraction in a high positive electric field.

FD (FIELD DESORPTION). A method for forming ions by electron extraction from the sample in a solid form coated on an activated emitter.

GC/MS (GAS CHROMATOGRAPHY/MASS SPECTROMETRY). The use of an instrument combining a gas chromatograph with a mass spectrometer via an interface. The interface can comprise a diverter valve and an enrichment device in an inlet line enabling only the effluent of interest to be allowed into the ionization source of the mass spectrometer, and removal of most of the carrier gas. With capillary GC/MS the total effluent is usually allowed into the source.

ISOTOPE EFFECT. Changes in rates of reaction caused by substitution of the common isotope by a stable or radioactive isotope. Large kinetic effects are found especially when substitution of deuterium for protium is carried out. The rate of cleavage of C—(²H) bonds can be up to 14 times slower than similar cleavage of C—(¹H) bonds.

STABLE ISOTOPE DILUTION ASSAYS

The requirement for a labelled internal standard is that it should be isotopically as pure as possible, and for this reason deuterium (99.9 atom %) is the best heavy isotope for this work. It also happens to be cheap and useful when using relatively large quantities of internal standards. Selected ion monitoring using a mass spectrometer has a number of advantages.

(1) Because the internal standard is chemically equivalent (as a first approximation) a stable isotope analogue of a compound is going to have similar extraction, chromatographic and ionization characteristics to the parent. Thus, a labelled standard can be added as the first step in an extraction sequence, minimizing errors due to procedural losses.

(2) The stable isotope can also be added in large excess, to act as a 'carrier'. If procedural losses remove 50 ng of compound, the analysis of 100 ng is going to be difficult due to loss of 50% material. However, procedural losses tend to be a constant weight, so if one adds 900 ng of label as carrier, the loss of 50 ng from 1000 now represents a loss of only 5% of material. Thus sensitivity and precision may be much improved.

The earliest reference to stable isotope dilution is work by Rittenberg and Foster¹⁹ on the analysis of amino acids and fatty acids. They were able to quantify palmitic acid in rat fat using palmitic acid containing 21.5 atom % deuterium, and glycine, glutamic acid and aspartic acid using the standard with 2 atom % ¹⁵N. However, most work had to await the development of the selected ion monitoring technique (Rittenberg and Foster compared peak height ratios on scanned spectra). The first multiple ion detector was described by Hammar²⁰ in 1971 following initial work by Sweeley in

1966.²¹ Kelly was able to quantify prostaglandin F_{2α} using the trideuteromethyl ester²² and described his work at the Elba meeting in 1971. At the conference two years later⁶ Draffan presented work on the determination of amobarbital and indoramin.²³ Other early reports included work on prostaglandin E²⁴ in 1970, and quantification of nortriptyline.²⁵ All of these early methods used EI mass spectrometry. Castagnoli was able to show enantiomeric metabolism of 1-(2,5-dimethoxy-4-methyl-phenyl)-2 aminopropane using selected ion monitoring and direct insertion CI mass spectrometry.²⁶ Since that time assays using gas chromatography/mass spectrometry and CI mass spectrometry have appeared, including an assay²⁷ for diphenoxylate (5 mg) using tetradeuterodiphenoxylate acid as internal standard. This assay showed good linearity over the range 10–400 ng ml⁻¹ in plasma and enabled the determination of diphenoxylate acid in plasma after a single oral dose of diphenoxylate (5 mg). The use of stable isotope dilution analysis tends to ignore the natural abundance of the stable isotope, and several authors have examined this mathematically to determine whether this assumption is valid or not. Pickup and McPherson²⁸ derived the equation

$$R = \frac{(X/Y)(p_1/M) + (q_1/M)}{(p_2/M)(q_2/M')}$$

where R is the measured ratio of natural labelled, X is the mass of naturally occurring isotope, Y is the mass of labelled form, M is the molecular weight of natural material, M' is the molecular weight of labelled material, and p, q are the probabilities of occurrence of natural and labelled material in natural or labelled sample.

A second approach has been proposed by Campbell.²⁹ He uses the enrichment factor (EF), defined as

$$EF = \frac{\text{intensity of } [M]^{++}}{\text{sum of intensities of } M}$$

where $[M]^{++}$ is the molecular ion for the labelled species, and M are the molecular ions for all natural and labelled species. The EF is corrected for the natural abundance of other isotopes, and here, using a low resolution instrument such as a quadrupole, the addition of ¹³C is equivalent to the addition of ²H (both increase mol. wt by 1). For high resolution instruments more accurate correction can be made. The EF is then

$$\begin{aligned} & \frac{\text{number of heavy isotopes in sample,} \\ & \quad \text{excluding naturally occurring}}{\text{total number of } ^{12}\text{C}/^1\text{H, excluding those} \\ & \quad \text{heavy isotopes naturally occurring}} \\ &= \frac{m - (np/o)}{n + m - (np/o)} = \frac{mo - np}{no + mo - np} \end{aligned}$$

where m is the number of added heavy isotope, n is the number of light isotope, p is the natural abundance of heavy isotope and o is the natural abundance of light isotope.

More recently still, Bush and Trager have taken up the work of Pickup and McPherson, modified by Colby and McCaman³⁰ and have produced a more generalized model suitable for multivariate analysis.³¹ All of these

models suggest the optimum stable isotopically labelled standard should contain three or more heavy atoms and have a high degree of purity to obtain good precision.

An introductory text to quantitative mass spectrometry which reviews some of the more practical aspects of stable isotope dilution has been written by Millard.³² Stable isotope dilution assays have been developed for a wide range of compounds, both endogenous and exogenous. Recent reports include assays for steroids,³³ ethynylloestradiol,³⁴ bile acids,³⁵ acetylcholine,³⁶ calcium,³⁷ prostaglandin,³⁸ tetrahydrocarboline,³⁹ trioxsalen,⁴⁰ diphenylhydantoin,⁴¹ and disopyramide.⁴² Nearly all rely on the classical approach, namely addition of stable isotope standard to the biological fluid, extraction, derivatization and GC/MS analysis with selected ion monitoring. This can impart a further advantage inasmuch that the stable isotope can act as a carrier⁴³ improving sensitivity. However, the 'carrier effect' has been questioned.^{32,44,45} No carrier effect was found on the analysis of octopamine using a deuterated internal standard.⁴⁴

In some cases a carrier effect has been shown to occur.⁴³ It is likely that the existence of a carrier effect depends very much on the site of labelling and the nature of the compound.

Summary of quantification using stable isotopes

(1) Stable isotopes enable one to use a chemically equivalent internal standard which can be added to the sample early in the assay, eliminating procedural errors.

(2) Stable isotopes can be also added in excess to act as carriers for trace amounts of drugs or metabolites, thus reducing variation in procedural losses.

(3) Deuterium labels are preferred because of their high isotopic purity and cheapness.

(4) The internal standard should contain three or more heavy atoms in order to reduce any corrections due to naturally occurring heavy isotopes to negligible proportions. Correction can be made for this, but it introduces extra steps into the computation.

(5) Heavy isotopes should also be clean and contain predominantly one labelled species (i.e. 100% $^2\text{H}_x$, not 20% $^2\text{H}_{x-1}$, 50% H_x , 30% H_{x+1}) in order to reduce the corrections necessary for natural isotopic abundance.

METABOLITE IDENTIFICATION

Natural isotopes

Certain elements, most noticeably the halogens, silicon and sulphur, comprise mixtures of two or more isotopes. Thus, chlorine comprises 76 parts ^{35}Cl and 24 parts ^{37}Cl ; bromine is almost equal proportions of ^{79}Br and ^{81}Br ; silicon contains 4.7% ^{29}Si , 3.1% ^{30}Si and sulphur contains 4.2% ^{34}S . ^{30}Si and ^{34}S are not too useful but the isotope patterns for chlorine and bromine have been used to identify drug related metabolites when the parent drug contains chlorine or bromine (e.g. chlor-

pheniramine, brompheniramine). Using the chlorine isotopes pattern as a tracer, Skinner *et al.*⁴⁶ were able to determine the metabolites of chlorpromazine and haloperidol in various organs from an overdose victim. In addition to haloperidol, dihydrohaloperidol, dehydroxyhaloperidol, chlorophenylhydroxypiperidine and chlorophenylpiperidine were all identified in the urine and kidney.

Deuterium label

Deuterium can be a poor choice of label for an *in vivo* experiment due to the large isotope effect associated with it (see below: biochemical isotope effects). However, it has advantages of being cheap (relative to ^{13}C or ^{15}N) and available with high isotopic purity (>99%). Provided the label is away from any obvious site of metabolism only secondary isotope effects will be observed, and these are usually sufficiently small to be neglected. If the object is to label recovered drug and metabolites to prove them to be drug derived, then even deuterium in a position where primary isotope effects could occur may be suitable. Indeed, distortion of the $(^2\text{H}_0)/(^2\text{H}_x)$ ratio away from the theoretical can give information about any isotope effects. In a recent study a mixture of $(^2\text{H}_0)/(^2\text{H}_3)$ propantheline bromide was administered to a single volunteer.⁴⁷ A trace amount of [^{14}C]propantheline bromide was also included to aid metabolite identification. The metabolism was determined to be largely hydrolysis to xanthonic acid. A number of similar studies have been reported in literature, including a study on sulotidil.⁴⁸ Farmer *et al.* reported on the metabolism of $(^2\text{H}_0)/(^2\text{H}_3)$ aniline mustard.⁴⁹ Baillie *et al.* were able to follow the metabolism and pharmacokinetics of 5α -pregnan-20-one and 5α -pregnan-3 β ,20 α -diol by giving deuterium labelled steroids to pregnant women.⁵⁰ A similar study on the turnover of catecholamines was also reported.⁵¹ One of the more exciting applications of deuterium label has been the simultaneous administration of equimolar amounts of (+)(^2H)propranolol and (-)(^2H)propranolol orally to dogs; and showing that metabolic excretion of the enantiomers was the same.⁵² Such a method can also be used to examine different metabolic pathways for enantiomers, and work by Jarman *et al.* has shown different pathways when a synthetic racemate of cyclophosphamide, where one enantiomer was labelled with deuterium, was administered to rats.⁵³

Carbon-13, nitrogen-15, oxygen-18

These three represent the remaining stable isotopes currently used in biomedical research, although far less extensively than deuterium. Both ^{13}C and ^{15}N can be obtained in 99% purity, but ^{13}C is usually 90–95% atom pure, and ^{15}N 95–97%. ^{18}O is available 90–95%. The isotope effects associated with ^{13}C , ^{15}N and ^{18}O are less than with ^2H , but ^{18}O is readily exchanged and tends not to be used *in vivo*. Both ^{15}N and ^{13}C have been used for metabolite tracer work (e.g. (^{13}C)cambendazole⁵⁴ and (^{15}N)cinoxacin,⁵⁵ and also for studying enantiomeric metabolism (^{13}C)- α -methyl dopa⁵⁶).

Summary use of as tracers

(1) Deuterium, due to its ready availability and low costs, has been used extensively to form artificial isotope clusters for metabolite identification. However, it has problems because of its relatively high isotope effect.

(2) Other heavy isotopes (^{13}C , ^{15}N) have been tried but not used extensively. They are more suitable for *in vivo* work because of their low isotope effects.

(3) Heavy isotopes have also been used to trace differences in the metabolism of enantiomers, where one form is labelled and the other is not.

(4) Despite its advantages, a mixture of stable isotopes for metabolite determination has not been utilized extensively.

BIOAVAILABILITY STUDIES

In the past bioavailability studies have usually been conducted on a cross-over basis, comparing two formulations or a formulation against a standard (usually an intravenous injection). The problems with this approach are many, not least being the large number of blood samples that require withdrawal from the volunteer and analysis, often using laborious methods to estimate low concentrations in small volumes. The assumptions are also made that the pharmacokinetic profile for the drug remains constant after the interval between doses, or that changes in terminal rate constants reflect changes in the elimination rate constant only, perhaps due to variation in urinary pH.

The simultaneous administration of a stable isotope labelled analogue of the drug offers the advantage that the kinetics of drug distribution and elimination are constant for both light and heavy drugs, and the absorption process can be studied free from corrections for changes. However, there are pitfalls. If a high circulating plasma concentration of drug affects its own absorption then the availability of an oral dose will be underestimated if an intravenous injection is given simultaneously. Perhaps a suitable injectable form is difficult to prepare, and the effort to produce one may outweigh the cost benefits of a simultaneous study. The most important pitfall is the possible isotope effect of a heavy analogue which may alter the metabolism or affect rates of metabolism, distribution and excretion. Therefore, the choice of a heavy analogue must be made carefully

to minimize isotope effects. Some studies reported to date are listed in Table 1.

The first report to appear was work by Atkinson *et al.* on *N*-acetylprocainamide⁵⁷ utilizing *p*-(acetamido-1- ^{13}C)-*N*-(2-diethylaminoethyl) benzamide (^{13}C -acetylprocainamide; (^{13}C)NAPA) for intravenous administration and normal *N*-acetylprocainamide (NAPA) for oral administration. Blood samples were withdrawn at intervals and the concentration of (^{13}C)NAPA and NAPA determined using penta-deutero-*N*-acetyl-procainamide as the internal standard. The curves obtained were analysed and the oral availability determined as 96.0, 74.0 and 78.1% in three subjects. Analysis of the excreted NAPA in urine suggested that 83% of the oral dose was absorbed.

The majority of the NAPA was excreted unchanged. Plasma concentrations after administering (^{13}C)NAPA i.v. (250 mg) and NAPA p.o. (500 mg) in a capsule were in the $\mu\text{g ml}^{-1}$ range. This study used a ^{13}C labelled derivative for i.v. administration, reducing the possibility of isotope effects; but depended on a single heavy atom in the molecule, necessitating a series of corrections for the natural abundance of ^{13}C in NAPA.

Another study reported by Draffan⁵⁸ examined the metabolism of clonidine ($^2\text{H}_0$) and ($^2\text{H}_4$) after oral co-administration of ($^2\text{H}_0$) and ($^2\text{H}_4$).

A further study⁵⁹ concerned co-administration of ($^2\text{H}_0$)SC-27166 and ($^2\text{H}_4$)SC-27166 to rats and the ratio of ($^2\text{H}_0$)/($^2\text{H}_4$) SC-27166 recovered in urine suggested that in the rat model $95.6 \pm 1.0\%$ of the oral dose was available. The study was carried out on a 'cross-over' basis in four animals, two receiving ($^2\text{H}_0$) p.o., ($^2\text{H}_4$) i.v., whilst the second pair received ($^2\text{H}_4$) p.o., ($^2\text{H}_0$) i.v.. Two baboons were also dosed; one received ($^2\text{H}_0$) i.v., ($^2\text{H}_4$) p.o. and the second ($^2\text{H}_4$) p.o., ($^2\text{H}_0$) i.v. The area under curve ratio suggested SC-27166 to be readily available in the baboon (54% and 81% respectively) and this was borne out by comparing the ($^2\text{H}_0$)/($^2\text{H}_4$) ratio in urine for free SC-27166 (53% and 123% respectively). The ($^2\text{H}_4$) label was carefully positioned in a metabolically inert portion of the molecule and the ($^2\text{H}_6$)analogue was used as the internal standard. A straight calibration line was obtained for mixtures of ($^2\text{H}_0$), ($^2\text{H}_4$) in plasma and urine using ($^2\text{H}_6$) as the internal standard.

In a study on mianserin, Vink and Van Hal co-administered ($^2\text{H}_4$) intravenously and ($^2\text{H}_0$) orally to female Beagle dogs.⁶⁰ They used a ($^2\text{H}_2$) internal standard. A discrepancy was found between the bioavailability measured in urine using this technique (42–44%) and that obtained by examining plasma (7–9%). Verbeeck *et al.*⁶¹ determined the bioavailability of meperidine (pethidine) using ($^2\text{H}_5$) as co-administered form and ($^2\text{H}_{10}$) as internal standard. The full results of this study are yet to appear.

The metabolism of co-administered theophylline has been studied in neonates suffering from primitive apnoea.⁶² Very little experimental detail is given.

A more concerted attempt to correlate the results from co-administration and classical cross-over studies was made by Haskins *et al.*⁶³ In this work rhesus monkeys were used. After co-administration the animals received each dosage in a single cross-over design. Discrepancies were found between the results, but these were not due to the influence of stable isotope, but

Table 1. Studies involving co-administration of stable isotope to determine bioavailability. This list is not necessarily complete

Date	Compound	Co-administered isotope	Internal standard	Reference
1975	<i>N</i> -acetylprocainamide	$^{13}\text{C}_1$	$^2\text{H}_5$	58
1977	Clonidine	$^2\text{H}_4$	?	59
1977	SC-27166	$^2\text{H}_4$	$^2\text{H}_6$	60
1979	Mianserin	$^2\text{H}_4$	$^2\text{H}_2$	61
1980	Meperidine	$^2\text{H}_5$	$^2\text{H}_{10}$	62
1980	Theophylline	$^{15}\text{N}_2$, ^{13}C	?	63
1980	Disopyramide	$^{15}\text{N}_1$, ^{13}C	$^2\text{H}_{14}$	64
1981	Verapamil	$^2\text{H}_3$	$^2\text{H}_7$	65

rather to the non-linear pharmacokinetics of the compound chosen for the study.

A study using verapamil was carried out⁶⁴ to avoid changes in the person's metabolic profile during a cross-over study.

Summary of the use of stable isotopes in bioavailability studies

(1) Simultaneous administration of a heavy analogue enables absolute availability to be determined in a single study with one set of blood samples.

(2) Caution must be exercised to ensure that no isotope effects occur. If possible, ^{13}C or ^{15}N analogues should be used for *in vivo* work; if deuterium is used then the deuterium should be carefully positioned in a metabolically inert position.

(3) A second labelled analogue is required as an internal standard. Preferably the administered light and heavy analogues and the internal standard should be separated by at least 3 u each, to reduce correction for natural abundance.

(4) Adverse pharmacological effects of the drug (i.e. inhibition of its own absorption, toxic overdosage after i.v. administration) should be borne in mind when planning such studies.

PHARMACOKINETICS IN CHRONIC ADMINISTRATION

The use of stable isotopes in chronic administration studies is a special case of isotope co-administration. It is usually difficult to estimate any changes in pharmacokinetics during long-term drug therapy, although such changes may be important. By differentiating a single dose by administering a stable isotope label one can perform a pharmacokinetic analysis.

An early study utilizing this technique was published by Klein *et al.*⁶⁵ A single dose of ($^2\text{H}_5$)methadone was substituted for the regular daily dose in a patient, and it was possible to follow the plasma disappearance of ($^2\text{H}_5$)methadone for 10 days.

A similar early study was carried out using ($^2\text{H}_2$)-propoxyphene, when a single dose was administered on day 20 of a chronic study in dogs.⁶⁶

When administering naturally occurring substances it is obviously difficult to distinguish between exogenous and endogenous compound. Again stable isotopes have been used in this case. A report concerned the metabolism of testosterone undecanoate⁶⁷ where ($^2\text{H}_2$)testosterone undecanoate was administered to volunteers. Similar studies have been carried out on other endogenous steroids, including pregnanolone sulphate.⁶⁸ The advantage of stable isotopes were fully exploited in this study, where healthy pregnant women were used as volunteers. Bile acid metabolism has also been studied,⁶⁹ as well as catecholamines,⁷⁰ tyramine⁷¹ and α -methyl dopa.⁷²

Another application has been in the study of differences in enantiomeric metabolism. The anti-cancer drug cyclophosphamide has been studied using liver microsomes by administering a racemic mixture of *R*-($^2\text{H}_4$)

and *S*-($^2\text{H}_0$) and *R*-($^2\text{H}_0$) and *S*-($^2\text{H}_4$).⁷³ It was found that the *S*-isomer was metabolized some three-fold faster than the *R*-antipode.

Methadone has also been administered to human volunteers as either *S*-($^2\text{H}_5$)methadone or *R*-($^2\text{H}_5$)methadone. The excretion of the enantiomers was followed during chronic dosage with *SR*-methadone.⁷⁴ It appears that pulse dosing with stable isotope now may be the method of choice for determining pharmacokinetic profiles during chronic dosing studies.

MEASURING BIOEQUIVALENCE

Bioequivalence measurements are a special case of co-administration. All absolute bioavailability studies can be used for bioequivalence, i.e. co-administration of an intravenous label and a formulation. However, more particularly bioequivalence can be measured by co-administering an oral solution of label and a formulation. If the pharmacokinetics of the drug are known then the analysis of an aliquot of a single urine collection over a suitable period to measure the ratio to parent, will be a measure of bioequivalence. Should more information be required, quantitative analysis of urine concentration of label and parent can be used.

The obvious advantage of such an approach are the non-invasive nature of the experiment and the single dosage regimen. Disadvantages may include the effect on tablet dissolution of a concentration of similar compound. A recent study used oral co-administration of a solution of phenobarbital.⁷⁵

An early study using this approach was carried out in female beagle dogs using benoxaprofen.⁷⁶ A standard solution contained ($^2\text{H}_3$)benoxaprofen, whilst the tested formulations comprised capsules containing crystal aggregates of particular mesh size.

A second study using methoxsalen failed because a saturable first pass effect was observed. Thus, co-administration of a standard labelled solution and a tablet caused a change in the pharmacokinetics of absorption.⁷⁷

BIOCHEMICAL STABLE ISOTOPE EFFECTS

The difference in mass between deuterium and protium causes the vibrational frequencies of C—D, O—D and N—D bonds to be lower than for corresponding C—H, O—H and N—H bonds. The more stable deuterium bond requires greater energy of activation and substitution of deuterium for protium can have a profound effect on the rate of chemical reactions involving bond cleavage. Generally values $k_{\text{H}}/k_{\text{D}}$ are in the range of 2–5, but the maximum effect can be as high as 7–10. These are PRIMARY isotope effects. Deuterium can also effect rates of reaction not directly involving cleavage of a deuterium bond, (e.g. hydrolysis of trideuteromethyl esters) and these are known as SECONDARY isotope effects. The ratio $k_{\text{H}}/k_{\text{D}}$ is usually 1–2 for these. Isotope effects occur for ^{13}C , ^{15}N , ^{18}O , etc., but in general these are negligible compared with the large effects seen for deuterium.

A review summarizes work carried out up to the end of 1975 on isotope effects observed for various drugs.¹⁷ For example, an extension of sleep time in mice administered 5-(3,3-dideuterio-*n*-butyl)-5-ethylbarbituric acid was observed. The major metabolite of butethal is 5-(3-hydroxy-*n*-butyl)-5-ethyl barbituric acid, so by slowing the rate of hydroxylation of the butyl side chain with deuteration at the 3 position, the active parent compound had an extended biological half-life.⁷⁸ Many other studies involving anaesthetics, antibiotics, alkaloids, amines and benzodiazepinones are also reviewed.¹⁷ Most of these studies show the effect of retarding a rate limiting step by substituting deuterium for protium. In a finely balanced situation where two competing metabolic pathways are involved, the use of a heavy analogue may cause a complete switch in the direction of metabolism. Horning *et al.* have studied the effects of deuteration upon the metabolism of caffeine and antipyrine,^{79,80} and in both cases were able to show that metabolism followed an alternative path after deuteration. The principal metabolites of caffeine are formed by *N*-demethylation to give either theophylline or theobromine in a 3:2 ratio. By administering 1-trideuteromethylcaffeine the ratio changed to 3:1 (loss of 1-CH₃ from caffeine yields theobromine). By administering 7-trideuteromethylcaffeine the ratio changed to 1:5 (loss of 7-CH₃ yields theophylline). In both cases a secondary isotope effect is probably taking place.

With antipyrine two deuterated antipyrines were prepared. Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) is metabolized by hydroxylation of the 4 position or the 3-methyl, or by *N*-demethylation. The latter is normally a minor pathway. However, substitution of a 3-trideuteromethyl group for the 3-methyl blocked formation of the 3-hydroxymethyl metabolite, and *N*-dealkylation became the dominant pathway. Replacement of the *N*-methyl groups by trideuteromethyl did not effect the overall pattern. Hydroxylation of the 3-methyl showed a primary isotope effect.

A number of other workers at the British Pharmacological Society Isotope Meeting in 1977⁴ also presented details of isotope effects. Farmer *et al.* showed that inhibition of 5-hydroxylation in 5-deuterocyclophosphamide reduced its efficiency as an anti-tumour agent. This work was reported fully later.⁸¹ Davies *et al.* showed a small isotope effect for tetradeuteroclonidine. Van den Heuvel announced that Merck were trying to market a deuterated drug based on the antibacterial 3-fluoro-D-alanine. By administering 2-deutero-3-fluoro-D-alanine the rate of *in vivo* metabolism is reduced several-fold. However, the heavy analogue has the same antibacterial activity as the parent, and thus the deuterated drug has an enhanced duration of activity.

Summary of biochemical stable isotope effects

(1) Isotopes effects are most apparent with deuterium, less so with ¹³C, ¹⁵N or ¹⁸O.

(2) Primary isotope effects occur when the cleavage of a C—²H, N—²H or O—²H bond is a rate limiting step in a reaction.

(3) Secondary isotope effects occur when the presence of deuterium affects a reaction (e.g. loss of *N*-trideuteromethyl, cleavage of trideuteromethyl esters).

(4) Careful substitution of protium by deuterium in a drug can lead to enhanced activity and reduced or altered metabolism.

TOXIC EFFECTS OF STABLE ISOTOPES

A review of deuterated drugs¹⁷ includes details of the toxic effects of deuterium oxide. In general, in rats, mice and beagle dogs a level of deuteration up to 15% of body water can be tolerated. Above 25% foetal viability ceases and death usually occurs at between 35 and 45% replacement of body water by heavy water. The ultimate cause of death from deuteration is not clear, but renal impairment, CNS disturbance, cardiac arrest and changes in enzymatic activity have all been observed.⁸² The toxic effects due to deuterium incorporated in a drug molecule are probably negligible as the dose of deuterium required to induce toxicity requires replacement of 15% of body water, which is a very high dose indeed in man. One report on man showed no toxic effects were apparent at the extent of 0.5% deuteration.

Other heavy isotopes may be expected to show even lower toxicity and in general this is the case. A review by Gregg¹⁸ described his work on ¹³C incorporation in mice. Mice were fed a diet of about 80 atom % ¹³C yeast for almost a year. At the end of this period about 60% of the body carbon of the mice was ¹³C. A further experiment on mouse embryos required them to be cultured to the blastocyst stage in a U-(¹³C)glucose medium.⁸³ After 48 h the embryos contained 20 atom % ¹³C. After implantation in pseudopregnant mothers, 44 apparently normal mice were successfully born and reared. Administration of U-(¹³C)glucose to pregnant mice during organogenesis leading to a 17 atom % ¹³C enrichment also failed to show any abnormal development in the foetuses.⁸⁴

Heavy oxygen (¹⁸O) may have greater toxicity than ¹³C due to its 2 u difference from the common isotope (¹⁶O). A parallel study on mice involved three groups of mice, one drinking H₂O, one drinking H₂¹⁸O (100 atom % ¹⁸O) and one drinking deuterium oxide (99 atom % ²H).⁸⁵ More mice in the same experiment drank H₂¹⁸O and breathed enriched air (96 atom % ¹⁸O). The mice drinking deuterium oxide died after 5 days. The mice drinking H₂¹⁸O had levels of enrichment of 38% in body water, 24% in dried tissue, whilst those breathing enriched air reached 54% enrichment in water, 33% in tissue. No gross pathological changes were observed in the ¹⁸O mice, and the pups born to a pregnant female showed no gross abnormalities either.

Summary of toxic effects of stable isotopes

(1) Deuterium appears to cause toxic effects in mammals when 15% incorporation into body water has taken place. When 35–40% occurs incorporated death usually results. The low levels of deuterium administered during a drug study are many factors below these levels and deuterium can be considered safe from this point of view.

(2) Carbon-13 and oxygen-18 at high levels of incorporation into mice (50–60%) have shown no toxic or teratological effects in studies to date.

(3) Stable isotopes can be regarded as non-toxic *per se* in the doses administered to man when incorporated in a test substance.

SUMMARY

The use of stable isotopes at present falls into two main categories: (1) their use in chemical analysis; (2) their use in biological situations. There is no reason to see these categories changing in the immediate future.

Use in chemical analysis

Stable isotopes are used as internal standards and as carriers. Any improvements in the area of chemical analysis are more likely to arise from developments in instrument techniques rather than the application of stable isotopes *per se*. These should include liquid chromatographic mass spectrometric systems, negative ion chemical ionization and quantitative capillary GC/MS. All of these are feasible at the present time.

The use of stable isotopes for quantitative analysis may expand into new areas; for instance, estimation of hormone pool sizes in the body can be accomplished by administering a known quantity of labelled hormone and using a second label to quantify this. More accurate assays may be devised by using one label as an internal standard for quantification and a second label as a carrier. By bringing the standard:sample ratio nearer to unity a more precise assay should result.

A new application of stable isotope quantitative analysis is to use them as standards against which the performance of simpler assays can be measured. This is due to a very high specificity accuracy and precision which can be obtained using mass spectrometry. An elaborate mass spectrometric method has been used to determine various steroids and triglycerides as an 'absolute' method. Recently, the US Food and Drug Administration decided to adopt such GC/MS assays as the method of choice.

However, in the gross area not much will occur and the use of labels in this field will remain much as it has for the last five to six years. There should be a rapid expansion of their use (for instance, Volume 7 of *Bio-medical Mass Spectrometry* contained 28 papers

describing some kind of isotope dilution assay). Procedures are becoming more routine, and a large and growing need at the present time is for a manufacturer to provide deuterium, carbon-13, nitrogen-15 labelled compounds rather than tritium, carbon-14 labels are provided at present. This is especially true if labels can be used routinely in the clinic to estimate hormonal pool sizes, or deficiencies in metabolism.

Use in biological situations

The use of labels is expanding rapidly at present, and the use in metabolism and pharmacokinetic studies is only now being fully explored. Perhaps the greatest area of expansion will be in the determination of metabolites using the 'isotope cluster' technique in subjects where the use of radiolabels is banned. For instance, the metabolism of the sulphates of pregnanolone and pregnanediol has been studied in pregnant women using deuterium labelled standards. The measurement of muscle synthesis in man has been followed by giving a 30 h infusion of L-(α - ^{15}N)lycine. In such a study chronic administration of a radiolabel would lead to unacceptable levels of radioactivity being given. The excretion of $^{13}\text{CO}_2$ was measured in normal and diabetic children after the administration of U- ^{13}C glucose.

New techniques for absolute availability relying on the co-administration of one label by one route, a different label by a second route and the measurement of the plasma ratio using another label sounds complex, but in fact has considerable advantages over earlier techniques. The most important of these are the withdrawing of only one set of blood samples, and a saving in analytical time by having only half as many samples to analyse. The disadvantages are the use of expensive equipment and the non-routine assays involved.

A further novel application could be used in the examination of sustained release formulations. If a compound can be given in a pelleted form such that a proportion of the dose is absorbed in the stomach and the remainder—having a different coating—is absorbed in the intestine, then labelling either would enable one not only to determine any differences in plasma concentration—time curves, but also to differentiate between plasma levels derived from absorption in the stomach and/or intestine.

The administration of a labelled drug during a chronic dosage schedule can give information on the pharmacokinetics and whether any changes have occurred due to enzyme induction, or saturation of an enzyme system, or overloading of a compartment.

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