Ann. Rev. Pharmacol. Toxicol. 1980. 20:609–21

STABLE ISOTOPES IN PHARMACOKINETIC STUDIES

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INTRODUCTION

The combination of gas chromatography–mass spectrometry (GC/MS) with an ever-increasing availability of stable isotopes has resulted in a dramatic expansion of the use of these isotopes in pharmacokinetic studies. The high resolving capabilities of gas chromatography and high pressure liquid chromatography, coupled with the specificity and sensitivity of quantitative mass fragmentography, provides in many instances a unique method of analysis with unsurpassed sensitivity. The mass fragmentation pattern of a compound provides a multiplex of information allowing an investigator to choose the most distinctive and/or prominent ion(s) for use in analyses. The principles of mass fragmentography or selected ion monitoring and their applications to the analysis of isotopically labeled compounds have been recently reviewed (1–5).

Automation of the routine functions involved in mass spectrometric analyses has permitted the use of this sophisticated instrumentation as a routine detector for multiple analyses. The increased use of computers for data collection, processing, and storage permits more rapid and accurate analysis of compounds in mixtures of chemical or biological origin. Comparison of unknown spectra to those contained in the ever-expanding libraries often provides information sufficient to separate irrelevant gas chromatographic peaks from the peaks of interest. Automatic focusing of the spectrometer and rapid scanning of peaks help diminish time spent in mechanical operations.

In this review we present a representative grouping of the applications

0362-1642/80/0415-0609$01.00

of stable isotope methodology to kinetic analyses. We have concentrated on those areas where stable isotopes provide a unique tool to investigate hitherto unresolved analytical problems.

QUANTITATIVE ANALYSIS

The advantage of utilizing compounds labeled with stable isotopes lies in their similarities in both physical and chemical characteristics to compounds under investigation. The similarity of chemical properties ensures similar if not identical efficiencies in the extraction and isolation procedures. The isotopically labeled compound thereby provides a nearly ideal internal standard for quantitative analysis. Chromatographic separation of isotopically labeled compounds from their unlabeled counterparts is slight or nonexistent in situations where the differences in molecular weight are small (<2%). Where greater molecular weight differences exist one can expect to see slight modifications of the chromatographic behavior particularly in high resolution systems. By focusing the mass spectrometer on selected pairs of ions or a multiplex of ions, many of the problems with background interference are eliminated and relevant compounds in samples that have undergone little purification may be delineated within the milieu of endogenous compounds. Maximum sensitivity, however, can often only be obtained by preanalytical separation from contaminating compounds. In general, the lowest detectable limits obtained with GC/MS are in the region of 200–500 pg/ml when analyzing 1 ml of plasma. A more routine level of analysis is in the area of 1–10 ng/ml. Representative applications of the use of isotopically labeled internal standards and mass spectrometry for analysis are shown in Table 1. Although there is a wide range in the indicated sensitivities of the individual methods, one must take into account the biological system that is being analyzed. The trade-off between sensitivity and sample preparation time is one that always has to be considered in any type of biological analyses. In many situations where plasma levels are on the order of 50–1000 ng/ml sample preparation can be decreased significantly. However, in situations where the compound has to be measured in the range of 1 to 50 ng/ml a significant amount of sample preparation is usually necessary. As shown in Table 1 the types of derivatization and ionization methods vary from compound to compound. While sensitivity, specificity, and ease of analysis have provided the major emphases behind the expanding use of stable isotopes for analytical purposes, stable isotopes also provide some unique opportunities for analysis in situations where discriminating methods are necessary. A number of these specific uses of stable isotope methodology are discussed below.

Table 1 Quantitative analysis using stable isotopes and mass spectrometry[a]

Compound	Derivative used	Internal standard	Analytical method	Sensitivity	Reference
Clonidine	Methyl	d_4-Clonidine	GC/MS-EI	0.15 ng/ml	6
Cyclophosphamide	None	d_4-Cyclophosphamide	GC/MS-CI	20.0 ng/ml	7
Phosphamide	Methyl	d_4-Phosphamide	GC/MS-CI	20.0 ng/ml	7
Nornitrogen mustard	Methyl	d_4-Nornitrogen mustard	GC/MS-CI	20.0 ng/ml	7
Pethidine	None	d_4-Pethidine	GC/MS-EI	100.0 ng/ml	8
Norpethidine	Trifluroacetyl	d_5-Norpethidine	GC/MS-EI	50.0 ng/ml	8
Testosterone	Heptafluorobutyl	T_2-Testosterone	GC/MS-EI	0.2 ng/ml	9
Cortisol	Dimethoxime and trimethylsilyl	^{14}C-Cortisol	GC/MS-EI	5.0 μg/ml	10
PGA_2	Methyl and trimethylsilyl	d_4-PGA_2	GC/MS-EI	0.2 ng/ml	11
Nabilone	None	d_6-Nabilone	GC/MS-EI	0.5 ng/ml	12
Nabilone metabolite	None	d_6-Metabolite	GC/MS-EI	0.5 ng/ml	12
Phencyclidine	None	d_5-Phencyclidine	GC/MS-EI	0.1 ng/ml	13
Quinidine	None	d_2-Quinidine	MS-EI (direct probe)	1.0 μg/ml	14
Lidocaine	None	d_4-Lidocaine	MS-EI (direct probe)	1.0 μg/ml	14
Monoethylglycine-xylidide of lidocaine	None	d_3-Monoethylglycine-xylidide of lidocaine	MS-EI (direct probe)	1.0 μg/ml	14
Δ_9-Tetrahydrocannabinol	None	d_3-Δ_9THC	GC/MS-CI	1.0 ng/ml	15
Diphenylhydantoin (DPH)	Trimethylsilyl	d_5-DPH	GC/MS-EI	1.0 μg/ml	16
p-Hydroxydiphenyl-hydantoin (HDPH)	Trimethylsilyl	d_5-HDPH	GC/MS-EI	0.1 μg/ml	16
d-Propoxyphene-d_7	None	Pyrrolidine analogue	GC/MS-CI	1.0 μg/ml	17
d-Norpropoxyphene-d_7	None	α-d-d_3-Norpropoxyphene	GC/MS-CI	1.0 μg/ml	17

Imipramine	Trifluoroacetyl	d_4-Imipramine	GC/MS-CI	10.0 ng/ml	18
Desipramine	Trifluoroacetyl	d_4-Desipramine	GC/MS-CI	10.0 ng/ml	18
l-Propoxyphene-d_0	None	d_7-Propoxyphene	GC/MS-EI	5.0 ng/ml	19
d-Propoxyphene-d_2	None	d_7-Propoxyphene	GC/MS-EI	5.0 ng/ml	19
Pirbuterol	Trimethylacetyl	d_9-Pirbuterol	GC/MS-EI	1.0 ng/ml	20
Salbutamol	*t*-Butyl-dimethylsilyl	d_3-Salbutamol	GC/MS-EI	5.0 ng/ml	21
Tolbutamide	Methyl	d_2-Tolbutamide	GC/MS-I	200.0 ng/ml	22
Lidocaine	None	d_4-Lidocaine	MS-CI (direct probe)	10.0 ng/ml	23
Methadone	None	d_5-Methadone	GC/MS-CI	1.0 ng/ml	24
Phenacetin	Methyl	d_3-Phenacetin	GC/MS-CI	10.0 ng/ml	25
Acetaminophen	Methyl	d_3-Acetaminophen	GC/MS-CI	10.0 ng/ml	25
Amitriptyline	None	d_3-Amitriptyline	GC/MS-CI	1.0 ng/ml	26
Nortriptyline	None	d_3-Nortriptyline	GC/MS-CI	0.5 ng/ml	26
Acetylmethadol	None	d_4-Acetylmethadol	GC/MS-EI	20.0 ng/ml	27
Cocaine	None	d_3-Cocaine	GC/MS-EI	2.0 ng/ml	28
Benzoylecgonine	Ethyl	d_3-Benxoylecgonine	GC/MS-EI	5.0 ng/ml	28
Scopolamine	Heptafluorobutyl	d_3-Scopolamine	GC/MS-EI	2.0 ng/ml	29
Cytosine arabinoside	Acetyl and methyl	d_3-Cytosine arabinoside	GC/MS-EI	20.0 ng/ml	30
Clonazepam	None	N^{14}-Clonazepam	GC/MS-CI	1.0 ng/ml	31
16α-CN-3β-HO-pregn-5-en-6-one cyclopentylether	Oxime and *t*-butyl-dimethylsilyl	d_9-Analogue	GC/MS-EI	1.0 ng/ml	32
PGA_2	Methyl	d_4-PGA_2	GC/MS-EI	5.0 pg/ml	33
Debrisoquin	Hexafluoroacetyl	d_{10}-Debrisoquin	GC/MS-EI	1.0 ng/ml	34

[a] CI = chemical ionization; EI = electron impact; GC = gas chromatography; MS = mass spectrometry.

BIOAVAILABILITY

Absolute Bioavailability

Bioavailability studies in humans generally require a large number of subjects to obtain reliable estimates of systemic availability. The number of subjects needed varies with the intrinsic properties of the compound under study. To get statistically significant results, 10–20 patients are generally a minimum requirement for satisfactory analysis. While it is important to determine the intersubject variation for any given drug preparation, comparisons of two forms of a drug are mainly concerned with the inherent differences between these formulations. When one overlays the differences between preparation with subject variability, bioavailability studies become large, cumbersome, and in many instances difficult to interpret. The use of GC/MS and stable isotope labeling provides a means of analysis which overcomes the problems of interindividual variability.

Absolute bioavailability can be determined by administration of isotopically labeled drug by one route (e.g. i.v.) and nonlabeled drug by the test route (e.g. oral). Plasma levels from both preparations are measured simultaneously using the mass spectrometer to distinguish the source of the circulating drug.

The determination of the absolute bioavailability of N-acetylprocainamide (NAPA) was made by administration of intravenous N-acetylprocainamide-^{13}C at the same time as an unlabeled NAPA capsule was given orally (35). This study showed not only that there were large interindividual differences in the kinetics, but also that the kinetics were dependent upon the occasion and the timing of administration. Despite these individual differences and variations with time, the calculated maximum bioavailability differed only by 10% or less. The oral absorption of the NAPA was found to be the range of 75-95%.

Simultaneous oral and intravenous administration of procainamide and NAPA-^{13}C permitted the comparison of the distribution kinetics of these compounds under identical conditions in the same subjects. Under these conditions it was found that the elimination half-life of NAPA was 2.5 times that of procainamide in normal subjects (36).

Oral administration of a novel antidiarrhea agent, SC-27166, was examined using unlabeled SC-27166 and the corresponding tetradeutero compound. The preparations were administered simultaneously orally and intravenously, and plasma levels were compared. In the rat the bioavailability was calculated as 96%. This figure was only 26% in the dog—this study was complicated by the fact that the dog vomited after administration. In the baboon the bioavailability was 81% in one animal and 50–55% in another (37).

Relative Bioavailability

Relative bioavailability can be determined by administration of two distinct preparations, one of which is labeled with heavy isotopes, one of which is unlabeled. The preparations are given by the same route, and plasma levels are measured by using the mass spectrometer.

Tofranil ® was compared to generic imipramine, using deuterium labeled imipramine as an internal standard. There was no difference observed in the bioavailability of the two preparations within a given subject (38). Intersubject comparisons, however, would have led to the erroneous conclusion that one preparation was superior to the other. In this study the deuterium-labeled imipramine was administered in aqueous solution and led to earlier and higher peak plasma levels. However, the availability over the time span examined was similar between the tablet and the aqueous solution. The average intersubject variation was approximately 26%.

The absorption characteristics of two salt forms of propoxyphene, napsylate and hydrochloride, were compared using preparations of each individual salt containing d_7-propoxyphene (17). Mixtures of the two salts containing a single deuterium labeled salt were administered. The absence of an isotope effect was confirmed using a mixture of unlabeled hydrochloride with labeled hydrochloride and unlabeled napsylate with labeled napsylate. The results indicated that the napsylate salt is absorbed more slowly during the early time period with little effect on overall blood levels when compared to the hydrochloride salt.

The apparent bioavailability of different formulations of benoxaprofen was measured in dogs using deuterium-labeled benoxaprofen (39). Preparations of different size crystals were compared to an oral solution as well as intravenous administration. Comparisons of the oral dosage form to the oral solution using labeled drug in the oral solution indicated that the size of the crystals seemed to be particularly important for availability with the smaller crystals having 97% availability vs 86% for the larger crystals.

Thus the use of stable isotopes in bioavailability studies has great promise for decreasing the number of subjects necessary for any given study and eliminating the need for a homogenous sample of subjects of large enough number to account for intersubject variability.

METABOLISM OF ISOMERS

Many useful therapeutic agents are marketed as racemic mixtures because separation techniques were either not available or were not considered important enough to demand their use in the large scale necessary for therapeutic applications. Nonetheless, increasing literature shows that enantiomeric compounds may not be handled in the same manner by a given species. Therefore, a thorough investigation of new optically active

drugs requires a knowledge of the fate of the individual enantiomers. Of particular importance is the evaluation of the pharmacokinetic parameters of each enantiomer. Studies of the optical isomers separately cannot take into account either the variation in the animals between the test times, individual variations between animals, or effects of one enantiomer on the fate of the other or on its own kinetic parameters. For example, a compound that increases blood flow will, by this pharmacological effect, alter the rate and perhaps the route of metabolism. The enantiomer of this compound which may not alter blood flow would thereby have a different metabolic fate. By using synthetic racemic mixtures containing a single isotopically labeled enantiomer, it is possible with mass spectral analysis to determine the fate of both enantiomers during concurrent administration.

The (–) isomer of cyclophosphamide is known to be twice as effective as the (+) form. Disposition of the two isomers were studied in microsomal systems using racemic mixtures of deuterium- and nondeuterium-labeled cyclophosphamide (40). The (–) isomer was found to be metabolized approximately 10% faster than the (+) isomer in rat microsomes. However, the opposite was true in mouse microsomes. In the rabbit the disappearance of the (–) isomer was three times that of the (+) isomer.

Studies with synthetic mixtures of trideuterated amphetamine containing a single deuterated isomer showed that disappearance of the R-isomer was inhibited by the presence of the S-isomer or one of its metabolites (41). Thus, not only is there a difference in the rate of metabolism of the enantiomers of amphetamine, but one enantiomer markedly affects metabolism of the other.

The kinetics of a new antiarrhythmic agent, drobuline, were examined in the dog using synthetic racemic mixtures (42). The major route of metabolism of this compound was glucuronide formation. Using specific deuterium-labeled racemic mixtures, it was found that the levels of free *l*-drobuline were three times higher than those of free *d*-drobuline. The levels of conjugated drobuline were higher with the *l*-isomer than with the *d*. The differences in the levels of the free compound were apparently caused by the more rapid disappearance of *d*-drobuline due to either uptake or metabolism.

Synthetic racemic mixtures of propranolol containing a single deuterated isomer were administered intravenously to dogs (43). The analysis of the urine by GC/MS showed ratios similar to that of the starting material. When plasma levels of (+) and (–) propranolol were measured after oral administration of synthetic racemic mixtures, the (+) propranolol was found to have higher levels than that of (–) propranolol, whereas the levels of the (–) conjugate glucuronide were found to be higher than those of the (+) conjugate (44, 45).

The *l*-isomer of propoxyphene has been shown to affect the plasma levels and pharmacologic activity of *d*-propoxyphene in mice and rats (46, 47).

To investigate the fate of individual isomers after administration of the racemic mixture, synthetic racemic mixtures of propoxyphene in which one enantiomer was labeled with deuterium were prepared. In dogs (48) and man (17) the levels of *d*-propoxyphene were found to be higher than that of the *l*-isomer. Moreover the levels of *d*-propoxyphene were higher when coadministered with the *l*-propoxyphene as opposed to when they were administered alone. Thus, in situations where a single isomer has specific pharmacological activity, plasma levels measurements after administration of racemic mixtures can often be misleading. Stable isotope labeling of the single isomers where little or no alteration in the metabolic fate is made can lead to distinction of the levels of the isomers and, therefore, a better correlation of plasma levels and pharmacological activity. Caution must be used, however, in this approach in that the position of the labeling can also have an effect on the rate and routes of metabolism.

ISOTOPE DILUTION

The techniques of isotope dilution have been utilized for many years for examining pool sizes and turnover rates. The ability to use stable isotopes for these types of studies lends increased sensitivity to the measurements as well as providing an excellent margin of safety for human studies. Some of the more recent applications of stable isotope labeling are discussed.

Glycine-^{15}N was used to examine the pool sizes of glycine and the flux of plasma glycine in rabbits (49). Plasma samples were analyzed for the amino acid which had been converted to the trifluoroacetyl-N-butyl ester derivatives. The results obtained were comparable to similar studies using radiolabeled glycine. Thus the stable isotope methodology would be suitable for use in human studies.

The effects of chronic dosing on the metabolic fate of a drug is often a prime consideration in development of new therapeutic entities. However, it has been difficult in the past to examine the fate of a drug after chronic dosing of the same drug because there may still be residual levels of administered drug present. By using different stable isotope labeled analogues of individual drugs, it is possible to measure the fate of one analogue after chronic administration of a second analogue or unlabeled material. In one application (50), one daily methadone dose to a subject maintained at 70 mg/day for one year was substituted with 70 mg of d_3-methadone. Plasma levels of methadone and d_3-methadone were determined. Steady state levels of methadone were higher than the peak concentration of d_3-methadone obtained 2 hr after dosing. An elevation of plasma levels of unlabeled methadone was seen upon administration of the d_3-labeled analogue indica-

tive of displacement of the unlabeled species from tissue binding sites and of existence of multiple pools of methadone in the body. In studies on 1-butyryl cinnamylpiperizine the d_2-labeled drug was administered for a period of three days (51). During this time period the rats developed tolerance to the effect of this agent. On the fourth day d_5-labeled drug was administered and the fate of this compound was examined. Tissue levels of d_2 and d_5-labeled drug were measured as well as plasma levels. The results indicated that the tolerant rat showed greater extent of metabolism of the parent drug than did the nontolerant animals.

Systemic glucose production was measured using ^{13}C as a tracer (52). Glucose was oxidized in an enzyme-linked assay to CO_2 and the ^{13}C analyzed as the ratio of $^{13}CO_2$ to that of CO_2. Administration of tracer-labeled glucose 1-^{13}C increased the ^{13}C to ^{12}C ratio from 1.1 to 1.3% of carbon^{13} 1-glucose. A comparison to similar studies using glucose 1-^{14}C indicated that the results obtained were comparable. Stable isotope labeled glucose is therefore a viable alternative for use in situations where the radioactivity exposure could be potentially hazardous. The metabolism of ^{13}C-glucose in vivo has been examined in normal and diabetic children (53). Glucose oxidation was monitored by measuring the ratios of $^{13}CO_2$ to $^{12}CO_2$ after administration of ^{13}C-glucose. The diabetics oxidized glucose at a slower rate than the normals. This deficit could be decreased by the administration of insulin.

Since catecholamine turnover is a most important determinant of neuronal activity, methods have been developed for the determination of the flux of catecholamines in brain. Although previous studies have been performed using radiolabeled catecholamines and their precursors, such studies are not feasible for extensive work in humans. A large number of stable isotope labeled catecholamines are currently available (54). The catecholamines as well as their metabolites can be used for examining the catecholamine turnover in man. Administration of the isotopically labeled precursor allows one to measure the decline in half-life of a single dose even though steady state levels may not change over the time period examined. In addition to measuring the fate of an ingested isotopically labeled catecholamine, the rate was also investigated by continuous infusion of deuterated tyrosine. Examination of the product catecholamines for deuterium content showed the rate of conversion of tyrosine to these various catecholamines (54).

The attempt has been made to measure dopamine turnover in the brain using ^{18}O in the atmosphere (55). Assuming that ^{18}O would be incorporated into catechols only via tyrosine hydroxylase during the short time of exposure of the animal, one could obtain an exact measure of the total incorporation for a given time period by following the ^{18}O content of dopamine.

However, during the course of the experiment there was significant synthesis of ^{18}O-containing tyrosine as well as ^{18}O-containing dopamine. This fact complicates the studies and does not permit an easy interpretation in terms of total dopamine turnover.

PLASMA KINETICS OF ENDOGENOUS COMPOUNDS

The same principles used for the analysis of drug levels have been applied to analysis of endogenous compounds. The tetradeutero analogues of 15-keto-13,14 dihydro metabolites of PGE_2 and $PGF_{2\alpha}$ were used as internal standards and carriers for measuring the corresponding prostaglandin metabolites (56) using methods similar to those developed by Green & Steffenrud (57). A similar internal standard was used for measuring PGA_2 in human plasma (11).

DL-Phenylalanine-4-d_1 and 1-tyrosine-d_7 were used as internal standards for the measurement of the corresponding unlabeled amino acids (58). When L-phenylalanine-d_5, an in vivo measurement of phenylalanine, was used, monooxygenase was made by quantitating the resultant tyrosine-d_4 and remaining phenylalanine-d_5.

The analysis of 5-hydroxytryptamine (5HT) and 5-hydroxyindole-3-acetic acid (5HIAA) was performed using tetradeutero 5HT and d_2-5HIAA as internal standards (59). This sensitive and relatively rapid assay permits ready analysis of brain 5-hydroxytryptamine metabolism.

CONCLUSION

Routine analysis using stable isotopes is currently not the general procedure in most clinical and pharmacological laboratories. Further development of instrumentation, the advent of an HPLC-mass spectrometer linkage, and other developments may lead to the time when stable isotopes will rival ^{14}C in both their utility and versatility and surpass radioactive isotopes in terms of safety and convenience. The expanding use of stable isotopes in pharmacology and clinical chemistry is proving to be a true challenge to the instrumentation manufacturers. The need for a reliable, easy to operate, easy to maintain, mass spectrometer is greater now than ever before. Our dependence upon stable isotope measurements for development of drugs and analysis of endogenous and exogenous compounds will be ever increasing. The variety of isotopes that are currently available along with the promise of increased availability of many of those that are currently scarce portends a bright future for the use of the stable isotope technology in both biological and biomedical research.

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