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COMMENTARY

SELECTED ION MONITORING IN PHARMACOLOGY

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During the past decade, integrated gas chromatography–mass spectrometry (g.c.–m.s.) has become recognized generally as one of the most versatile and powerful instrumental systems available to the chemical pharmacologist, and has been the subject of a number of reviews [1–8] and an excellent monograph [9]. This commentary will be concerned with one mode of operation which has proved to be particularly valuable in this context, and is most commonly known as selected ion monitoring (SIM)*. In this mode, the mass spectrometer is used as a fixed mass filter to monitor the gas chromatographic effluent and respond selectively to molecules which give rise to ions of the predetermined mass or masses. Molecules which do not generate ions at these specific masses are not detected. Because of this selectivity, and the time-averaging of the signal achievable in a fixed focus mode, the noise level is greatly reduced and the usable sensitivity is increased by a factor of 10^2–10^3 compared to that obtained for the same ions during a scan. Sensitivities for certain compounds in the femtomole (10^{-15} mole) range have been reported. This sensitivity can only be achieved when the experimenter knows beforehand what masses must be monitored, since the remainder of the mass spectrum is not measured. The system is analogous to a fixed wavelength monitor for a high performance liquid chromatography system, which is useful only if an informative absorption or emission peak is known before the experiment. It is important to distinguish a selected ion profile obtained by SIM from a mass chromatogram [12], which is a selected ion profile reconstructed from repetitive scanning. The latter, of course, provides only as much sensitivity as the original scans from which it is constructed, which is considerably less than that provided by SIM unless the mass range is very small.

Because SIM lends itself to precise, essentially simultaneous measurement of two or more ion currents, it is particularly valuable for quantitative applications in which the information sought is an ion current ratio, although it has also proved useful for confirmation of the identity of a compound whose mass spectrum is known. The facility with which accurate measurements of ion current ratio may be made by SIM on minute quantities ($\geq 10^{-12}$ mole) of a compound has stimulated, in turn, the application of stable isotopes as qualitative and quantitative tracers in biological and ecological systems (see section on "Stable Isotopes in Pharmacology"). We present below a summary of the instrumental requirements for SIM, and an illustrated discussion of the areas of pharmacology in which SIM, in conjunction with stable isotopic labels, has been particularly promising and productive.

*The term mass fragmentography was used originally to describe this mode of operation [2, 10, 11]. This phrase is now obsolescent and has been replaced by "selected ion monitoring" and "selected ion profile."

Instrumentation for SIM

Precise isotope ratio measurements to one part in 10^5 are commonplace in geochemical and environmental applications. The sample size is generally not a limiting factor, and the sample is usually combusted and bled at a constant rate into a mass spectrometer of special design, usually having two or more collectors which simultaneously integrate the current (or count the ions) at different masses. Although this approach can also be used in some biomedical problems, notably $^{13}CO_2$ breath tests [13], pharmacological applications generally provide very small samples, and the only feasible approach is to monitor continuously the effluent from a gas chromatograph. Since the concentration changes continuously as the peak elutes, all the ions of interest must be monitored repetitively at intervals which are short compared to the duration of the peak ($< 1:10$). This imposes limits on the accuracy achievable (1 part in 10^4–10^3) and on the instrumentation used.

For applications in which the masses to be monitored fall in a narrow range, the relevant part of the spectrum may be scanned repetitively. However, it is more efficient to switch discontinuously between the tops of the mass peaks of interest. The original methods to achieve this with magnetic instruments were based on alternating changes in accelerating voltage (AVA [14]); quadrupole instruments are more readily programmed to operate in this mode [15, 16], and the output can be simultaneously demultiplexed and smoothed to provide several channels each of which describes the ion current at a single mass as a function of time. Quadrupole instruments are also more amenable than magnetic sector instruments to the fast switching required, and are now most commonly used for SIM.

Although dramatic results have been reported with some new and still experimental ion sources [17, 18], those almost universally employed for routine SIM are electron impact (EI) and chemical ionization (CI), in which a reagent gas (commonly methane, isobutane or ammonia) is admitted to the ion source at relatively high pressure (approximately 1 mm Hg) where it is ionized by an electron beam and, in turn, transfers charge to molecules of the sample. In comparison to EI, a CI spectrum usually shows less fragmentation, sometimes showing only a single abundant ion representing the unfragmented molecule, to which a fragment of the reagent gas is usually

added (quasimolecular ion) (e.g. Ref. 19). For some compounds, CI may yield an abundant quasimolecular ion while EI gives only a small peak at the mass of the molecule ion; in such cases the sensitivity achievable by CI quantitation may be substantially greater than by EI. However, this is not always the case, and EI may give better results when an abundant fragment ion is monitored. Reliance on a fragment ion for quantitation imposes constraints on the site in the molecule at which an isotopic label is placed in a tracer or internal standard, since it must be retained in the fragment in order to be detected separately; however, this is usually not a serious problem. It is likely to become more serious in double-labeling experiments designed to determine whether a compound is metabolically split and reconstituted or remains intact, since in this case both labels must be retained in the monitored fragment and their positions are determined, in part, by the metabolic characteristics of the compound. Fragmentation of the molecule ion may be reduced, using EI, by lowering the electron energy (see for example, Ref. 20); however, this results in a decrease in the efficiency of ionization and, therefore, of sensitivity. CI, therefore, appears to be clearly preferable for certain specific applications; nevertheless, it has some disadvantages which make it undesirable as the sole ion source in a g.c.–m.s. installation. The fact that fragmentation is minimal necessarily means that the mass spectrum contains less information on which to base a positive identification, although the prominent quasimolecular ion may be very informative. Use of the CI spectrum for identification is limited further by the fact that most libraries of reference spectra were obtained primarily by electron impact. More important from the point of view of quantitative applications is the cluster of ions (typically including $[M + 1]$ and $[M - 1]$) which is generally seen in the vicinity of the molecular ion. This complicates interpretation of ion current ratio data when isotopic internal standards or tracers are used since, for example, an $(M - 1)$ ion containing two ^2H atoms would have the same mass as the $(M + 1)$ ion without the label. This problem is aggravated further by the fact that the ion current ratios within the molecular cluster may vary during a working day as a result of temperature and pressure changes or accumulating background in the ion source. A final point of some practical importance is the frequency with which a CI source must be cleaned compared to EI, presumably because of the higher pressure at which it operates, and the higher background normally associated with a CI source. This is offset only partially by the higher mass range in which it is frequently possible to operate for quantitative SIM.

The rate at which information can be generated by a g.c.–m.s. system is so large that it cannot be utilized completely without an automatic data system for its acquisition and analysis. Most commercial g.c.–m.s. systems are now available with an interactive data system for control and analysis. This need is greatest for acquisition, reduction and interpretation of scans, but even for SIM the analysis of data is tedious without a data processor and for multiple variant experiments it is essential (see section on "Quantitative Applications"). In addition, the computer takes over the function of the switching device used to program the mass spectrometer and demultiplex the output. The "housekeeping" functions of calibration, standardization, organization of data and even trouble-shooting can be accomplished more reliably and efficiently with the aid of a computer. While a computer and data interface substantially augment the cost, this increase is more than offset in the long term by greater efficiency of operation. The trend is now toward integrated systems comprising gas chromatograph, mass spectrometer, computer and interfaces which together constitute a functioning unit. A unit of this type is now available for less than $50,000 [21].

Identification by gas chromatography–mass spectrometry

This is an application for which g.c.–m.s. is most well known. It is frequently possible to identify specific compounds in the environment [1, 22] or in body fluids in amounts smaller than with any other technique. In general, such rigorous identification requires a reference sample of the authentic compound and comparison of mass spectra under identical conditions. However, comparison of a spectrum with a reference file may provide strong presumptive evidence of identity, and extensive libraries of mass spectra are available for this purpose. Unfortunately, the utility of most of these libraries is limited by variations in spectra, due to impurities or different instruments or conditions, and by the increasingly large number of derivatizing methods in use, many of which are quite new. For identification of drug metabolites, presumptive evidence of identity can be obtained frequently by inspection of the mass spectrum because of the relatively limited number of possible metabolic transformations known to occur in similar molecules. A major limitation of g.c.–m.s. in metabolite identification is the requirement of volatility, which precludes its direct use for some of the more polar conjugated metabolites, although identification of (derivatized) glucuronides without prior hydrolysis has been reported [23]. Despite these limitations, several hundred new drug metabolites and endogenous compounds have been identified by g.c.–m.s. in the last few years, and it is frequently possible to reduce the time required for such identification from several months to a few days.

When the quantity of a compound is too small to obtain a complete spectrum, it may still be possible to verify its identity by SIM if a sample of the authentic compound is available. A set of the more abundant ions in the spectrum of the putative substance is selected, numbering three or more. The mass spectrometer is programmed to monitor these ions, and the sample and standard are chromatographed alternately under identical conditions. Ideally, sufficient replicates are obtained to assess the variation in relative abundance. Identity is then established on the basis of the relative ion current at each of the selected masses, and the chromatographic retention time. The certainty of the identification increases as the number of monitored ions increases, but the variability is likely to increase also if the sample is very small, because of the statistical effect of reducing the residence time on each ion. In any event, the

sensitivity which can be expected is substantially greater (approximately 10^2) than with a complete scan. This increased sensitivity can only be achieved, of course, when the probable identity is already known, so that an appropriate selection of ions can be made. This is frequently the case in drug metabolism studies, in which the number of likely metabolites may be limited and identity can sometimes be tentatively established on the basis of chromatographic retention time. This technique was originally used by Hammar *et al.* [11] to establish some of the metabolites of chlorpromazine; it has since been used in a number of studies including, for example, identification of acetylcholine in the chick ciliary ganglion [24] and Schwann cell sheath of the squid giant axon [25], and *in vitro* metabolites of amphetamine [26].

Quantitation by gas chromatography–mass spectrometry

Quantitation requires the accurate measurement of ion current at one or more masses. This information can be extracted from repetitive scans, but the signal/noise ratio can be enhanced enormously by SIM. Although quantitative g.c.–m.s. methods have been described, based on the absolute ion current at specific masses under fixed conditions [27], it is good practice to employ an internal standard to control against variable losses in the work up and analysis. This is even more important than in g.c. with more conventional detectors, because m.s. detection also involves losses in the interface and ionizer which are likely to change significantly during a series of analyses. The internal standard in g.c.–m.s., as in conventional g.c., is frequently a homolog or related compound. A homologous internal standard allows chromatographic resolution; if it generates an ion of the same composition and mass as the compound to be analyzed (e.g. methylphenidate [28], some potential advantage is afforded since it eliminates the need for alternate switching between masses, and may, as a result, improve accuracy and reliability [29]. However, this advantage is highly dependent on the equipment used and we have found it to be very small.

Errors arising from different losses of the internal standard and the compound to be analyzed can be minimized by using a stable isotope labeled variant* of the compound as an internal standard [32]. Isotope dilution assays of this type have been described for several hundred different compounds, and are capable of great precision and sensitivity with detection limits extending down to a few femtomoles.

In a number of published procedures, it has been recommended that the labeled variant be added in great excess (100:1–1000:1) so that it will serve as a carrier as well as an internal standard (e.g. Green [33]). In our experience, it is better not to exceed the expected quantity of the unlabeled compound by such a large ratio because of "crosstalk," i.e. generation of some ion current at both of the masses being monitored by both of the variants [34, 35], which greatly increases the error at large molar ratios. It has been shown [36] that at least for one analytical problem it is preferable to use as an internal standard a homolog with a fragment ion identical to that of the compound to be measured, and to add as a carrier a stable isotope labeled variant in which the mass of the ion of corresponding structure is augmented. This procedure appears to be subject to the same potential source of error, in addition to a possible switching error [29]. There seems little doubt that the optimal procedure is dependent on the compound to be analyzed, its workup and derivatization, and on the stability and noise characteristics of the instrumentation to be employed. Particularly significant factors are likely to include instrumental crosstalk between channels of a multiple ion detector, resolution of the mass spectrometer, and stability of the overall system sensitivity as a function of time.

As in conventional gas chromatography, quantitation is based on the ion current ratio and the quantity of internal standard added, by reference to a standard curve. It should not be assumed that equimolar amounts of the labeled and unlabeled variants will yield identical currents, since there may be an isotope effect on fragmentation, as in the case of acetylcholine [35]. Crosstalk is a further complication which does not occur when the internal standard is resolved in time. A blank, due to the internal standard, yields a standard curve with an intercept which can simply be subtracted, but if the unlabeled compound contributes ion current at the base peak of the internal standard, a conventional plot of ion current ratio against mole ratio will be convex upward, and fitting a straight line may introduce a significant error. This is likely to be small unless the crosstalk is large or the mole ratio is large. It can be avoided by solving the simultaneous equations for ion current at each mass [34, 35] rather than assuming this straight line relationship. The expected error structure, and therefore the correct weighting procedure for a linear regression analysis, is likely to be dependent *inter alia* on the type of internal standard used [37].

Stable isotopes in pharmacology

Quantitative applications. The use of stable isotopic labels as tracers dates back to the introduction of isotopic fractionation techniques in the 1930's. Pharmacological applications of stable isotopic labeling have been reviewed recently [38–42]. The explosive growth of this approach in the past five years is attributable to the recent increase in availability of stable isotopes, including easily utilized chemical intermediates, and more specifically the recent development of g.c.–m.s. and SIM as means of measuring abundance accurately in very small samples. Many examples may be found in the proceedings of three International Conferences on Stable Isotopes in Chemistry, Biology and Medicine [43–45] of uses of stable isotopic labels as quantitative tracers,

*Although the isotopic label is usually a stable isotope such as ^2H, ^{13}C, ^{15}N or ^{18}O, ^3H [30] and ^{14}C [31] have also been used for reasons of availability. This appears to pose some danger of increased background from contamination of the instrument if a large number of analyses is to be conducted. Crosstalk problems are likely to be more significant, since most radioisotopically labeled compounds are not available in the high isotopic purity available for ^2H-labeled intermediates. The detection sensitivity with a mass spectrometer is, of course, very similar to that achieved with stable isotopes if the label is appropriately located in the molecule and crosstalk is not excessive.

particularly in estimating pool sizes and kinetics of endogenous metabolites. This approach is particularly valuable because the safety of stable isotopes allows these methods to be used both experimentally and in a clinical setting [46].

Great care must be taken in deciding upon the position(s) in the molecule in which the isotopic label is to be introduced, particularly if the compound is to be a tracer. It should be retained, of course, in the molecule throughout the metabolic pathways to be studied; it should not produce a significant isotope effect in distribution or metabolism; it must be retained during fragmentation in one of the more abundant ions, and there should be little, if any, crosstalk between the masses selected for monitoring the unlabeled and labeled compounds if the two are to be estimated simultaneously in the same sample. Some of the requirements can, of course, be relaxed when selecting a labeling site for the internal standard.

The simultaneous use of different isotopic variants of a compound as a tracer and an internal standard has been reported from this research group in experimental studies [20, 35, 47] and has been described also in a clinical experiment on bioavailability [48, 49]. Data analysis can be simplified by using a homologous internal standard in tracer experiments [50], or by splitting the sample, using one part to estimate the mole ratio of tracer variants in each compound and adding labeled internal standards to the other for absolute quantitation [51]. One of the advantages offered by stable isotopic tracers and g.c.–m.s. analysis over radioisotopic tracers is the capacity to expand the number of tracer variants which can be distinguished from one another by their mass spectra. Multiple labeling experiments are possible which allow a kinetic analysis of events from a single analysis [20]. Multiple labels have also proved valuable to compensate for the formation or loss during sample workup of a compound being analyzed [52, 53].

In a simple assay employing an internal standard, a conventional standard curve with linear regression analysis usually suffices for calibration and interpretation of relative peak sizes to yield estimates of the mole ratio of assayed compound to internal standard (see, however, the previous section). Interpretation of multiple ion current measurements for quantitative analysis of multiple isotopic variants is not so simple since, in general, each of the variants contributes some current at each of the masses monitored. The solution requires that a set of simultaneous linear equations be solved, and that an appropriate set of standards be measured to determine the linear coefficients. When n isotopic variants are used and corresponding mass peaks of fixed elemental composition are monitored, the mole ratio of the ith variant to the internal standard (1st variant) is given by the expression [15,34]:

$$y_i/y_1 = \left(\sum_{j=1}^{n} b_{ij}x_j\right)\left(\sum_{j=1}^{n} b_{1j}x_j\right)^{-1}$$

where x_j is the ion current at the jth mass and the b_{ij} are coefficients that can be calculated by inverting the matrix of relative ion currents at each monitored mass for each variant. Estimation of the $(n \times n)$ matrix requires measurement of all $(n - 1)$ relative ion currents in response to each of (n) linearly independent combinations of the n variants and one mixture containing all the variants in known proportions. These calculations can be extremely tedious when multiple variants are used unless some simplifying assumptions are possible, and an automatic data reduction system is virtually essential.

Among the oldest and commonest applications of stable isotopes in biology is the estimation of pool size and turnover rate of endogenous compounds [4, 38, 39, 42]. These include total body water [54], amino acids, bile acids, and neurotransmitters and their metabolites. These techniques offer a number of advantages over radioisotopic methods, particularly when used in conjunction with g.c.–m.s. Perhaps the most significant of these is their potential or actual application in clinical medicine. No significant hazard from stable isotopes has been demonstrated with quantities likely to be encountered in experiments of this kind, and the normal body content of ^{2}H, ^{13}C, ^{15}N and ^{18}O is usually much larger than the quantity which would be used for tracer purposes. A number of breath tests based on enrichment of $^{13}CO_2$ have been devised for indirect, non-invasive investigations which are of particular value in metabolic studies on children [55]

Stable isotopic labels provide one of the most direct methods ever devised for the assessment of absolute bioavailability or the comparison of bioavailability in different preparations or by different routes. This approach has been employed by Strong *et al.* [49] to assess the bioavailability of *N*-acetylprocainamide (NAPA). [^{13}C]NAPA was administered intravenously at the same time that an unlabeled NAPA capsule was given orally. Concentrations of both isotopic variants were estimated at various times in plasma and urine by SIM, using [$^{2}H_5$]NAPA as an internal standard.

Bioavailability was assessed by deconvolution of the plasma level profiles from intravenous and oral administration, and by comparing the urinary excretion of unmetabolized NAPA. Oral absorption of NAPA was found to be 87–92 per cent within the first 12 hr after administration. This method appears to be applicable quite generally to studies of this kind, and has the advantage of completely eliminating errors due to differences between subjects or differences within subjects at different times. A similar method has been used by McMahon and Sullivan [56] to compare the fates of the *d*- and *l*-isomers of propoxyphene. One of the isomers was labeled with two deuterium atoms in the benzylic group, and an artificially reconstituted racemic mixture was administered orally and intravenously to dogs. SIM was used to monitor plasma levels at various times after administration, using [$^{2}H_7$]propoxyphene as an internal standard. Plasma levels of the *d*-isomer were consistently higher than those of *l*-propoxyphene, and the half-life of *d*-propoxyphene was longer. Isotope effects on metabolism were ruled out by showing that the isotopic composition in plasma remained constant as a function of time after administration of either enantiomer containing an artificial mixture of [$^{2}H_0$]- and [$^{2}H_2$]propoxyphene.

Stable isotope tracers and SIM also have considerable potential in the analysis of compartmentation

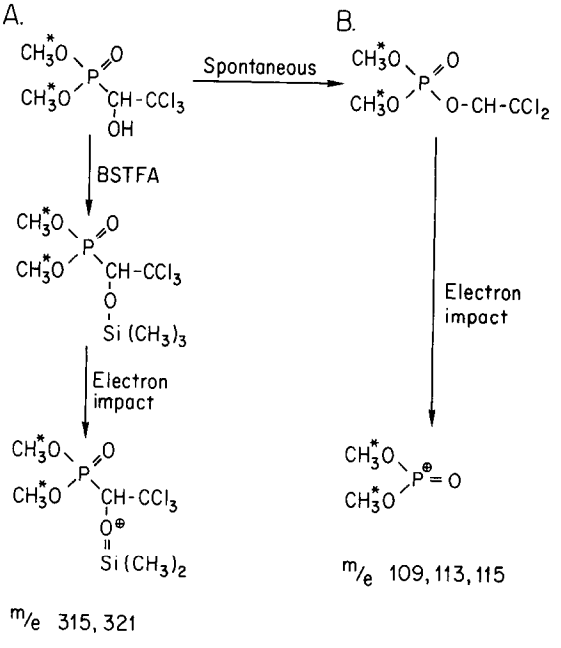
problems in steady state pharmacokinetics. After establishing a steady state by repeated administration of an unlabeled compound, a molar equivalent of a labeled variant of the same compound is substituted for a single dose, after which the unlabeled compound is resumed at the same dosing interval. Serial analyses of plasma and other fluids by SIM permit measurement of the time course of a single dose (of the labeled compound) during a steady state, and its comparison with that of a single dose in a naive subject. The effect of the pulse dose on the elimination kinetics of the unlabeled compound can also be studied. A difference between this disappearance curve and that seen after discontinuation is an indication of non-linearity in one of the processes controlling the pharmacokinetics. This approach has been used both experimentally [57] and clinically [50, 58] and is likely to be of particular value when evidence from other sources suggests that the distribution or rate of metabolism of a drug may change during chronic administration.

Two examples will serve to illustrate uses of multiple stable isotopic labels in the investigation of drug metabolism: a study of deanol in this laboratory [52, 59, 60] and of metrifonate by Holmstedt *et al.* [53,61].

Deanol (*N*,*N*-dimethylaminoethanol) has been used for many years in clinical states thought to be associated with hypofunction of cholinergic mechanisms, with the rationale that it could enter the brain more readily than choline and be converted there to acetylcholine [62]. This rationale has become increasingly suspect [63], and an unequivocal study of the metabolism and distribution of this compound as a possible precursor of choline and acetylcholine was needed. A g.c.–m.s. approach presented the problem that choline and acetylcholine are not volatile and must be *N*-demethylated for gas chromatography by sodium benzenethiolate or by pyrolysis [19], yielding deanol and acetyldeanol. One of the solutions to this problem was to use [$^2H_6$]deanol as a tracer in studies of its distribution and metabolism. This can be distinguished from the unlabeled compound formed by derivatization of choline by a shift in the base peak from *m/e* 58 to *m/e* 64 (Scheme 1). Additional ^2H-labeled variants of deanol and choline were used as internal standards, yielding base peaks at *m/e* 62 and *m/e* 66 respectively. When [$^2H_6$]deanol is methylated metabolically to form choline, only two of the three methyl groups of choline are deuterated. Random (approximately) demethylation by benzenethiolate yields a mixture of [$^2H_3$]- and [$^2H_6$]deanol with prominent mass peaks at *m/e* 61 and 64 in the ratio of about 1:2. The concentration of this variant can be determined by monitoring at *m/e* 61 but of course, allowance must be made for its contribution at *m/e* 64. Finally, [$^2H_4$]- or [$^2H_2$]choline was used as a kinetic tracer to assess the effect of deanol on choline transport and acetylcholine synthesis, by monitoring *m/e* 60. All the variants of deanol, choline and their acetyl esters were measured simultaneously by monitoring *m/e* 58, 60, 61, 62, 64 and 66 (Scheme 1). Additional masses were also monitored to confirm the identity of the compounds [52]. These studies led to the conclusion that deanol is not methylated or

NUMBER	ISOTOPIC VARIANT	N-DEMETHYLATION PRODUCT	STRUCTURE OF MONITORED ION	m/e	EXPERIMENTAL USE OR ORIGIN
I	$CH_3-\overset{\oplus}{N}(CH_3)(CH_3)-CH_2-CH_2-OR$	$N(CH_3)(CH_3)-CH_2-CH_2-OR$	$\overset{\oplus}{N}(CH_3)(CH_3)=CH_2$	58	Endogenous choline (or deanol) and acetylcholine
II	$CH_3-\overset{\oplus}{N}(CH_3)(CH_3)-C^2H_2-C^2H_2-OR$	$N(CH_3)(CH_3)-C^2H_2-C^2H_2-OR$	$\overset{\oplus}{N}(CH_3)(CH_3)=C^2H_2$	60	Kinetic tracer for choline transport and acetylation
III		$N(C^2H_3)(C^2H_3)-CH_2-CH_2-OR$	$\overset{\oplus}{N}(C^2H_3)(C^2H_3)=CH_2$	64	Deanol tracer to distinguish exogenous source
IV	$CH_3-\overset{\oplus}{N}(C^2H_3)(C^2H_3)-CH_2-CH_2-OR$	$N(CH_3)(C^2H_3)-CH_2-CH_2-OR$ (1) $N(C^2H_3)(C^2H_3)-CH_2-CH_2-OR$ (2)	$\overset{\oplus}{N}(CH_3)(C^2H_3)=CH_2$ $\overset{\oplus}{N}(C^2H_3)(C^2H_3)=CH_2$	61 64	Choline and acetylcholine formed metabolically from administered deanol (III)
V		$N(CH^2H_2)(CH^2H_2)-CH_2-CH_2-OR$	$\overset{\oplus}{N}(CH^2H_2)(CH^2H_2)=CH_2$	62	Internal standards for deanol and acetyldeanol
VI	$C^2H_3-\overset{\oplus}{N}(C^2H_3)(C^2H_3)-C^2H_2-C^2H_2-OR$	$N(C^2H_3)(C^2H_3)-C^2H_2-C^2H_2-OR$	$\overset{\oplus}{N}(C^2H_3)(C^2H_3)=C^2H_2$	66	Internal standards for choline and acetylcholine

Scheme 1. Summary of isotopic variants of deanol, choline and their acetate esters used in studying effects of deanol on cholinergic neurochemistry [52, 59, 60].



Scheme 2. Spontaneous conversion of metrifonate (A) to dichlorvos (B), and the structure of ions monitored. An asterisk indicates site of labeling of internal standards, which contained three and two ^2H atoms for A and B, respectively, in each methyl group [53, 61].

acetylated in the brain to a significant degree, but is converted slowly to choline after incorporation in the phospholipid pathway in the liver and released into plasma. Plasma and brain choline levels are elevated only after huge doses of deanol, and high affinity choline transport is inhibited by deanol [59, 60].

Metrifonate (Scheme 2, A), a drug used to treat schistosomiasis, undergoes a spontaneous base-catalyzed rearrangement to form dichlorvos (Scheme 2, B), an organophosphate cholinesterase inhibitor [53, 61]. This conversion occurs non-enzymatically during the sample workup, and in order to compensate for dichlorvos formed during the workup procedure, both [$^2H_6$]metrifonate and [$^2H_4$]dichlorvos were added as internal standards. These variants were labeled with three and two ^2H-atoms, respectively, in the groups marked with an asterisk. Both [$^2H_0$] dichlorvos and [$^2H_6$]dichlorvos were estimated by SIM, monitoring a fragment ion at *m/e* 109 and 115, respectively; the internal standard was monitored at *m/e* 113. [$^2H_6$]dichlorvos could have been formed only during the sample workup, from the [$^2H_6$]metrifonate added as an internal standard, and required the calculation of a correction in both concentrations to compensate for this artifact. Metrifonate was silylated with BSTFA and an $(M\text{-}15)^+$ ion was monitored at *m/e* 315 and 321 for the unlabeled compound and internal standard respectively. This ion is represented by a cluster of peaks in the mass spectrum, which is due to the naturally abundant heavy isotopes of chlorine (^{37}Cl) and silicon (^{30}Si). This phenomenon, which is inevitable in compounds containing certain elements, can cause significant loss of sensitivity in SIM assays because a single abundant peak is replaced by several smaller peaks, and requires that the internal standard be multiply labeled to avoid excessive cross-talk. In the present instance it was handled by monitoring the most abundant ion in the cluster (*m/e* 315) and by including six ^2H atoms in the internal standard. This led to the conclusion that metrifonate is, in effect, a slow release formulation of dichlorvos, which achieves peak tissue concentrations shortly after those of the parent compound [53, 61].

Qualitative tracers. Some of the most valuable uses of stable isotopic labels do not require precise quantitation but yield solutions to problems from qualitative or semiquantitative data. The classical use of isotopic labels to trace metabolic pathways and reaction mechanisms is a common application of both radioactive and stable isotopes. SIM has the enormous advantage of providing, in one measurement, information regarding both the presence of a label in a specific molecule and its position in the molecule. Radioisotopic methods generally require extensive degradative studies to yield the same information. A more qualitative application of stable isotopes is in the generation of recognizable mass spectral labels (e.g. doublets) to facilitate recognition of metabolic products of a labeled compound. This section summarizes some of these applications of stable isotopes as qualitative tracers.

Stable isotopes are used commonly to label pools of reagents or intermediates to assess their role in metabolic reactions. ^{18}O has been used in this context in studies on catechol formation catalyzed by the hepatic P-450 mixed function oxidase. In early experiments investigating mechanisms of ring hydroxylation, Holtzman *et al.* [64] employed $^{18}O_2$ and found that 1 g atom each of ^{18}O and ^{16}O appeared, found in the isolated napthalene-1,2 diol. This observation was interpreted as evidence of the involvement of an epoxide which is hydrolytically opened to a dihydrodiol. More recently, analogous experiments utilizing $^{18}O_2$ and butamoxane [65] have yielded evidence for an alternative hydroxylation mechanism, the sequential addition of two atoms of oxygen from the atmosphere. When the oxygen isotope content of 6,7-dihydroxybutamoxane was examined by g.c.–m.s., 90% of the oxygen was ^{18}O, indicating that little or no oxygen had added from a hydrolytic process. Thus, these types of experiments provide evidence for the existence of at least two different oxygen insertion mechanisms catalyzed by the P-450 system.

When the formation of the same glutathione conjugate of acetaminophen and phenacetin was studied in $^{18}O_2$, two different mechanisms were proposed to account for the absence of $^{18}O_2$ in the acetaminophen metabolite and its presence in the phenacetin metabolite [66]. *N*-hydroxylation was proposed to account for the absence of ^{18}O in the glutathione conjugate of acetaminophen and the arene oxide was invoked in the formation of the phenacetin metabolite. In these studies, the metabolites were isolated by thin-layer chromatography and introduced directly into the mass spectrometer. Deuterium-labeled compounds have been used in this laboratory for mechanistic studies on the formation of phenylacetone [67]. Amphetamine labeled with deuterium was incubated with liver microsomes in the presence of a small amount of *N*-hydroxyamphetamine, and the time course of phenylacetone

formation and its isotope enrichment was determined. The results indicated that essentially all of the phenylacetone originated from amphetamine without participation of the hydroxylamine, since no dilution of the deuterium label was observed in the phenylacetone.

The visually recognizable feature provided by the natural abundance of the isotopes of chlorine in mass spectra of components of uring extracts was utilized as a means of recognizing possible metabolites of chlorpromazine [32], since chlorine-containing organic compounds are not commonly found in urine. This led Knapp *et al.* [68,69] to create artificial isotopic clusters by partial substitution of deuterium for protium in nortryptiline, and allowed the recognition of a new metabolite in an extract of urine. A similar approach has been employed by McMahon *et al.* [70] and by Bush *et al.* [71,72]. In a clinical study [58], artificial doublets were created in the mass spectrum of eterobarb (*N*,*N*-bis[methoxymethyl]-phenobarbital) in an attempt to identify metabolites in urine by their doublet-containing mass spectra. A program was written to search for doublets automatically in a continously scanned gas chromatographic effluent [73]. This allowed an automatic and reliable search to be substituted for the virtually impossible task of visually inspecting 1000–1500 mass spectra per g.c.–m.s. run for doublet features. This led to the identification of monomethoxymethylphenobarbital, phenobarbital and hydroxyphenobarbital. No unique metabolites were found which might account for the reported properties of eterobarb, and these are probably attributable entirely to its conversion to phenobarbital, which unlike the parent compound accumulates on repeated administration of eterobarb because of its long half-life [58].

In the course of these studies, certain major limitations were recognized in the use of the doublet approach, particularly when this is implemented automatically. The most troublesome is chromatographic resolution of the isotopic variants. An efficient gas chromatographic column frequently shows significant separation of isotopic variants when the base peaks are monitored by selected ion monitoring (see, for example Ref. 14), and this effect is demonstrable with a conventional detector on a capillary column An automatic doublet searching algorithm is likely to examine each component of the peak with negative results, rather than the pair of peaks which taken as a whole would show doublets. Although this problem, in principle, can be resolved by analyzing a moving segment of the multiple mass chromatogram without regard to peak resolution, this defeats the purpose of using a high efficiency column, and makes it virtually impossible to search a complex mixture such as urine or blood, in which any arbitrary segment of a chromatogram is likely to be contaminated by an extraneous peak. At least one example was found in which an extraneous peak eluted between the two components of an isotopic mixture, making it virtually impossible to detect a doublet in any single scan during its elution. This difficulty can probably be reduced by using ^{13}C, ^{15}N or ^{18}O to create the doublets rather than ^{2}H, since isotope effects on retention time are less likely with heavier elements. Another difficulty arises from quantization errors, which tend to create doublets among less abundant ions in small peaks when the relative abundancies are not in fact equal. This may be remedied partially by extending the dynamic range of the analog/digital conversion system [74], but the basic problem is inherent in the discontinuous or quantized nature of the information reaching the electron multiplier during a scan, since individual ions are the indivisible units of the signal. A very small peak will tend to show doublets on less abundant ions for this reason, regardless of the A/D system.

Isotope effects in mechanistic studies. In most of the applications described above, isotope effects are undesirable and tend to obscure the information sought if they are substantial. In other areas, advantage can be taken of isotope effects to gain specific information regarding the rate-limiting step in a metabolic reaction involving C–H bond fragmentation. For example, the cleavage of a C–H bond may be rate-limiting in the hydroxylation at the benzylic carbon of ethylbenzene [75] but not that of tolbutamide [76].

The so-called kinetic isotope effect is a change in rate that results from substitution of a normally abundant isotope with another. The isotopes most commonly employed are those of hydrogen because of the large change in relative mass. The difference in zero point energies between C–H and C–D bonds reflects the relative difference in the masses of H and D, which is 1:2. In contrast, the relative difference between ^{12}C and ^{13}C is only 1:1.08. This difference in zero point energy results in a higher activation energy for C–D bond cleavage so that reactions in which C–D bond cleavage is rate-limiting would be expected to proceed at a lower rate, thus exhibiting a deuterium isotope effect. Operationally, the decrease in rate seen with deuterium substitution is interpreted to reflect C–H bond cleavage in the rate-determining step, although this is not conclusive evidence for such a mechanism because the change in hydrogen isotope can also affect nonkinetic parameters such as base or acid strength, and additional control experiments are a necessary part of this type of study.

Deuterium isotope effects have been described for a number of mixed function oxidase reactions, such as aromatic ring hydroxylation [77], *O*-dealkylation [78–80], *N*-dealkylation [81, 82] and *N*-hydroxylation [83]. The values for the ratio (rate ^{1}H/rate ^{2}H) vary substantially with the substrate as well as the reaction, indicating that widely different mechanistic details must be involved in C–H bond cleavage by the P-450 enzyme system A "reverse" isotope effect (rate ^{1}H/rate $^{2}H < 1$) was noted in the formation of *N*-hydroxyamphetamine [83] in the formation of the 455 nm complex by *N*-hydroxyamphetamine [84] when the α-hydrogen was substituted with deuterium. One explanation for these observations is the decreased rate of α-carbon metabolism that might be expected as a result of deuterium substitution. This decreased rate would allow *N*-hydroxyamphetamine to accumulate instead of being further metabolized to the oxime or ketone. If the P-455 complex is a result of subsequent oxidation at the nitrogen as postulated [85, 86], then the same argument could apply, i.e. a higher "steady state" level of *N*-hydroxyamphetamine would result in greater levels of higher *N*-oxidation states.

Conclusions

Gas chromatography–mass spectrometry and SIM have emerged from an era in which they were considered highly specialized techniques available to a few investigators only. Present instruments are more reliable, more versatile, more automatic and less expensive, relative to their capabilities, than 5–10 years ago. Their use in many laboratories is now routine for isotope dilution methods of quantitation, and applications to stable isotopic tracer experiments are becoming more common, partly because of the increasing availability and range of stable isotope labeled intermediates and drugs*. Critical appraisal of the strengths and limitations of g.c.–m.s./SIM is required relative to other methods such as high performance liquid chromatography and radio-immunoassay which may be more suitable for specific applications. Typical factors to be considered include the volatility of the compound to be analyzed, a need for fast turnaround of very large numbers of samples, economic considerations, and the relative importance of the analytical rigor which is a feature of g.c.–m.s. analyses. SIM provides unique opportunities for the solution of certain kinds of problems, such as the analysis of stable isotopic tracers in very small quantities. This broad capability awaits greater exploitation particularly in the context of clinical investigation, where it is capable of providing solutions currently obtainable in no other way.

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*A national Stable Isotope Resource to help meet this need has been established at the Los Alamos Scientific Laboratories.

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