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Use of stable isotope mixtures as a labeling technique in drug metabolism studies*

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IN USING the combination gas chromatograph–mass spectrometer (GC-MS) in drug metabolism studies, it would be very helpful to have some means of easily determining which of the chromatographic peaks in a complex biological mixture arose from the administered drug. We present here a labeling technique using stable isotope mixtures which allows for identification of the labeled compounds by simple inspection of the mass spectrum for the presence of characteristic doublets.

Stable isotope labeling in itself is not new in biochemical studies. Deuterium labels have been used widely in biosynthetic studies.[1,2] Carbon-13 has seen use in biosynthetic studies[3] and in mechanistic studies of enzymic conversions.[4] Oxygen-18 has been quite useful in studying enzymic oxidation.[5] Nitrogen-15 has been used both as a tracer in the study of normal metabolic processes[6] and in drug metabolism studies.[7–10] All of these applications, however, have made use of quantitative determinations of isotopic abundances.

Compounds labeled with stable isotopes have also been used as internal standards in quantitative GC-MS work.[11,12] These uses substantiate the assumption that the labeled compounds are chromatographically identical to their unlabeled counterparts. This assumption is not necessarily valid for extensively labeled compounds, however, as evidenced by the fact that d_{45}-penta-*O*-trimethylsilyl-D-glucose is chromatographically separable from its non-deuterated analog.[13]

In spite of the extensive use of stable isotope labels, little use appears to have been made of stable isotopes for artificially creating visually conspicuous mass spectral "isotope clusters" as markers. One such use has recently been reported in the area of peptide chemistry.[14,15] An equimolar mixture of acetic anhydride and perdeuteroacetic anhydride was used to acylate peptides as part of a derivatization procedure for mass spectral study. Any monoacetylated fragment, therefore, exhibits an M, M + 3 doublet in the mass spectrum which serves as an easily recognized marker. We present here a technique using the same concept whereby an M, M + 3 doublet in an administered drug serves as a tracer label to follow the fate of the drug and its metabolic products.

The M, M + 3 doublet is created by making an equimolar mixture of nortriptyline and trideuterium-labeled nortriptyline. The parent drug then, as well as any metabolites retaining the labeled site, will exhibit in the mass spectrum an M, M + 3 doublet for the molecular ion and any fragment ions containing the labeled site.

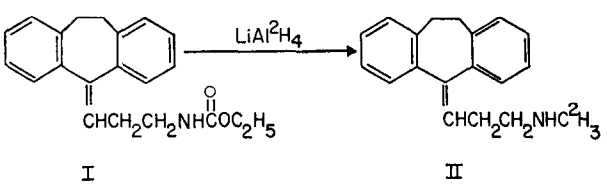

FIG. 1. Synthesis of trideuterium-labeled nortriptyline.

Methods†

The *N*-methyl trideuterium-labeled nortriptyline (II) was synthesized from 5-(γ-*N*-carbethoxyaminopropylidene)dibenzo[a,d]-cyclohepta-1,4-diene (I) (Fig. 1) by reduction with lithium aluminum deuteride‡ according to the method of Marshall and McMahon[16] and isolated as the hydrochloride salt. The labeled material showed a mass spectrum identical to authentic nortriptyline except for the

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† The gas chromatograph-mass spectrometer used in this work was the LKB 9000.

‡ Stohler Isotope Chemicals, Rutherford, N.J.

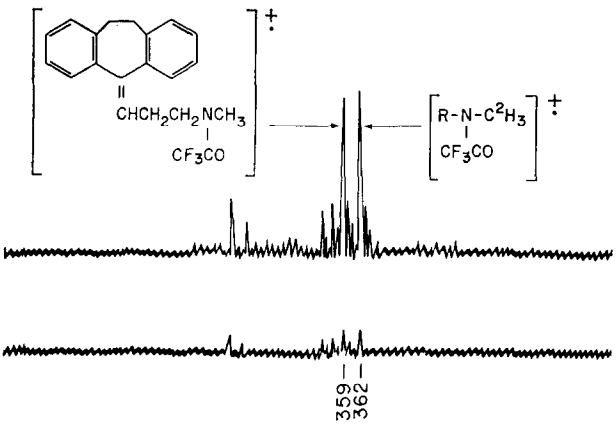


FIG. 2. Partial mass spectrum of the trifluoroacetylated nortriptyline isotopic mixture (20 eV).

shift of the molecular ion from *m/e* 263 to *m/e* 266. The M, M + 3 mixture was prepared by mixing equimolar quantities of the labeled and unlabeled nortriptyline hydrochlorides.

A sample of this mixture was dissolved in water, the solution was basified with aqueous sodium hydroxide to liberate the free bases, and the nortriptylines were extracted into benzene. The benzene extract was concentrated to a small volume by boiling and treated with trifluoroacetic anhydride and dimethylformamide to form trifluoroacetyl derivatives of the nortriptylines. The mass spectrum was obtained via the gas chromatographic inlet on the LKB 9000 (6 ft × 1/8 in. glass column, 1% OV-17 on Chromosorb W-HP, 200° isothermal, He flow 10 ml/min). Figure 2 shows the molecular ion region from the mass spectrum of the trifluoroacetylated mixture. Spectra are shown as the raw

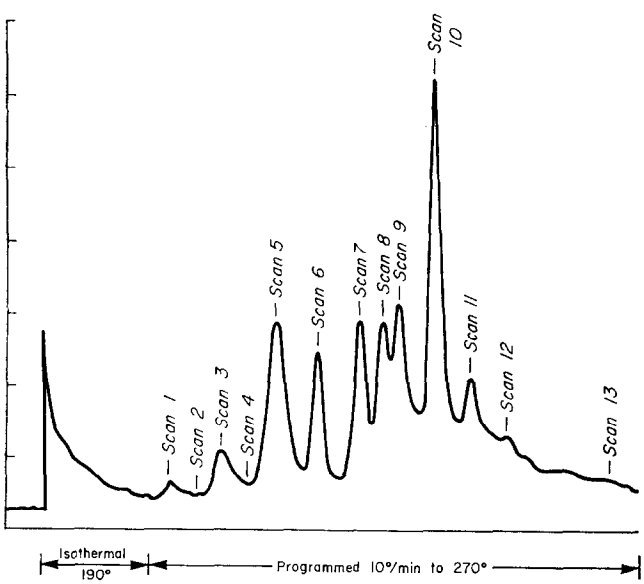


FIG. 3. Total ion current recording from separation of trifluoroacetylated basic urine extract showing mass spectral scan points.

oscillograph traces rather than normalized bar graphs, since the facility of the method stems from the easy recognizability of the doublets in the raw spectra.

For the metabolism study, the mixture of nortriptyline hydrochlorides was administered intraperitoneally in water to a rat at a dose of 20 mg/kg. The urine was collected for 24 hr. A 2-ml sample of the 24-hr urine was treated with β-glucuronidase at pH 5, adjusted to pH 13 with aqueous sodium hydroxide, and extracted with benzene. An aliquot of the concentrated benzene extract was treated with trifluoroacetic anhydride and dimethylformamide. A 2-μl portion of the derivatized extract, representing the material extracted from ca. 20 μl of urine, was injected into the GC-MS (same column and flow as above, 190° isothermal then linear 10°/min to 270°). The gas chromatographic separation trace from the total ion current detector is shown in Fig. 3. Mass spectral scans (20 eV) were taken at the points indicated. The spectra were then inspected for the presence of the M, M + 3 doublet. Scan 10 was found to contain the doublet as shown in Fig. 4. The region from *m/e* 230 to the doublet was essentially free of peaks.

A second rat was administered only trideuterated nortriptyline at the same dose and with the same work-up. The scan corresponding to scan 10 in this case showed only the *m/e* 360 peak of the doublet.

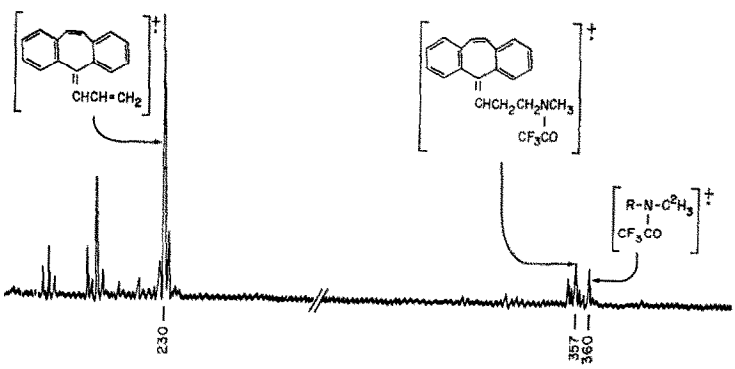


FIG. 4. Partial mass spectrum from scan 10 (20 eV).

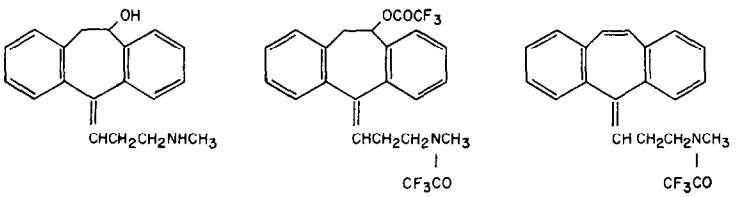
Results and discussion

The M, M + 3 doublet was found only in scan 10 and indicated that the compound present in that chromatographic peak came from the administered drug. Thus the mass spectrum serves the additional function of locating the label as well as the usual function of providing structural information.

The doublet appeared only at the high *m/e* extreme of the spectrum indicating that the label was lost in the first fragmentation. The base peak at *m/e* 230 is two mass units lower than the base peak from trifluoroacetylated nortriptyline. This information plus the knowledge of the previously reported metabolites[17] of nortriptyline led to the assignment of the structure V.

McMahon *et al.*[17] has reported that the major nondemethylated metabolites of nortriptyline in the rat are the *cis* and *trans* 10-hydroxy derivatives (III) (Fig. 5). Trifluoroacetylation of III (*cis* and *trans*) would be expected to give the derivatives IV (*cis* and *trans*) which presumably would be chromatographically separable and give rise to two peaks each having an M, M + 3 doublet at *m/e* 471, 474 in the mass spectrum. Loss of trifluoroacetic acid from either of the derivatives IV would give the same product V. Whether this loss occurs during the trifluoroacetylation or thermally in the gas chromatograph is uncertain, but the loss is not unexpected since McMahon *et al.*[17] observed the loss of acetic acid from the diacetyl derivatives of III under strongly acidic conditions. Since the efforts of Hammar *et al.*[18] to circumvent this loss by use of several other derivatives were without success, we did not pursue the problem further.

There is always the chance that an observed doublet might be coincidental and not stem from an administered isotopic mixture, although in the M, M + 3 case this would be quite unusual. The validity of an observed doublet as a tracer can be established by comparing the mass spectrum of the corresponding chromatographic peak from an animal administered only the labeled or the unlabeled drug. In this case, we should see only one of the peaks of the doublet. We tested the doublet in our example by administering only the trideuterated nortriptyline to a second rat. After the same work-up and derivatization, the scan from this rat, corresponding to scan 10 (Fig. 3) from the first rat, was



III (*cis* and *trans*) M = 279

IV (*cis* and *trans*) M = 471 M+3=474

V M = 357 M+3=360

FIG. 5. 10-Hydroxynortriptyline and trifluoroacetylated derivatives.

found to contain only the *m/e* 360 peak of the previously observed doublet. The doublet could just as easily have been tested by administering unlabeled nortriptyline to the second rat. In this case we would see only the *m/e* 357 peak.

The method is dependent upon chemical and physical identity of the labeled and unlabeled compounds under the experimental conditions. It is unlikely that physical differences associated with the change of 2 or 3 mass units in a normal sized drug molecule (molecular weight of 100 or more) would be sufficient to cause the doublet to be unrecognizably distorted during the experiment. Essential chemical identity can be assured by locating the isotopic label in a metabolically stable position thereby eliminating any problems with primary kinetic isotope effects. Secondary kinetic isotope effects again are unlikely to be sufficient to distort the doublet beyond recognizability.

It is obvious that our label, being in a metabolically labile position, was not in the best position for a thorough metabolic study. Our label position was chosen for synthetic convenience and merely to demonstrate the method. With the label in a metabolically more stable position, the method should be generally applicable for thorough qualitative metabolic studies. Many stable isotopes could conceivably be used; ^{13}C, ^{15}N and ^{18}O being particularly attractive. It should perhaps be pointed out that the utility of an M, M + 1 doublet would be limited by the distortion introduced by natural isotopic contributions.

The method offers certain definite advantages. Assuming sufficient structural information can be gleaned from the mass spectrum, the need for actually isolating the metabolites is eliminated by use of the GC-MS, and the label allows us to focus attention only upon those components containing the label. This can result in tremendous time savings in a drug metabolism study. Further refinements could be made by the use of the GC-MS computer techniques.[19] Of particular interest in this regard is the analysis of unresolved chromatographic peaks via "mass chromatography". Possibly the greatest advantage to the use of stable isotopic labeling, however, is the potential applicability to human studies. With stable isotope labels the radiation hazards attendant with the use of traditional labels is avoided. Thus one of the greatest obstacles to thorough drug metabolism study in humans, and in particular in children and pregnant women, can be eliminated.

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Stabilization of rat liver lysosomes by heparin *in vitro*

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WHEN a rat liver mitochondrial fraction is incubated in isoosmotic sucrose at pH 5 and 37°, the free activity of lysosomal enzymes gradually rises: there is an increase in permeability of the lysosomal membrane to exogenous substrates.[1] Certain steroids, retinol and tocopherol, are able to make lysosomes more sensitive to this treatment; on the contrary, cholesterol and, to a lesser extent, cortisone exert a protective effect on the granules.[2] In our search for other substances that might affect lysosome behavior during incubation at pH 5, we found that heparin is a potent lysosome protector under such conditions.

Mitochondrial fractions from the liver of Wistar rats, corresponding to the sum of fractions M and L of de Duve *et al.*,[3] were prepared in 0·25 M sucrose. Treatment of the granules at pH 5 and 37° was performed according to de Duve *et al.*[2] Acid phosphatase and acid ribonuclease were measured by the procedure of de Duve *et al.*,[3] and β-galactosidase was assayed according to Vaes.[4] Free activity was measured for 10 min in 0·25 M sucrose; total activity was measured in the presence of added 0·1% Triton X-100.[5] Experiments were carried out on mitochondrial preparations isolated from normal rats and in some cases, from rats injected with 170 mg Triton WR 1339 in saline 4 days before sacrifice, since it has been shown[6] that lysosomes from rats injected with this detergent are more stable at pH 5 and more sensitive to the stabilizing effect of cholesterol.

As illustrated in Fig. 1, free acid phosphatase activity increases during the incubation of mitochondrial fractions at pH 5 and 37°. When the granules are incubated with heparin, free activity does not increase as much. By way of comparison, the effect of cholesterol is also indicated. Heparin seems to be more effective than cholesterol with preparation from normal rat and slightly less effective with preparation from detergent-injected rat.

These results suggest that heparin is able to protect the lysosomes membrane to some extent during incubation of the granules at pH 5 and 37°. If this is true, one would expect that the free