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THE USE OF STABLE ISOTOPES IN GAS CHROMATOGRAPHY–MASS SPECTROMETRIC STUDIES OF DRUG METABOLISM

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SUMMARY

Internal reference compounds labeled with stable isotopes have been used to quantify drugs and drug metabolites in urine, plasma and breast milk. [2,4,5-$^{13}C_3$]Diphenylhydantoin, [2,4,5-$^{13}C_3$]phenobarbital and [1-C^2H_3]valium have been used to quantify diphenylhydantoin, phenobarbital and valium, respectively; [2,4,5-$^{13}C_3$]pentobarbital has been used to quantify amobarbital, secobarbital, butabarbital and pentobarbital. Analyses have been carried out in the picogram to nanogram range by selective ion detection with two gas chromatograph–mass spectrometer–computer systems. Instrumental measurements with picogram samples have also been made using an atmospheric pressure ionization mass spectrometer.

INTRODUCTION

In order to identify and quantify drugs and drug metabolites in the picogram to nanogram range, analytical methods with the sensitivity and selectivity of detection provided by gas chromatograph–mass spectrometer–computer (GC–MS–COM) systems are necessary; gas chromatographic (GC) procedures alone are usually not adequate. With such systems, the mass spectrometer is used as a detector and analyses are carried out by selective ion detection[1,2]. Two GC–MS–COM systems are in use in our laboratory, *viz.* an LKB 9000-PDP 12 operated in an electron impact mode (LKB, Rockville, Md., U.S.A.) and a Finnigan 1015-PDP 8/I operated in a chemical ionization mode[3] (Sunnyvale, Calif., U.S.A.). A third system, an atmospheric pressure ionization (API) mass spectrometer–computer[4], has also been used in these studies. Preliminary results with the API system indicate that estimations may be possible with femtogram to picogram samples[5].

For high-sensitivity detection, internal reference compounds are added to biological samples before the isolation of drugs and drug metabolites is initiated. Stable isotope-labeled analogs are the most satisfactory internal reference compounds, although structurally related compounds (homologs) can be used. If suitable

standards are not available, a calibration curve can be made each day using a reference solution of the drug to be measured[6].

EXPERIMENTAL

Isolation of drugs and drug metabolites

Simple, rapid procedures for isolating drugs and drug metabolites from biological samples (urine, plasma and breast milk) which involve salt–solvent pairs have been developed[7–9]. After saturation of the sample with powdered anhydrous ammonium carbonate, the drugs and drug metabolites were extracted with chloroform, ethyl acetate, methylene chloride, or isopropanol.

Plasma samples (100–200 μl) were diluted to 1 ml, saturated with powdered anhydrous ammonium carbonate and extracted twice with 2-ml portions of solvent, usually ethyl acetate. Urine samples (5–7 ml) were saturated with powdered anhydrous ammonium carbonate without dilution and then extracted twice with 5-ml portions of solvent. The extractions can be conveniently carried out in centrifuge tubes and the layers separated by centrifugation. For quantitative recovery of some compounds, it is necessary to use potassium carbonate as the salt and isopropanol as the solvent. With the proper selection of the salt–solvent pair, the recovery of drugs and drug metabolites using radioactive tracers was 90–100%.

The isolated drugs and drug metabolites were converted to derivatives (methylation followed by silylation) for analysis by Finnigan or LKB systems. No derivatization was necessary for analysis with the API mass spectrometer.

Internal standards were added to all of the biological samples at the beginning of the isolation procedure. The standards presently in use are [2,4,5-$^{13}C_3$]pentobarbital, [2,4,5-$^{13}C_3$]phenobarbital and 2,4,5-$^{13}C_3$]diphenylhydantoin. These compounds were provided by Dr. Monroe Wall of the Research Triangle Institute through a program sponsored by the National Institute of General Medical Sciences. [1-C^2H_3]Valium was a gift from Hoffmann La Roche (Basle, Switzerland).

Selective ion detection

Most studies with stable isotopes were carried out with the Finnigan 1015-PDP 8/I operated in a chemical ionization (CI) mode. Methane was used as the carrier gas; the GC separations were carried out isothermally or by temperature programming. The effluent from the gas chromatograph passed directly into the ion source at a pressure of 1 Torr; a separator was not employed. The ions to be monitored, usually MH^+, were detected by repetitive scan, and the data were stored on magnetic tape under control of the program supplied with the system.

A special program was then used to read the data and to integrate responses for the selected masses over the duration of the GC peaks due to the compounds to be measured. [2,4,5-$^{13}C_3$]Pentobarbital (N,N-dimethyl derivative, $MH^+ = 258$) was used as the internal standard for measuring the N,N-dimethyl derivatives of amobarbital ($MH^+ = 255$), pentobarbital ($MH^+ = 255$) and secobarbital ($MH^+ = 267$) in biological samples; the ions at 258–259, 255–256, and 267–268 amu were monitored. [2,4,5-$^{13}C_3$]Phenobarbital (N,N-dimethyl derivative, $MH^+ = 264$) was used as an internal standard for measuring phenobarbital (N,N-dimethyl derivative, $MH^+ = 261$).

When diphenylhydantoin (N-methyl derivative, $MH^+ = 267$) was monitored in biological samples, [2,4,5-$^{13}C_3$]diphenylhydantoin (N-methyl derivative, $MH^+ = 270$) was used as the internal standard and the ions at 267–268 and 270–271 amu were monitored. [1-C^2H_3]Valium ($MH^+ = 288$) was used as the internal standard for measuring Valium ($MH^+ = 285$).

An LKB 9000-PDP 12 system equipped with an accelerating voltage alternator (AVA) and peak matcher was used for multiple ion detection by electron impact[3]. A PDP 12 computer controls the relays of the AVA and the peak matcher, and by means of a digital-to-analog converter can superimpose a 20-V ramp on the accelerating voltage of the mass spectrometer. This permits the computer program to compensate for the drift which normally occurs as the magnet warms up over a period of hours. Each data point is obtained by sampling and summing the signal for one ion up to 4095 times. Each ion (up to four) can be measured in this way at 2-sec intervals. Selective ion monitoring was usually carried out under isothermal conditions. For example, analyses of diphenylhydantoin (N-methyl derivative) were carried out at 205°; the ions at *m/e* 266 (M) for diphenylhydantoin and at *m/e* 269 (M) for [2,4,5-$^{13}C_3$]diphenylhydantoin were monitored (Fig. 1). For the analysis of phenobarbital in plasma, the base peaks occurring at *m/e* 232 (M–28) for phenobarbital and at *m/e* 235 for [2,4,5-$^{13}C_3$]phenobarbital were monitored, and the analyses were carried out at 175°.

The operation of the API mass spectrometer has been described[4]. This system can be operated in both positive and negative ion modes. For the estimation of barbiturate and diphenylhydantoin concentrations in plasma, the negative ion mode was preferred and ions at M-1 were monitored. When biological samples were analysed, an aliquot of the solvent extract (usually methylene chloride) containing underivatized drugs and drug metabolites and one or more internal standards was injected directly into the external source at atmospheric pressure. The M–1 ions monitored were at *m/e* 231 (phenobarbital), 234 [2,4,5-$^{13}C_3$]phenobarbital, 251 (diphenylhydantoin) and 254 ([2,4,5-$^{13}C_3$]diphenylhydantoin).

RESULTS AND DISCUSSION

Quantitative studies of diphenylhydantoin and phenobarbital concentrations in plasma have been carried out with three types of mass spectrometric systems. Plasma samples were obtained from several young adults maintained on daily doses of diphenylhydantoin and phenobarbital. For the analyses, 100- to 200-μl samples of plasma saturated with ammonium carbonate were extracted with ethyl acetate or methylene chloride. The final volume of the derivatized sample was 25 μl and 1–2 μl were used for analysis by selective ion detection. When underivatized samples were analyzed by the API system, it was not necessary to concentrate the solvent extract; 1- to 2-μl samples out of a total volume of 4–5 ml of a methylene chloride extract were injected into the ion source. Figs. 1–9 illustrate the types of data obtained with the three systems.

Fig. 1 shows the electron impact spectra of the N-methyl derivatives of diphenylhydantoin and [2,4,5-$^{13}C_3$]diphenylhydantoin obtained with an LKB 9000-PDP 12 system. The molecular ions at *m/e* 266 and 269 were monitored for purposes of quantification. The base peak (*m/e* 180) in the spectrum of diphenylhydantoin

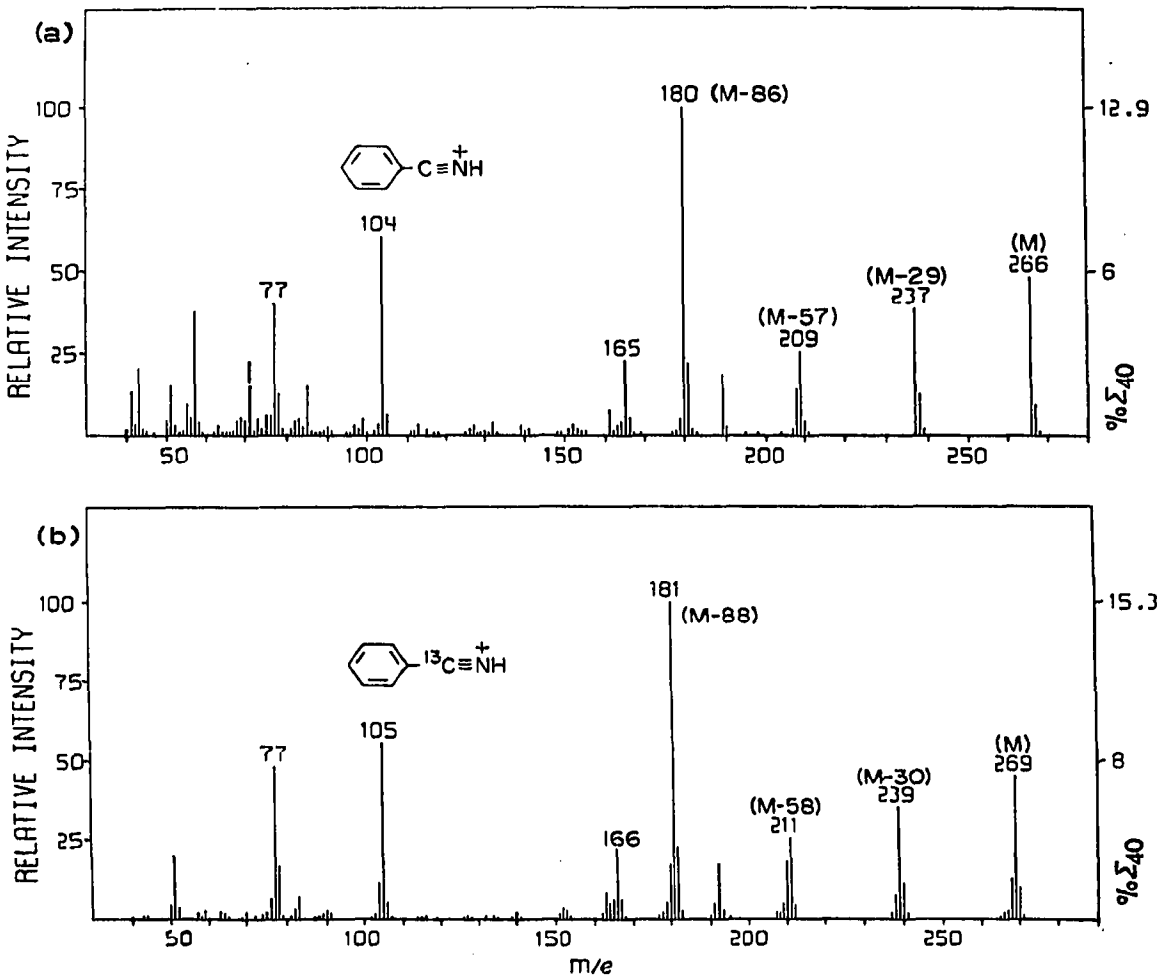


Fig. 1. Electron impact spectra (LKB 9000) of (a) the N-methyl derivative of diphenylhydantoin and (b) [2,4,5-$^{13}C_3$]diphenylhydantoin.

was not used for selective ion detection because the corresponding peak in the spectrum of the labeled drug occurred at *m/e* 181, and this ion would have a contribution due to the natural abundance of ^{13}C in the unlabeled drug.

An analysis of a plasma extract obtained with an LKB 9000-PDP 12 system is shown in Fig. 2. The plots were computer drawn. The concentration of diphenylhydantoin was calculated from the ratio of the peak heights of the molecular ions of diphenylhydantoin and ^{13}C labeled diphenylhydantoin. The concentration of the drug could also be calculated from the ratio of the areas of the two peaks.

The chemical ionization spectra of diphenylhydantoin and [2,4,5-$^{13}C_3$]diphenylhydantoin obtained with a Finnigan 1015-PDP 8/I are shown in Fig. 3. The protonated molecular ions (MH^+, N-methyl derivative) of the drug (*m/e* 267) and the internal standard (*m/e* 270) were monitored. The ion at *m/e* 269 in the spectrum of the ^{13}C-labeled compound was due to a protonated molecular species containing two instead

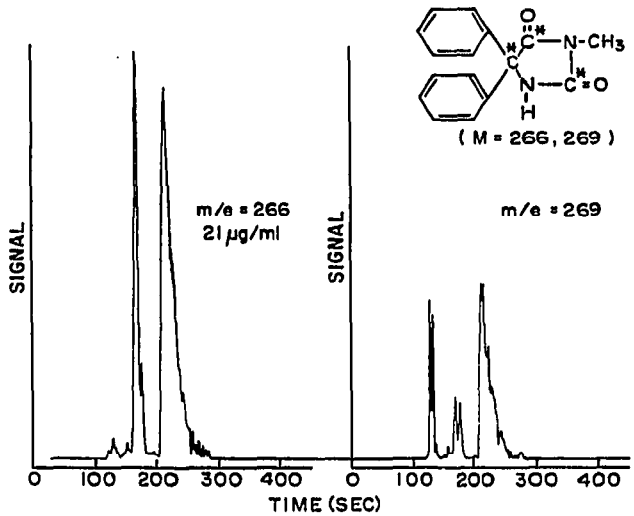
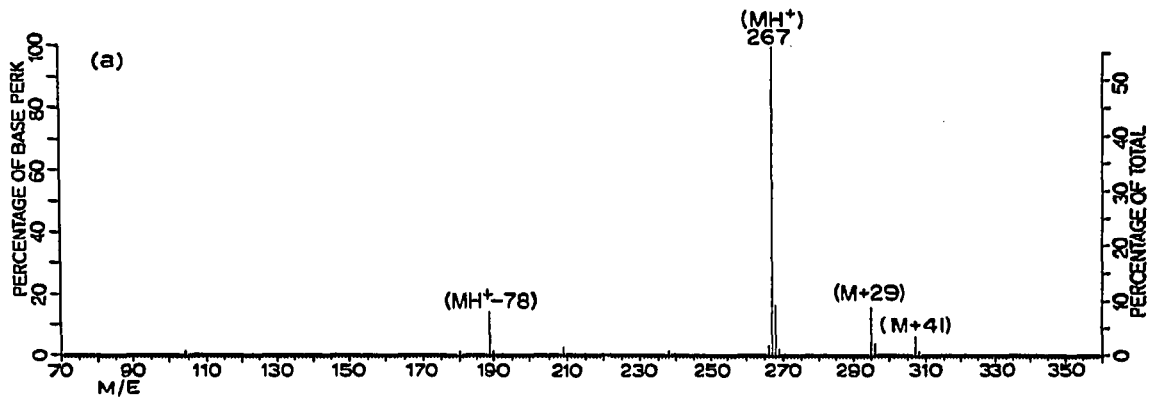


Fig. 2. Analysis of an ammonium carbonate–ethyl acetate extract of 200 μl of plasma by selective ion detection using the LKB 9000-PDP 12 system. The ions at *m/e* 266 (M^+, diphenylhydantoin) and 269 (M^+, [2,4,5-$^{13}C_3$]diphenylhydantoin) were monitored.



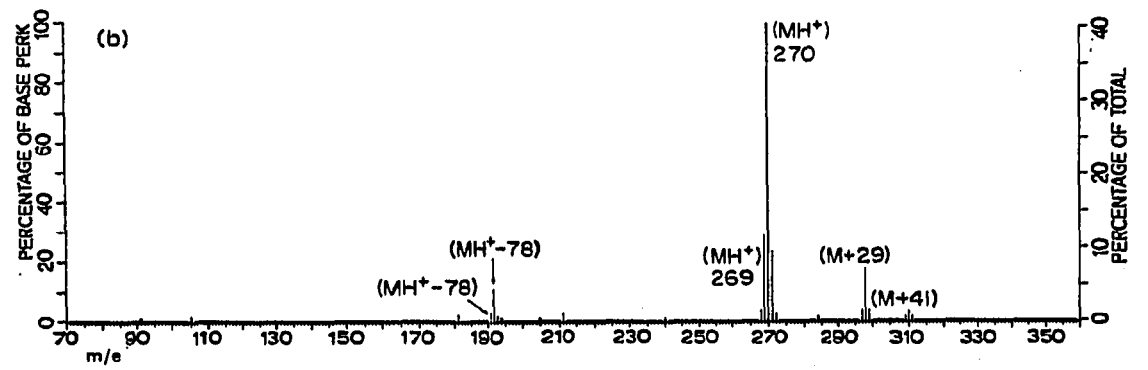


Fig. 3. Chemical ionization spectra (Finnigan 1015-PDP 8/I) of the N-methyl derivative of (a) diphenylhydantoin and (b) [2,4,5-$^{13}C_3$]diphenylhydantoin. The ion at *m/e* 269 is the MH^+ ion of doubly labeled diphenylhydantoin.

of three labeled carbon atoms in the heterocyclic ring. The ratio of the ion at *m/e* 270 to that at *m/e* 269 was 4:1. Suitable corrections were made, therefore, when calculating plasma concentrations since 1 μg of the internal standard contained only 0.8 μg of the triply labeled diphenylhydantoin ($MH^+ = 270$).

An analysis of an ammonium carbonate–ethyl acetate extract of plasma obtained with the Finnigan 1015–PDP 8/I system is shown in Fig. 4. The ratio of the peak heights of the ions at *m/e* 267 and 270 was used to calculate the concentration of diphenylhydantoin in plasma. Since the two compounds, labeled and unlabeled N-methyldiphenylhydantoin, chromatograph exactly together and any losses affect them equally, it is unnecessary to use peak areas or even to construct a calibration curve (although this was in fact done as a precaution, and the ratio of peak heights was found to be the same as the ratio of labeled and unlabeled drugs in reference mixtures).

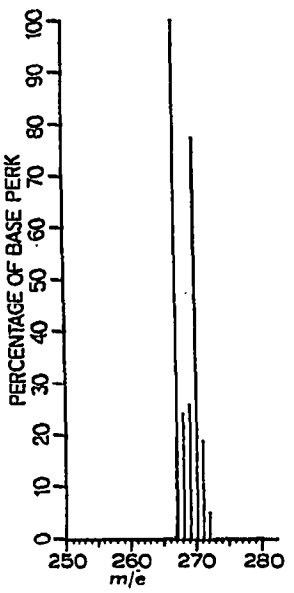


Fig. 4. Analysis of an ammonium carbonate–ethyl acetate extract of 200 μl of plasma by selective ion detection using the Finnigan 1015-PDP 8/I system in the chemical ionization mode. The ions at *m/e* 267 (MH^+, diphenylhydantoin) and 270 (MH^+, [2,4,5-$^{13}C_3$]diphenylhydantoin) were monitored.

The sensitivity of detection of the two systems in their present configuration for barbiturates and anticonvulsant drugs is approximately the same. The sensitivity of the Finnigan system is illustrated in Fig. 5. It is possible to measure as little as 80 pg of phenobarbital with a precision of ± 5% when a reference solution of the drug is employed. When a plasma extract is used, slightly larger samples are needed (150–200 pg). Increasing the voltage on the multiplier increases the sensitivity of detection. This can be seen by comparing the peak areas or peak heights obtained for 400 pg with multiplier settings of 2400 and 2500 V (Fig. 5).

The time required for a single analysis with either system is about 10–15 min.

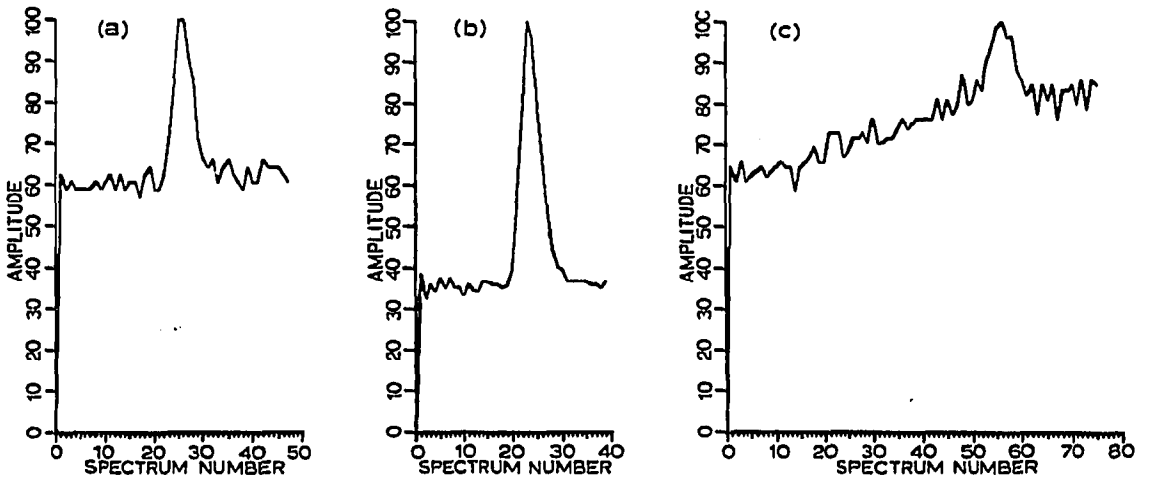


Fig. 5. Response of the Finnigan 1015-PDP 8/I (chemical ionization mode) to 400 pg (a, 2400 V; b, 2500 V) and 80 pg (c, 2700 V) of [2,4,5-$^{13}C_3$]phenobarbital monitoring the ions at *m/e* 263–266.

This includes the time necessary to purge the column before injecting the next sample. Thus, between twenty and forty analyses can be carried out daily.

With the Finnigan system, it is possible to quantify several drugs in a single-programmed gas chromatographic analysis. With the computer program now in use, it is possible to monitor eight different masses. Thus, four drugs and their internal standards could be quantified in one analysis. However, because of the time spent in scanning each mass, better results were obtained when only four or five masses were monitored in a single GC analysis. Fig. 6 shows an analysis of plasma obtained from a young adult female maintained on mephobarbital (N-methyl-5-ethyl-5-phenyl-barbituric acid) in which three masses were monitored. Mephobarbital and its metabolite, phenobarbital, were present in the plasma extract as well as [2,4,5-$^{13}C_3$]pheno-

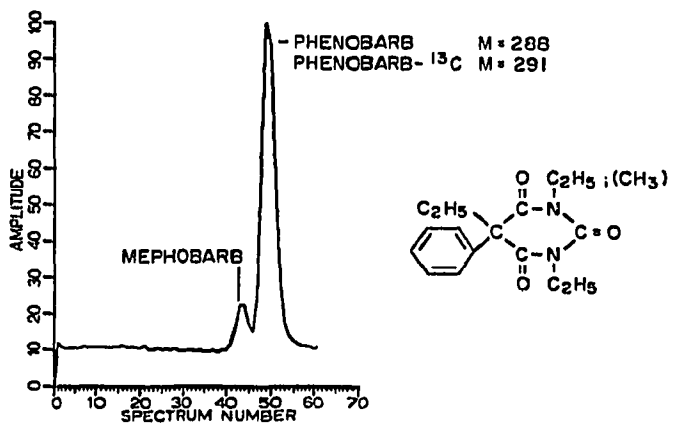


Fig. 6. Analysis of an ammonium carbonate–ethyl acetate extract of 200 μl of plasma by selective ion detection using the Finnigan 1015-PDP 8/I system (chemical ionization mode). The ions at *m/e* 274–277 and 288–294 were monitored.

barbital, which had been added as the internal standard. In order to separate mephobarbital from phenobarbital, the sample was ethylated with diazoethane. (Methylation with diazomethane converts mephobarbital and phenobarbital to the same N,N-dimethyl derivative.) The ions monitored were at *m/e* 274–277 (N-ethylmephobarbital), 288–291 (N,N-diethylphenobarbital) and 291–294 [2,4,5-$^{13}C_3$]N,N-diethylphenobarbital). The concentration of mephobarbital (6 μg/ml) and phenobarbital (38.5 μg/ml) was calculated from the ratio of the peak heights.

Although phenobarbital and [2,4,5-$^{13}C_3$]phenobarbital were present in the same GC peak (Fig. 6) they can be plotted separately by the computer by calling for the appropriate limited mass range (Fig. 7).

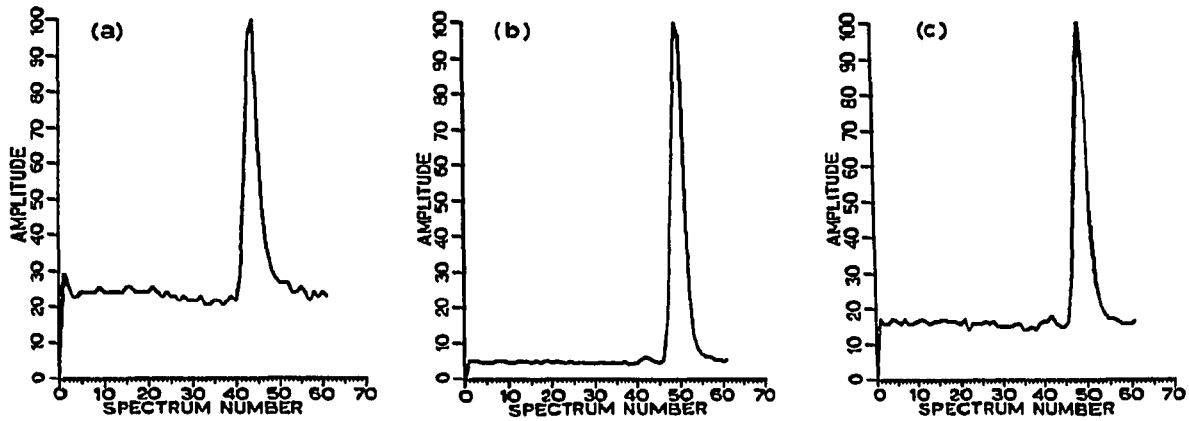


Fig. 7. Limited mass range scans of the sample shown in Fig. 6. (a) Mephobarbital, *m/e* 274–277; (b) phenobarbital, *m/e* 288–291; (c) [2,4,5-$^{13}C_3$]phenobarbital, *m/e* 291–294. Each mass chromatogram has been normalized to an arbitrary height of 100 units.

Nanogram quantities (20–200 ng) of phenobarbital and diphenylhydantoin were injected into LKB or Finnigan systems in these studies. With the API mass spectrometer, however, drugs and drug metabolites can be measured reliably and rapidly in picogram quantities. The time required for a single analysis is on the order of 1 min or less. The limit of detection with the present configuration of the system is in the range of 30–50 femtograms of compound injected into the source.

In Fig. 8, the API negative ion mass spectrum of a plasma extract obtained from a male patient maintained on a daily dose of 300 mg of diphenylhydantoin and 100 mg of phenobarbital is shown. [2,4,5-$^{13}C_3$]Diphenylhydantoin was added to the plasma (100 μl) before extraction by the ammonium carbonate–methylene chloride procedure. The mass range was scanned from 0–750 amu. The only masses detected in addition to those arising from the solvent (Cl^- and $Cl^-(CH_2Cl_2)$) were at 231 (M—1, phenobarbital), 251 (M—1, diphenylhydantoin) and 254 amu (M—1, [2,4,5-$^{13}C_3$]diphenylhydantoin). Fig. 9 shows a plot obtained when another aliquot of the same sample was scanned over a limited mass range (245–260) for quantification. The concentration of the internal standard was equivalent to 24 μg/ml (2.4 μg in 100 μl of plasma) and the concentration of diphenylhydantoin in plasma was 10.4 μg/ml.

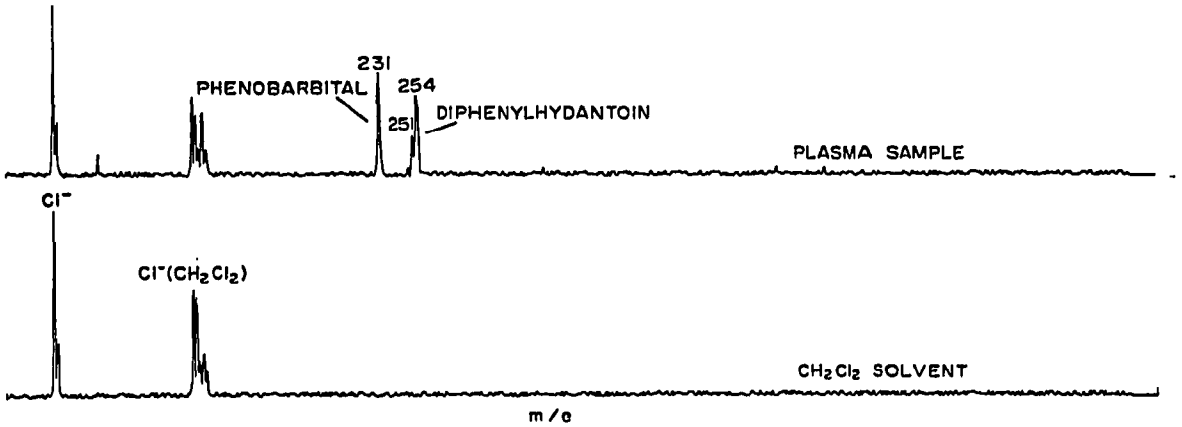


Fig. 8. Negative ion profile observed for a methylene chloride extract of plasma from a patient receiving diphenylhydantoin and phenobarbital. Diphenylhydantoin labeled with ^{13}C was added as an internal standard. The scanned spectrum from 0–750 amu was taken with the API mass spectrometer–computer system.

The peak at *m/e* 254 represents 480 pg of injected standard or the equivalent of 20 nl of plasma. When the scan was carried out over this limited mass range, unit resolution was obtained.

In a preliminary study, a comparison was made of the analytical results obtained with the three systems. It was found that the most precise measurements were made with the API system at the picogram level. The greater precision of the API

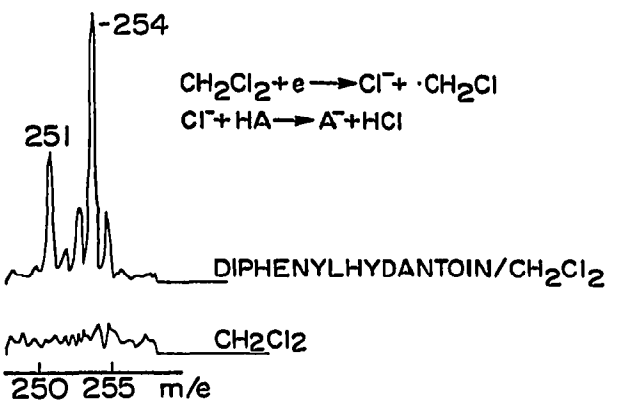


Fig. 9. Narrow range scan of negative ions (245–260 amu) of the plasma extract used in Fig. 8.

system may be due to the pulse counting and signal averaging techniques employed with this system.

Although most of the studies have been concerned with measurements of phenobarbital and diphenylhydantoin in plasma, analyses have been carried out on urine, amniotic fluid and breast milk. The result of a study of drugs in breast milk is summarized in Table I. The analyses were carried out by selective ion detection using

TABLE I

DRUGS IDENTIFIED IN HUMAN BREAST MILK AND COLOSTRUM USING GC–MS–COM PROCEDURES

Drug	*Concentration (μg/ml)*	*Hours after administration*	*Days after delivery*
Phenobarbital	2.74*	16	6
	3.3*	—	3
Pentobarbital	0.17*,**	19	4
Butabarbital	0.37*,**	1.5	4
Secobarbital	+§	24	4
Valium	0.10§§	25	1
Diphenylhydantoin	4.5†	3.5	38
Ethosuximide	+	—	36
Codeine	+	8	2
Methadone	+	1.25	3
	+	1.5	5
Tolbutamide	+	—	25
Caffeine	+	1	††

* [2,4,5-$^{13}C_3$]Phenobarbital added as internal standard.
** [2,4,5-$^{13}C_3$]Pentobarbital also used as internal standard.
§ + denotes drug present but not quantified.
§§ [1-C^2H_3]Valium added as internal standard.
† [2,4,5-$^{13}C_3$]Diphenylhydantoin added as internal standard.
†† Sample collected two months prior to delivery.

the Finnigan system. With this technique it was possible to show that all of the drugs listed were transferred from the maternal circulation to breast milk and as a result were ingested by the neonate. Ethosuximide, codeine, methadone, tolbutamide and caffeine were not quantified because satisfactory internal standards were not available. Thus the study illustrates not only the usefulness of stable isotope-labeled standards but also points out the need for additional reference compounds labeled with either ^{13}C, ^{2}H or ^{15}N.

Although analysis by selective ion detection involves expensive instrumentation, the procedure has several compensating advantages. Very small plasma samples can be employed to quantify drugs present in relatively high concentrations (μg/ml) such as phenobarbital and diphenylhydantoin or larger samples of plasma (100–200 μl) can be used to measure drugs present in very low concentration (pg–ng/ml). Furthermore, identification and quantification are obtained in the same analysis, and several drugs and internal standards can be monitored in a single analysis. Most other analytical procedures which provide high sensitivity, such as radioimmunoassay, measure only a single component in each analysis. Our preliminary experience with the API system indicates two particular advantages of this instrument for drug analysis: (1) the high degree of selectivity of ionization obviates the need for GC introduction and hence permits very rapid analysis; (2) the reaction chamber or ion source, being continuously flushed with hot gas and having no electric fields, will operate for months without becoming contaminated.

In these studies, drugs labeled with ^{13}C have been used only as internal standards for quantification. However, drugs labeled with stable isotopes can also be used

as tracers in studies of drug metabolism in man. The isolation procedures and analytical systems described make it possible to carry out studies of pool size, pharmacokinetics, and drug–drug interaction with very small biological samples.

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