# Application of Quantitative GC-Mass Spectrometry to Study of Pharmacokinetics of Amphetamine and Phentermine

ARTHUR K. CHO▲, BARBARA J. HODSHON, BJÖRN LINDEKE, and GERALD T. MIWA

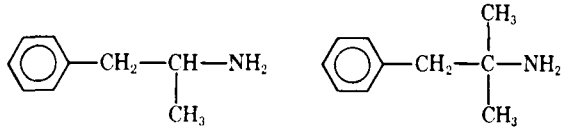
**Abstract** □ Quantitative GC–mass spectrometric assays were used to determine plasma and brain levels of amphetamine and phentermine. Deuterium-substituted variants of the two amines were used as internal standards in the procedures, which were sensitive to 10 pmoles/ml. The levels in rats given doses of 0.5 mg./kg. i.v. were examined in terms of a two-compartment open model. Levels of amphetamine were also examined after bilateral nephrectomy.

**Keyphrases** □ Amphetamine plasma and brain levels—GC-mass spectrometric analysis, rats □ Phentermine plasma and brain levels—GC-mass spectrometric analysis, rats □ GC-mass spectrometry—analysis, amphetamine and phentermine, plasma and brain, rats

The phenylisopropylamines are an interesting class of pharmacological agents because of their similar behavioral effects and their differing actions on the biogenic amines. For example, *p*-chloroamphetamine [1-(4-chlorophenyl)isopropylamine] and amphetamine (1-phenylisopropylamine, I) are qualitatively similar in their ability to cause central stimulation and anorexia. Various related compounds exhibit similar behavioral actions (1). The actions of these compounds on the biogenic amines are more diverse and, while amphetamine reduces steady-state levels of brain norepinephrine (2), *p*-chloroamphetamine reduces levels of 5-hydroxytryptamine (3).

An additional example of the differences observed within this class of compounds is the multiple pathways of metabolism, reflecting both chemical and species differences. In general, there are three initial sites of metabolism: the aromatic ring, the $\alpha$-carbon, and the amino nitrogen. Metabolites resulting from oxidation at each position have been described, and the relative proportions of each metabolite differs with species (4). In the rat, the major metabolite of amphetamine *in vivo* is the *p*-hydroxy compound; in the rabbit, phenylacetone and benzoic acid resulting from deamination are the major metabolites. The role of divergent metabolic pathways in the pharmacology of these compounds has been studied by numerous investigators (2, 5–7).

Phentermine (2-methyl-1-phenylisopropylamine, II) is another structural variant of amphetamine which has similar anorexic action; but like *p*-chloroamphetamine, it has reduced central nervous system (CNS) stimulatory activity (1). The structural differences between phentermine and amphetamine are reflected in their chemical and biochemical differences. The base strength and partition coefficient of phentermine are higher than those for amphetamine, and the $\alpha$-methyl group should prevent the $\alpha$-oxidation–deamination pathway of metabolism (8). These differences could also result in differences in the distribution of the two drugs which, in turn, might account for some pharmacological differences. In the present study, the pharmacokinetics of these two compounds in rats were examined after intravenous administration.



I



II

The compounds were estimated by quantitative GC–mass spectrometry using procedures developed in these laboratories. The procedure for amphetamine was described elsewhere (9), and the procedure for phentermine was developed during these studies using analogous techniques. The assays involve extraction of the amines from the biological fluid, derivatization, and GC, using a mass spectrometer to monitor the effluant. The spectrometer is set to monitor mass fragments that are characteristic for the substance to be analyzed and for an isotopically enriched variant of the substance, which is the internal standard. Quantitation is based on the ratio of spectrometer response to unknown and to internal standard. The data collected were analyzed according to the two-compartment open kinetic model described for plasma decay curves (10).

## EXPERIMENTAL

**Extraction and Sample Preparation**—The amines were extracted from the biological fluids and converted to their *N*-trifluoroacetyl derivatives for analysis by GC–mass spectrometry. Thus, a 2-ml. volume of plasma or a 4-ml. volume of brain extract was charged with 500 pmoles of deuterium-labeled amine as internal standard, alkalinized with 0.5 ml. of 5 *M* NaOH, and extracted with 6 ml. of benzene by shaking for 20 min. on a mechanical shaker. After centrifugation to separate layers, 5 ml. of the benzene layer was transferred to another extraction tube and shaken with 0.6 ml. of 1 *N* HCl. A 500-$\mu$l. aliquot of the aqueous phase was then transferred to another extraction tube containing 1.2 ml. of benzene and 0.2 ml. of 5 *M* NaOH. The resulting mixture was again shaken, 1 ml. of the benzene layer was transferred to a microcentrifuge tube, and 20 $\mu$l. of trifluoroacetic anhydride was added. The acetylation was allowed to proceed overnight at 4° and then the benzene was evaporated under a stream of nitrogen. The overnight acetylation time is not necessary but was carried out for convenience in scheduling the mass spectrometer. The residue from the evaporation step was dissolved in 20 $\mu$l. of acetonitrile for introduction into the GC–mass spectrometry system.

A standard curve was routinely prepared by adding 50–500 pmoles of amine to a series of extraction tubes containing 2 ml. of a 5% solution of plasma proteins[1] and 500 pmoles of internal standard. These tubes were then carried through the extraction and quantitation procedure.

**Analysis**—The GC–mass spectrometry system[2] utilized an ionizing energy set at 70 ev. with the emission current at 250 $\mu$amp. The GC column was a 2-m. × 2-mm. (i.d.) silynized glass column packed with 3% OV-17 on Gas Chrom Q (80–100 mesh), operated at 130°; the injection port and separator were operated at 180 and 185°,

[1] Plasmanate, Cutter Laboratories, Berkeley, Calif.
[2] EAI Quad 300 quadrupole mass spectrometer coupled to a Varian 1400 gas chromatograph with a glass frit separator.

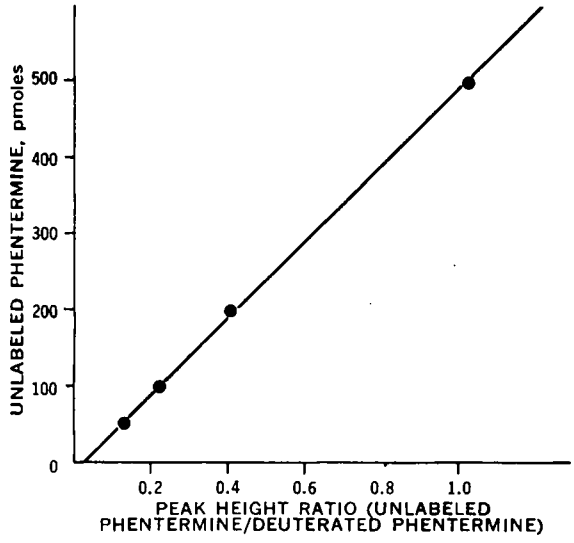



**Figure 1**—*Standard curve for phentermine determination.*

respectively. The mass spectrometer was operated in its specific ion detection mode (11) focused on masses 140 and 143 for amphetamine and 154 and 157 for phentermine. Quantitation was achieved by comparing the mass spectrometer responses at 140 and 143 (internal standard) for amphetamine and 154 and 157 (internal standard) for phentermine. The GC retention times for the trifluoroacetyl derivatives of amphetamine and phentermine were approximately 2.3 and 1.9 min., respectively. The internal standards used in the assays were 1-phenyl[3,3,3-$^2H_3$]isopropylamine (12) and 2-methyl-1-phenyl[3,3,3-$^2H_3$]isopropylamine (13) of 99.9 and 99.8% isotopic purity, respectively.

**Animal Procedures**—Male Sprague–Dawley rats, weighing 250–300 g., were given an intravenous injection of amine salt equivalent to 0.5 mg. free base/kg. at 0.25 ml./100 g. The animals were lightly anesthesized with ether, and blood was collected by cardiac puncture with a heparinized syringe. The brains were then removed and immediately frozen. The whole blood was centrifuged (3000 × *g*), and aliquots of plasma were taken for analysis. The brain was homogenized in four volumes of 0.1 *N* HCl, and the protein was precipitated by the addition of 0.2 ml. of 60% perchloric acid and centrifugation. A 4-ml. aliquot of the supernate was then extracted as described for plasma.

Bilateral nephrectomized animals were prepared by ligation of both renal arteries and veins, followed by excision of both kidneys under ether anesthesia. The animals were allowed to recover overnight before administration of the drug[3].

## RESULTS AND DISCUSSION

A typical standard curve for phentermine is shown in Fig. 1. The standard deviation of the slopes from three curves was 1.3%. The standard deviation for the average slopes of five amphetamine curves was 0.3%. The 95% confidence limits calculated from these standard curves was 2–5 pmoles/ml. sample for amphetamine and 3–8 pmoles/ml. for phentermine.

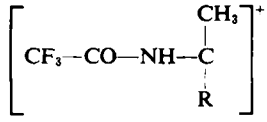
The specificity of the extraction procedure was established for phentermine by focusing the specific ion detector on three major mass peaks of the spectrum of the compound. Thus, the GC peak at 1.9 min. in the extracts from phentermine-treated animals was examined for the relative intensities at *m/e* 154, 132, and 59, major peaks in the spectrum of phentermine. As shown in Table I, the relative abundances of these fragments were identical to those of authentic compounds. A similar experiment was reported previously for amphetamine (9).

In quantitative GC–mass spectrometry, the spectrometer is alternately focused on two mass numbers, one representing a major fragment in the spectrum of the compound to be analyzed and the other representing the corresponding fragment from the internal standard. For these quantitative analyses, the specific ion detector was focused on *m/e* values corresponding to the base peak for each compound. The mass spectra of the trifluoroacetyl derivatives of amphetamine and phentermine have base peaks at *m/e* 140 and 154, corresponding to the fragment (III) that results from cleavage between the α- and

**Table I**—Relative Abundances of Key Fragments in the Mass Spectrum of Phentermine-Trifluoroacetamide in Standard and Extract[a]

| | *m/e* | | |
|---|---|---|---|
| | 154 | 132 | 59 |
| Standard | 1.000 | 0.164 | 0.959 |
| Plasma Extract | 1.000 | 0.169 | 0.959 |
| Brain Extract | 1.000 | 0.163 | 0.947 |

[a] A comparison of the relative abundances of key fragments of phentermine-trifluoroacetamide in extracts of biological fluids from phentermine-treated animals and in a standard solution of phentermine-trifluoroacetamide. The values represent relative ion current at each mass integrated over the appropriate GC peak.




III

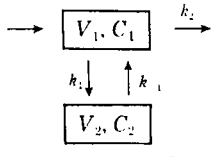
amphetamine: R = H
phentermine: R = $CH_3$

β-carbons (14). The internal standard is labeled with three deuterium atoms at the side-chain methyl group and the corresponding fragments for the labeled variants occur at *m/e* 143 and 157. The use of the base peak is desirable for maximal sensitivity, but it is more important that the peak chosen for detection is relatively isolated in the spectrum (9).

The internal standard serves to quantitate the spectrometer response so that the unknown concentration can be obtained by the ratio of responses. In addition, by its presence at levels of 500 pmoles, it acts as a "carrier" to minimize losses of the compound to be analyzed by absorption into glass columns, *etc.*, during extraction and chromatography. These losses become significant when picogram levels of amines are being handled but are minimized with this technique, since the addition of internal standard is equivalent to operating at nanogram levels where the losses are not significant.

The changes in brain and plasma levels of amphetamine and phentermine with time are plotted in Figs. 2*a* and 2*b*. Brain levels are consistently higher than plasma for both drugs, with the ratio of brain to plasma level gradually decreasing with time. Early (<1 hr.) levels of the two amines were very close, but plasma phentermine declined more rapidly after 3 hr. Results (Table II) of an analysis of the data are based on a two-compartment open model (Scheme I), where $V_{dist}$ is the volume of distribution; $V_1$ and $V_2$ are the volumes of the central and second compartments, respectively; and the *k*'s refer to the rate constants for movements indicated by the arrows. The $t_{1/2}$ values are derived from the β term in the two-exponential equation: $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ (10). The data indicate that the transfers out of the different compartments were comparable but that the apparent volumes were different. The volume of distribution of phentermine was higher than that of amphetamine, suggesting that greater localization occurs with the more lipid-soluble amine.

The decay curves for plasma and brain were not single exponentials and were analyzed according to the two-compartment model discussed by Riegelman *et al.* (10), using a nonlinear regression analysis procedure (Table II). In the case of amphetamine, the differ-




*Scheme I*

[3] The amphetamine sulfate used in these experiments was obtained from Dr. Glenn Ullyot of Smith Kline & French Laboratories, and the phentermine was obtained from Dr. Aldo P. Truant of Pennwalt Corp.

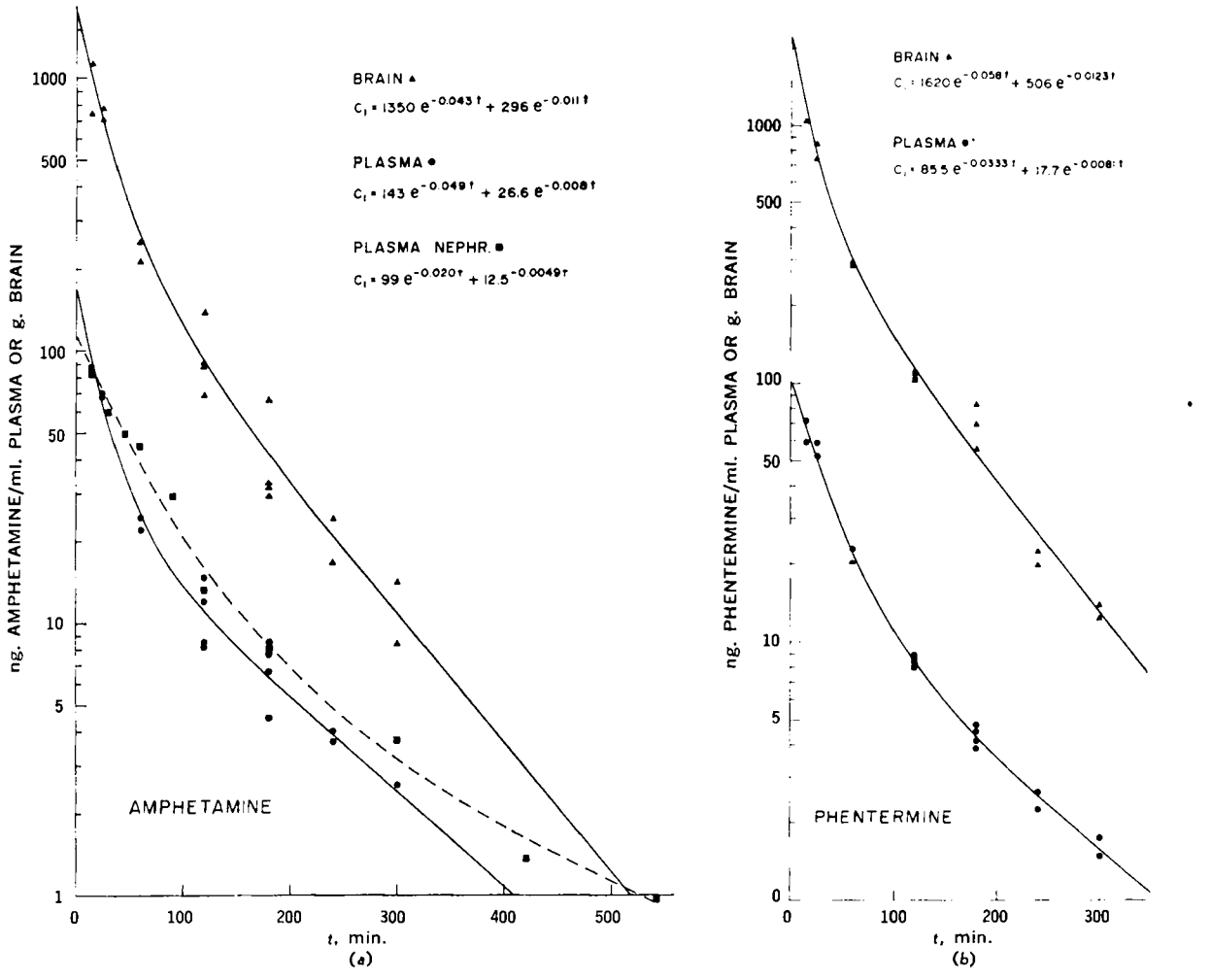



**Figure 2**—*Plasma and brain levels of amphetamine* (a) *and phentermine* (b). *The data are superimposed on curves representing the double-exponential equation shown in the upper right corner of each figure. The dotted lines in* 2a *represent the corresponding curves for data from nephrectomized animals.*

ing volumes ($V_1$ and $V_2$) calculated for the two compartments reflect the differences in tissue concentrations, with the plasma volume about 10-fold greater. The rate constants are comparable for the two tissues, indicating that they are both part of the central compartment. This would be expected from the high perfusion rate of the brain and the ease with which amphetamine would penetrate the blood–brain barrier.

Analysis of the phentermine data gives similar results, with the ratios of the plasma and brain volumes differing by a factor of 20. The values of the rate constants were somewhat different, perhaps reflecting the greater deviation of the 3- and 4-hr. brain levels from the calculated curve representing two-compartment kinetics (Fig. 2*b*).

The plasma volume of distribution calculated for phentermine was larger than that for amphetamine, reflecting the difference in the volume of $V_1$, the central compartment. The difference is consistent with the higher organic–aqueous partition coefficient (1) for phentermine, since highly lipid-soluble compounds tend to be localized and give larger volumes of distribution. Another example of this phenomenon is the larger volume of distribution of thiopental compared to its more polar analog, pentobarbital (10). However, in contrast to the amines, it is the volume of the second compartment that reflects the differing volumes of distribution of these weakly acidic compounds.

The role of excretion in controlling the volume of distribution and half-life of amphetamine was examined in nephrectomized rats. The changes in plasma level are shown in Table III and are compared to the levels from control animals.

Nephrectomy caused a small increase in the plasma half-life as calculated from $\beta$, the disposition rate constant (10). This increase, from 87 to 141 min., reflected differences in plasma levels at later time points (Fig. 2*a*). In contrast, inhibition of metabolism increased brain levels (2) and the brain half-life (15) of amphetamine by a factor of 5. A corresponding increase in the plasma half-life of the drug can be assumed since both brain and plasma belong to the central compartment. These data indicate that metabolism is considerably more important than renal excretion as the route of elimination of amphetamine.

**Table II**—Analysis of Phentermine and Amphetamine Levels[a]

| | —Amphetamine— | | —Phentermine— | |
|---|---|---|---|---|
| | Plasma | Brain | Plasma | Brain |
| $V_{diat}$, l. | 0.81 | 0.084 | 1.34 | 0.065 |
| $V_2$, l. | 0.85 | 0.088 | 0.78 | 0.046 |
| $V_1$, l. | 1.66 | 0.172 | 2.12 | 0.111 |
| $k_1$, min.$^{-1}$ | 0.015 | 0.010 | 0.007 | 0.016 |
| $k_{-1}$, min.$^{-1}$ | 0.016 | 0.017 | 0.012 | 0.023 |
| $k_2$, min.$^{-1}$ | 0.028 | 0.025 | 0.022 | 0.029 |
| $t_{1/2}$, min. | 86.7 | 62.3 | 85.6 | 56.3 |

[a] The plasma and brain levels were fitted to the two-exponential function describing the two-compartment model of Riegelman *et al.* (10). A Wang desk computer programmed for nonlinear regression analysis was used.

Little information is available on the *in vivo* metabolism of phentermine. Vree *et al.* (16) reported that phentermine is not metabolized in man but is excreted unchanged in the urine. In preliminary experiments with rat liver microsome preparations, we found little if any *p*-hydroxylation occurring but observed *N*-hydroxylation in the rabbit (17). However, it is difficult to demonstrate *p*-hydroxylation of amphetamine *in vitro* (18) and a similar situation may exist

Table III—Plasma Levels of Amphetamine and Phentermine

| $t$, min. | Amphetamine[a], ng./ml. | Amphetamine[a] (Nephrectomized), ng./ml. | Phentermine[a], ng./ml. |
|---|---|---|---|
| 15 | 86.5 (1.8)[b] | 83.8 | 65.1 (2.1) |
| 25 | 69.4 (2.1) | 59.5 | 55.7 (6.9) |
| 45 | — — | 49.0 | — — |
| 60 | 23.0 (2.5) | 44.6 | 21.1 (1.9) |
| 90 | — — | 29.4 | — — |
| 120 | 10.8 ± 3.1[c] | 12.0 | 8.5 ± 0.4[c] |
| 180 | 6.8 ± 1.8[c] | 7.8 | 4.4 ± 0.4[c] |
| 240 | 3.8 (0.9) | — | 2.4 (0.4) |
| 300 | 2.5 | 3.62 | 1.6 (0.2) |
| 420 | — — | 1.35 | — — |
| 540 | — — | 0.95 | — — |

[a] All animals were given 0.5-mg./kg. i.v. doses of drug and lightly anesthetized with ether; blood was collected as described in the *Experimental* section. [b] Numbers in parentheses are the range of two values, and the numbers given ± *SD* are the mean of four values. [c] Standard deviation.

with phentermine. Experiments examining this possibility are in progress.

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## ACKNOWLEDGMENTS AND ADDRESSES


Received February 1, 1973, from the *Department of Pharmacology, School of Medicine, University of California, Los Angeles, CA 90024*

Accepted for publication May 14, 1973.

Supported by U. S. Public Health Service Grants MH 20473-01 and GM 02040.

The authors thank R. W. Silverman and J. A. Steinborn for their advice and assistance and Dr. G. Ullyot and Dr. A. P. Truant for the drugs.



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# Novel Method for Bioavailability Assessment


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**Abstract** □ An alternative strategy is proposed for the assessment of bioavailability. It is based on estimates of renal clearance, plasma clearance, and urinary excretion of unchanged drug. The method is totally compatible with pharmacokinetic methods but lends itself to a more flexible sampling schedule and is model independent.



**Keyphrases** □ Bioavailability—determination with flexible, model-independent method based on estimates of renal clearance, plasma concentration, and urinary excretion of unchanged drug □ Plasma concentration and urinary excretion data—used in flexible, model-independent method for assessing bioavailability □ Urinary excretion and plasma concentration data—used in flexible, model-independent method for assessing bioavailability


There are many ways to estimate bioavailability; each one entails a set of assumptions, some of which can be experimentally verified. Oser *et al.* (1) assumed that the same fraction of the absorbed compound from different preparations is excreted unchanged in the urine. Hence, the ratio of the urinary recovery following a test preparation to that following the same dose administered as a solution is taken to be a measure of relative absorption, which they defined as "physiologic availability."

Pharmacokinetic methods of assessing bioavailability from blood plasma concentrations or urinary excretion data were summarized by Wagner (2). In essence, methods based on plasma concentration data are predicated on comparisons of the products of plasma clearance, $\dot{V}_{cl,p}$, and the total area under the plasma concentration curve, $(AUC)_\infty$. If one assumes $\dot{V}_{cl,p}$ to be constant for a given subject from one test dose to another, then the ratio of $(AUC)_\infty$ is a measure of relative absorption. But if there are intrasubject variations in elimination, the assumption of constant $\dot{V}_{cl,p}$ will not hold and an adjustment is indicated. The nature of such an adjustment depends on one's ability to estimate the terminal plasma $t_{1/2}$ in a region free from the influence of continued absorption and on one's conception of the pharmacokinetic model (3, 4). In any event, a proper