ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 1311–1323

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

A capillary GC-MS method for analysis of phenytoin and [$^{13}C_3$]-phenytoin from plasma obtained from pulse dose pharmacokinetic studies[1]

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Received 10 September 1997; received in revised form 21 November 1997; accepted 21 November 1997

Abstract

Stable isotope analogues of phenytoin are useful for pulse dose pharmacokinetic studies in epilepsy patients. A simultaneous assay was developed to quantitate phenytoin (5,5-diphenylhydantoin) and its stable isotope analogue [$^{13}C_3$]-phenytoin (5,5-diphenyl-2,4,5-$^{13}C_3$-hydantoin) from plasma. Quantitation was achieved by GC-MS analysis of liquid/liquid extracted plasma samples, with [$^{2}H_{10}$]-phenytoin (5,5-di(pentadeuterophenyl)-hydantoin) as an internal standard. The total coefficients of variance (C.V.$_t$) were $<7\%$ for phenytoin (2.5–40 μg ml^{-1}) and $<10.3\%$ for [$^{13}C_3$]-phenytoin (0.1–6.0 μg ml^{-1}). The accuracy of the assay varied from 87.8–100.1% (phenytoin, 2.5–40 μg ml^{-1}) and 89.6–116.3% ([$^{13}C_3$]-phenytoin, 0.02–6.0 μg ml^{-1}). The assay was tested under in vivo conditions by administration of a pulse dose of the stable isotope analogue to a single rat dosed to steady-state with fosphenytoin, a phenytoin prodrug. The results of the in vivo experiment demonstrate the usefulness of this assay for future pharmacokinetic studies in special population epilepsy patients.

Keywords: Phenytoin; Stable isotope; Pulse dosing; Gas chromatography-mass spectrometry; Pharmacokinetics

1. Introduction

5,5-Diphenylhydantoin (phenytoin, PHT, Dilantin®, Parke Davis) is a widely used antiepileptic medication whose metabolism has been well studied in the past several decades [1] The pharmacokinetic variability observed from patient to patient is thought to arise from differing rates of formation of the major oxidative phenytoin metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH) [2]. HPPH formation is catalyzed by enzymes in the CYP2C family, which includes CYP2C9, CYP2C18, and CYP2C19 [3]. A major difficulty in studying the pharmacokinetics of phenytoin in persons with epilepsy is that the drug must be maintained at steady state blood levels to protect the patient from recurring seizures. Therefore, studies in which drug administration is ceased and the rate of disappearance of the drug is followed are not feasible in epilepsy patients maintained on phenytoin [4]. One method to over-

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0731-7085/98/$ - see front matter
PII S0731-7085(98)00018-1

come this obstacle is to administer a part of the daily phenytoin dose as phenytoin labeled with a stable isotope (pulse dosing) [5].

Stable isotopes are extensively used in life sciences in areas of research such as drug metabolism, determination of drug bioavailability, and pharmacokinetic studies [6,7]. Stable isotopes provide a safe means of studying the kinetics of a drug at steady state, as their use enables the researcher to follow the levels of the pulse dose separately from the unlabelled drug [6]. In 1980, Browne et al. first reported a GC-MS method for the quantitation of stable isotope labeled phenytoin analogues [4,8]. This method accurately measured both phenytoin and HPPH, and their stable isotope analogues [5,5-diphenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin and 5-(4-hydroxyphenyl)-5-phenyl-2-^{13}C-1,3-$^{15}N_2$-hydantoin] in both urine and plasma after an intravenous dose given to humans [4]. Subsequent studies demonstrated that in epilepsy patients chronically treated with phenytoin, certain pharmacokinetic parameters such as half-life and clearance, had changed with chronic therapy, as compared to single dose studies in untreated patients [9]. Browne et al. also determined the lack of phenobarbital interference with phenytoin steady-state concentration and other pharmacokinetic parameters by the tracer-dose method of phenytoin administration in epilepsy patients [10].

Other uses have been demonstrated for stable isotope-labeled phenytoin in clinical studies, e.g. determination of bioavailability. Absolute bioavailability of an oral dose of phenytoin was measured in humans by co-administration of an intravenous dose of the stable isotope analogue, [$^2H_{10}$]-phenytoin [11]. This method avoided the disadvantage of a necessary washout time period between oral and i.v. doses. A method for the determination of phenytoin mass balance in humans with stable isotope analogues was demonstrated by Browne et al., which eliminated the need for the use of potentially dangerous radioisotopes [12].

In order to study the single-dose kinetics of phenytoin in elderly persons with epilepsy who are at steady state, an analytical method for analysis of phenytoin and several stable isotope analogues was needed. To validate the technique, preliminary in vivo experiments were performed in the rat and thus a method capable of accurate analysis of small plasma volumes was necessary. Most previously published methods for GC-MS analysis of phenytoin and stable-isotope analogues have utilized packed-glass columns. We have developed a method to monitor the concentrations of both phenytoin and its stable isotope analogue in plasma by capillary-column gas chromatography/mass spectrometry (GC-MS). For the stable isotope of phenytoin, 5,5-diphenyl-2,4,5-$^{13}C_3$-hydantoin ([$^{13}C_3$]-PHT) was selected. Since the stable isotope is placed in the hydantoin ring the possibility of an isotope effect altering the rate of phenytoin hydroxylation is avoided [2]. For the internal standard, 5,5-di(pentadeuterophenyl)-hydantoin ([$^2H_{10}$]-PHT) was used. Our capillary column GC-MS assay was tested in a rat by infusion of unlabeled fosphenytoin (a water soluble pro-drug of phenytoin) to steady-state, followed by i.v. administration of a single pulse dose of the stable isotope of phenytoin ([$^{13}C_3$]-PHT).

2. Materials and methods

2.1. Chemicals

5,5-Diphenyl-2,4,5-$^{13}C_3$-hydantoin ([$^{13}C_3$]-PHT) was purchased from CDN Isotopes, Quebec Canada, and 5,5-di(pentadeuterophenyl)-hydantoin ([$^2H_{10}$]-PHT) was purchased from Cambridge Isotopes, Andover, MA. Methanolic stock solutions of the stable isotopes were prepared at 1.0 mg ml^{-1}. Standard solutions of [$^{13}C_3$]-PHT were prepared by serial dilution to concentrations of 150, 50, 12.5, 5, 2.5 and 0.5 μg ml^{-1}. [$^2H_{10}$]-PHT was diluted to 100 μg ml^{-1} for use as the internal standard. 5,5-Diphenylhydantoin (PHT) was purchased from Sigma (St. Louis, MO). A 1 mg ml^{-1} methanolic stock solution was prepared from PHT. This PHT stock solution was serially diluted with methanol to calibration standard solutions of 1000, 500, 375, 125, 62.5 and 12.5 μg ml^{-1}. Trimethylphenylammonium hydroxide (TMAH) was purchased from Supelco, (Bellefonte, PA) as a 0.2 M methanolic solution. Fos-

phenytoin was a generous gift of Dr Ronald Sawchuck. All other chemicals were of reagent grade.

2.2. GC/MS analysis

The GC/MS system was comprised of a HP5890 series II gas chromatograph connected to a HP5971 mass selective detector. The system was controlled by Chemstation software supplied by Hewlett Packard. The GC column was a DB-5MS 30 m × 0.25 mm i.d. capillary of 0.5 micron film thickness (J&W Scientific, Folsom, CA). The temperature program was set at 270°C for 1 min and then raised to 300°C at 10°C min^{-1}. The final temperature of 300°C was held for 1 min. The carrier gas was 99.95% high purity helium with a head pressure of 1.5 kg cm^{-2} and a split ratio of 30:1. Electron current was set to 2000 emV. The transfer line temperature was 300°C and the injection port temperature was set to 290°C. Two mirolitres of the sample was injected onto the column. Typical retention times were: 3.43 min ($[^2H_{10}]$PHT), 3.44 min ($[^{13}C_3]$PHT) and 3.45 min (PHT). Ions monitored were: m/z 194 (PHT), m/z 206 ($[^{13}C_3]$PHT) and m/z 290 ($[^2H_{10}]$PHT).

2.3. Extraction of plasma samples

Blood samples (0.5 ml) were drawn at each time point from the experimental rat. From this a volume of 100–200 μl of plasma was procured by centrifugation of the heparinized blood at 2000 g for 5 min. To maintain consistency, 100 μl of rat plasma were extracted. Plasma standards were prepared from a human plasma sample (donated from a phenytoin negative volunteer). Standard curve samples were processed after addition of 20 μl of the appropriate calibration standard (PHT and $[^{13}C_3]$PHT). Internal standard solution (20 μl of 100 μg ml^{-1} $[^2H_{10}]$-PHT) was added to all samples and standards, except blanks. Samples and standards were vortexed initially with 1 ml of hexane, and the aqueous layers frozen on a dry ice-acetone bath. The hexane layers were discarded, removing lipophilic impurities, and the aqueous layers allowed to thaw. Once the aqueous layers had thawed they were extracted twice with 2 ml of ethyl acetate. The ethyl acetate layers were combined and evaporated to dryness under nitrogen at 40°C in a nitrogen evaporating apparatus (N-EVAP®, Organomation Associates, South Berlin, MA).

2.4. Derivatization of phenytoin and stable isotope derivatives

Each sample and standard extract was reconstituted with 100 μl of TMAH. This procedure led to permethylated derivatives of phenytoin and the stable isotope derivatives of phenytoin upon injection into the heated GC insert [8]. Each reconstituted sample or standard was transferred into borosilicate glass inserts contained in crimp-topped injection vials (Hewlett Packard) for use in the HP7673 automated injector.

2.5. Animals

A male, white Sprague-Dawley rat weighing 299 g was anesthetized with 12.5 mg of pentobarbital and cannuli were inserted into the left femoral artery, left ileac vein, and the right femoral artery. Twenty-four hours after surgery, an infusion of fosphenytoin (75 mg ml^{-1}) was begun at a rate of 0.04 ml h^{-1} (3 mg h^{-1} of fosphenytoin, equivalent to 2 mg h^{-1} of phenytoin) into the left ileac vein. After 12 h a bolus dose of $[^{13}C_3]$PHT (5 mg kg^{-1}) was given over several minutes. The stable-isotope was formulated for intra arterial infusion in propylene glycol: ethanol: distilled water (4:1:5) with the pH adjusted to between 10 and 11 with 1 M NaOH. Serial blood samples (0.5 ml) were drawn from the right femoral artery at 5, 15, 30, 60, and 120 min. after administration of the bolus dose. Samples were collected in heparinized Vacutainers®, centrifuged, and the plasma collected. The plasma samples were analyzed along with a set of standards prepared from blank human plasma as described above.

2.6. Assay validation

Peak-area ratios (PHT/internal standard and $[^{13}C_3]$PHT/internal standard) were used to calcu-

late PHT and [$^{13}C_3$]PHT concentrations from extracted plasma standards and samples. Standard curves were prepared at the following concentrations: PHT 0, 0.5, 2.5, 5, 15, 20 and 40 μg ml^{-1} and [$^{13}C_3$]PHT 0, 0.02, 0.1, 0.2, 0.5, 2 and 6 μg ml^{-1}. Standards were prepared in triplicate at each concentration.

The estimated total variability of the plasma assay for a single sample on different days was determined by one-way analysis of variance (ANOVA) from calculated concentrations of multiple triplicate weighted (inverse square of x) standard curves [13]. The within-day (S^2_{wd}) and between-day (S^2_b) components of variance were determined by the following equations:

$$S^2_{wd} = [MS_{wd}]$$

$$S^2_b = [MS_b - MS_{wd}]$$

where MS_{wd} and MS_b are the within-day and between-day mean squares from the ANOVA table, respectively. The total variance (S_t) of a given observation was determined by calculating the square root of the sum of within-day (S_{wd}) and between-day (S_b) variance. The percentage coefficient of variation (C.V.) was calculated by dividing the S_t, S_{wd}, and S_b by the grand mean of the calculated concentration values multiplied by 100.

3. Results

3.1. Gas chromatographic mass spectrometry

A mass spectrometric scan of phenytoin and the individual stable isotope analogues was performed to determine the optimal ions to use for selected ion monitoring in this assay. Figs. 1–3 show fragmentation patterns for PHT, [$^{13}C_3$]-PHT, and [$^2H_{10}$]-PHT, respectively. Phenytoin gave the expected permethylated parent ion at m/z 280, with major fragment ions of m/z 203, 194, 165 and 118. [$^{13}C_3$]-PHT gave the expected permethylated parent ion m/z 283, with major fragment ions of m/z 206, 166 and 119. [$^2H_{10}$]-PHT gave the expected permethylated parent ion m/z 290, with major fragment ions of m/z 208, 203 and 123. We chose to monitor [$^2H_{10}$]-PHT at m/z 290, as this is the highest possible mass that could arise from the three compounds. For phenytoin and its stable isotope analogue we chose ions several mass units away from ion fragments arising from the other analogues to avoid interference and maintain isotopic selectivity. Phenytoin was monitored at m/z 194, while [$^{13}C_3$]-PHT was monitored at m/z 206. At m/z 206, a small contribution to the total peak area arose from the fragmentation of incompletely deuterated internal standard, [$^2H_{10}$]-PHT. The percent ratio of m/z 206 to m/z 290 was determined by injection of triplicate plasma extracts containing only [$^2H_{10}$]-PHT prior to each run. The contributed area from the [$^2H_{10}$]-PHT varied from 6.1–6.7% of the total m/z 290 area, with a average of 6.42 ± 0.23% for the five validation curves. The m/z 206 area due to [$^{13}C_3$]-PHT monitored for during the assay was then corrected by subtraction of the contributed area from the [$^2H_{10}$]-PHT. Under the described chromatographic conditions typical retention times were: 3.43 min ([$^2H_{10}$]-PHT), 3.44 min ([$^{13}C_3$]-PHT) and 3.45 min (PHT), respectively. Fig. 4 shows the structures of the three analytes, while Figs. 1 and 3 show the proposed ion fragments monitored. Figs. 5 and 6 show a typical extracted-ion chromatogram from a plasma standard and a plasma sample, respectively.

3.2. Assay validation

Five standard curves performed in triplicate on different days were used to validate this assay. The analytical method for the quantitation of PHT and [$^{13}C_3$]-PHT was both accurate and precise. Tables 1 and 2 show accuracy, within-day, between-day, and total C.V. for PHT and [$^{13}C_3$]-PHT. All of the C.V. values were equal to or less than 10%, except for the lowest concentration for both PHT and [$^{13}C_3$]-PHT (0.5 and 0.02 μg ml^{-1}, respectively). The accuracy for PHT quantitation in the range of 2.5–40 μg ml^{-1} varied from 87.8 to 100.1%. The accuracy for [$^{13}C_3$]-PHT quantitation in the range of 0.02–6 μg ml^{-1} varied from 89.6 to 116.3%. Table 3 shows the extraction recovery of PHT and [$^{13}C_3$]-PHT from human and rat plasma, and rat plasma subjected to three

Table 1
Total variability and the between-day and within-day variation for PHT in rat plasma

Concentration of PHT (μg ml^{-1})	n	Mean measured concentration (μg ml^{-1})	Accuracy (%)	S_{wd}[a] (μg ml^{-1})	C.V.$_{wd}$[b] (%)	S_b[a] (μg ml^{-1})	C.V.$_{wd}$[b] (%)	S_t[a] (μg ml^{-1})	C.V.$_t$[b] (%)
40	15	40.030	100.1	1.634	4.1	NAc	NAc	1.385	3.5
20	15	20.227	101.1	1.239	6.1	0.3079	1.5	1.277	6.3
15	15	14.795	98.6	0.5847	4.0	0.5803	3.9	0.8238	5.6
5	15	4.388	87.8	0.1231	2.8	0.1225	2.8	0.1735	4.0
2.5	15	2.355	94.2	0.0808	3.4	0.1612	6.8	0.1804	7.7
0.5	15	0.841	168.3	0.0326	3.9	0.1497	17.7	0.1530	18.2

[a] Standard deviation: S_{wd} = within-day; S_b = between-day; S_t = total.
[b] Coefficient of variation: C.V.$_{wd}$ = within-day; C.V.$_b$ = between-day; C.V.$_t$ = total.

Table 2
Total variability and the between day and within day variation for [$^{13}C_3$]-PHT in rat plasma

Concentration of [$^{13}C_3$]-PHT (μg ml^{-1})	n	Mean measured concentration (μg ml^{-1})	Accuracy (%)	S_{wd}[a] (μg ml^{-1})	C.V.$_{wd}$[b] (%)	S_b[a] (μg ml^{-1})	C.V.$_b$[b] (%)	S_t[a] (μg ml^{-1})	C.V.$_t$[b] (%)
6.0	15	5.379	89.6	0.2865	5.3	Na[c]	NA[c]	0.2688	5.0
2.0	15	1.905	95.2	0.1233	6.5	0.0787	4.1	0.1462	7.7
0.5	15	0.501	100.2	0.0249	5.0	0.0000	0.0	0.0256	5.1
0.2	15	0.204	101.9	0.0074	4.4	0.0000	0.0	0.0090	4.4
0.1	15	0.116	116.3	0.0119	10.3	0.0000	0.0	0.0116	10.0
0.02	15	0.019	96.3	0.0050	26.3	0.0000	0.0	0.0041	21.6

[a] Standard deviation: S_{wd} = within-day; S_b = between-day; S_t = total.
[b] Coefficient of variation: C.V.$_{wd}$ = within-day; C.V.$_b$ = between-day; C.V.$_t$ = total.

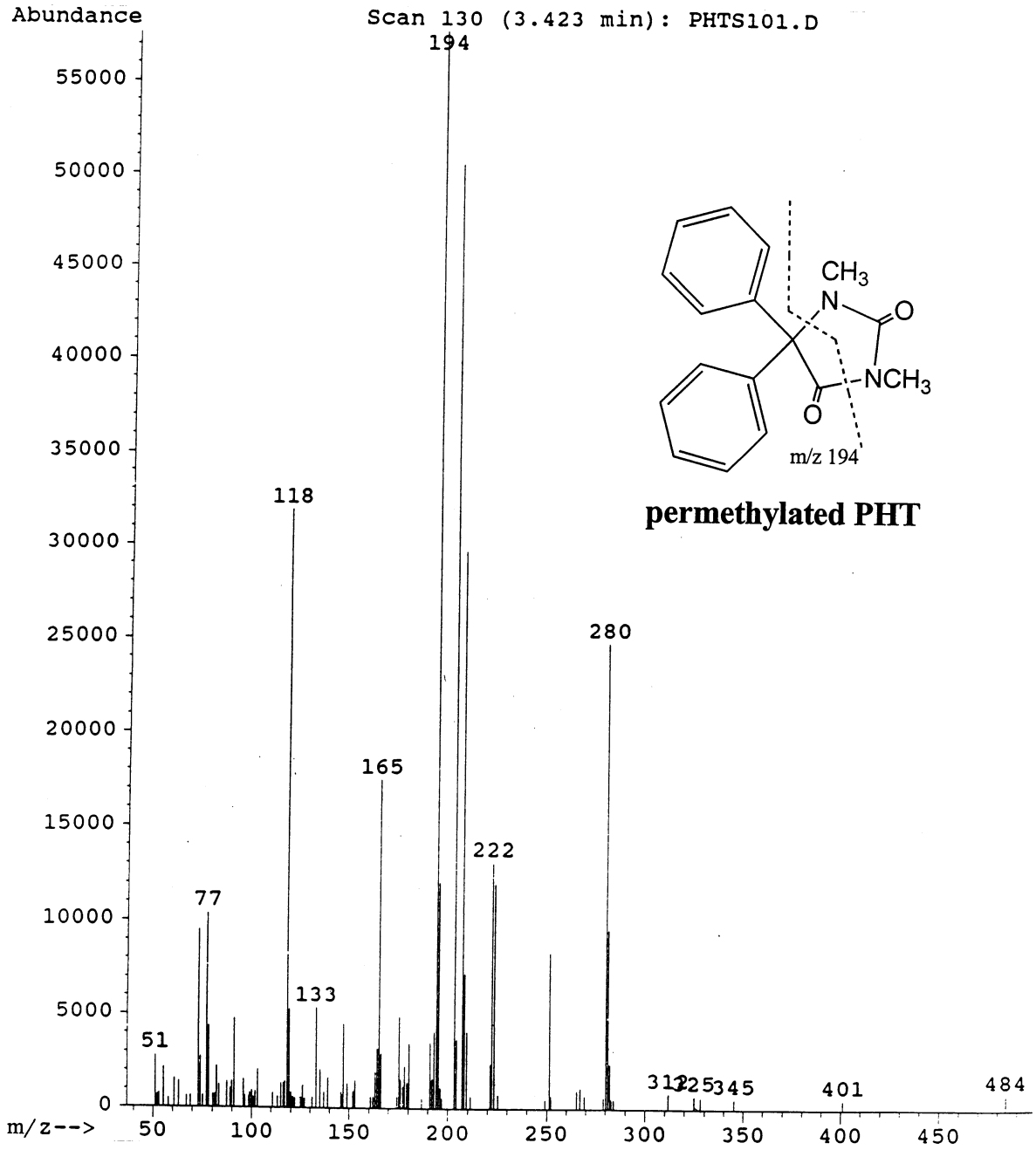


Fig. 1. MS fragmentation of an unextracted solution of PHT in methanol.

cycles of freeze-thawing. There was no significant difference in extraction recovery when human or rat plasma was used, or when rat plasma was frozen and thawed several times.

3.3. Pulse [$^{13}C_3$]-PHT dose in a rat at steady-state with phenytoin

To determine if our assay would measure both

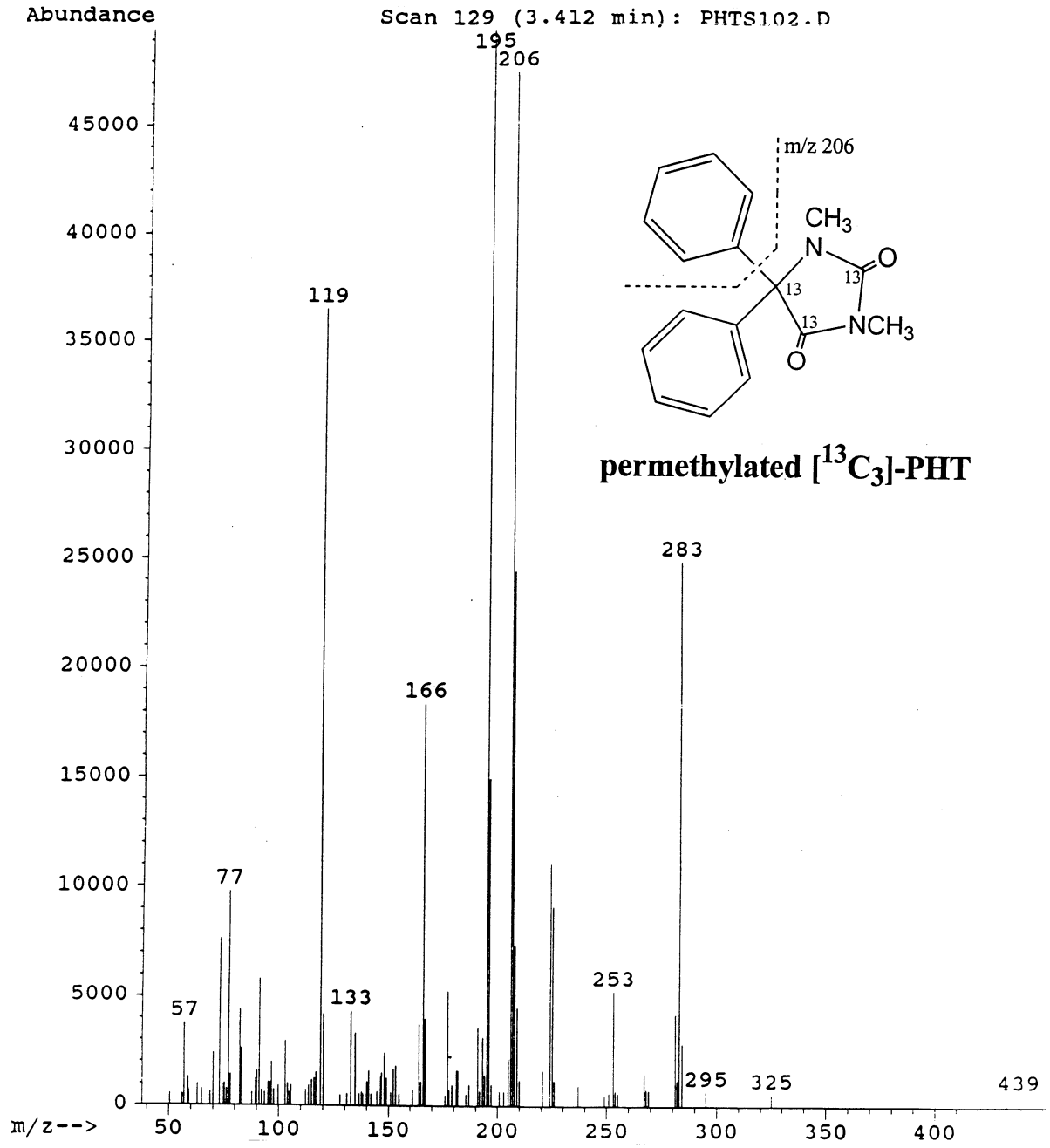


Fig. 2. MS fragmentation of an unextracted solution of [$^{13}C_3$]-PHT in methanol.

PHT and [$^{13}C_3$]-PHT plasma levels under in vivo conditions, a male Sprague–Dawley rat was dosed to steady-state with fosphenytoin (equivalent to 2 mg PHT h^{-1}) and then given a bolus dose of [$^{13}C_3$]-PHT (5 mg kg^{-1}) 12 h after the PHT infusion had started. Plasma samples were collected over 6 h and analyzed by the GC-MS method. The plasma-concentration profile of PHT and [$^{13}C_3$]-PHT

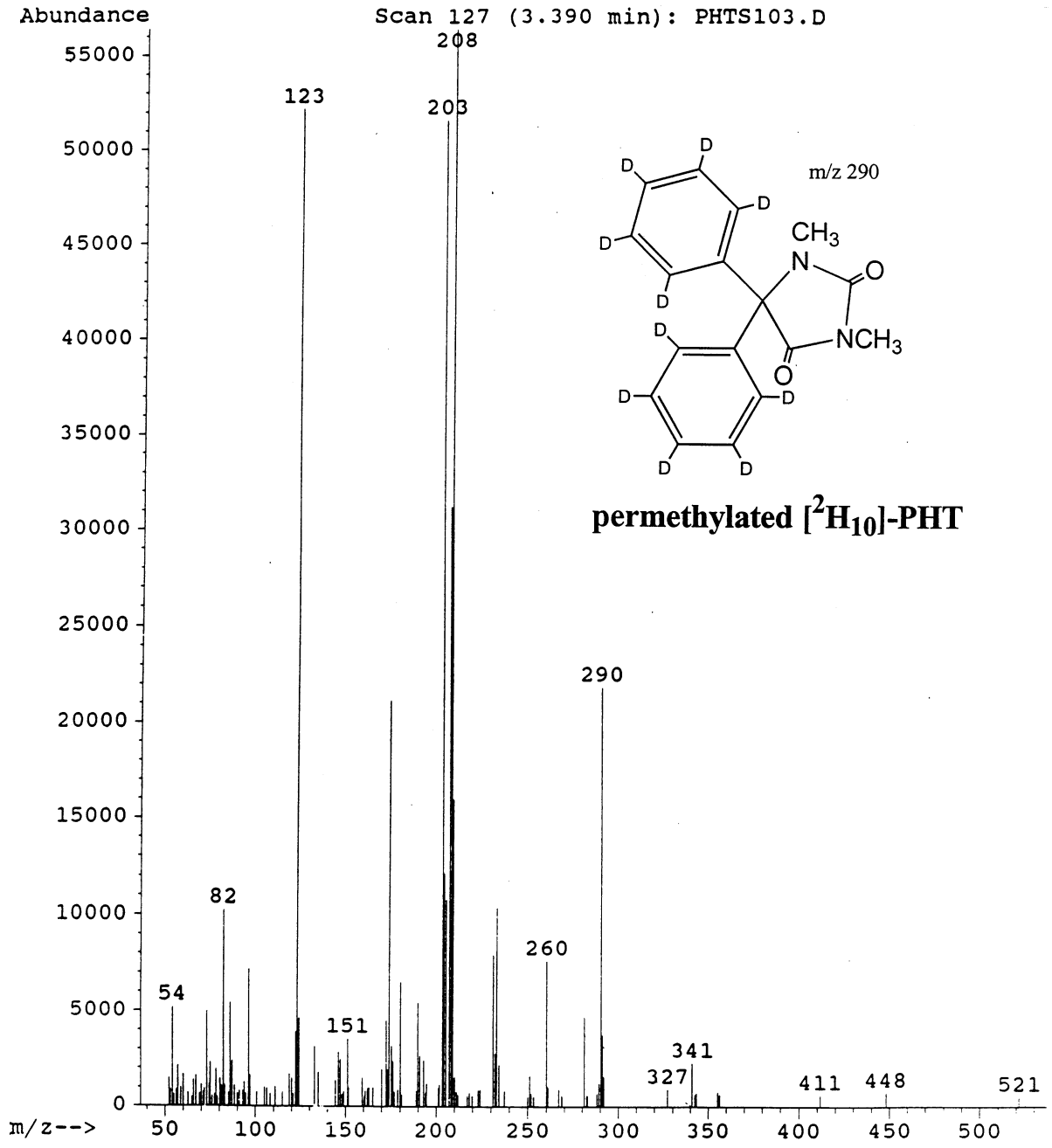


Fig. 3. MS fragmentation of an unextracted solution of $[^2H_{10}]$-PHT in methanol.

over 6 h is shown in Fig. 7. PHT plasma levels remained constant (near 20 μg ml^{-1}) for 4 h, after which an apparent increase in plasma concentration to 25.4 μg ml^{-1}. $[^{13}C_3]$-PHT plasma levels peaked at 7.1 μg ml^{-1} (5 min after the pulse dose) and decreased in an exponental manner over time.

4. Discussion

This methodology will be used to determine the effect of aging on the pharmacokinetics of phenytoin in elderly epilepsy patients. In addition, we intend to examine drug–drug interactions between phenytoin and antidepressants that are commonly used in the elderly.

Many examples of PHT analysis by GC-MS with glass-packed column GC columns are found in the literature [3,7,9,10,14]. Bailey and colleagues have published a capillary column assay for the simultaneous determination of plasma PHT and [$^2H_5$]-PHT levels with GC-NPD and GC-MS analysis [15]. This paper describes a method for the simultaneous quantitation of phenytoin and a stable isotope analogue on a capillary column. Capillary columns are inherently more stable, provide much better resolution, and may result in a more sensitive analysis. They are particularly well suited to GC-MS analyses because the column output can be placed directly into the MS source.

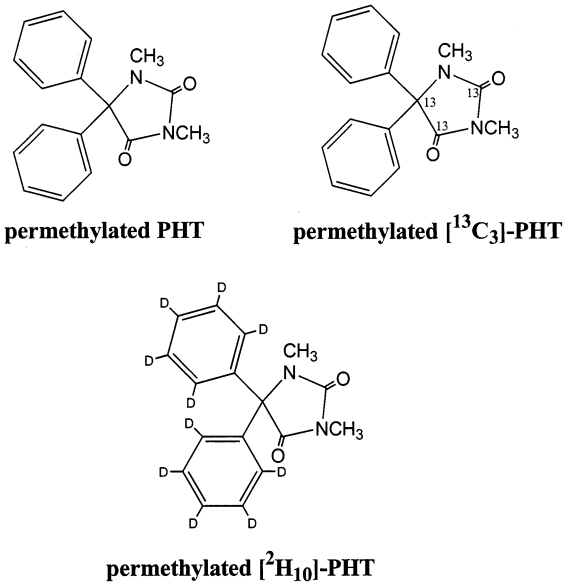


Fig. 4. Structures of dimethylated analytes PHT, [^{13}CD3]-PHT and [$^2H_{10}$]-PHT.

High sensitivity is necessary for the analysis of plasma samples obtained during pulse-dose studies, as the stable isotope is present at a fraction of the parent drug's concentration. The GC-MS assay presented here is sensitive to levels in rat plasma (0.5 μg ml^{-1} (PHT) and 0.1 μg ml^{-1} ([$^{13}C_3$]-PHT)) that are comparable to the sensitivity of previously published GC assays for phenytoin. Browne and colleagues were able to quantitate phenytoin, [$^{13}C_2$-1,3-$^{15}N_2$]-PHT, HPPH and [$^{13}C_2$-1,3-$^{15}N_2$]-HPPH at human plasma levels down to 0.1 μg ml^{-1} with a glass-packed column GC-MS assay [4,8]. With a similar assay, Dickinson and colleagues showed sensitivity in human plasma at 0.5 μg ml^{-1} for phenytoin and 0.1 μg ml^{-1} for [^{13}C-1,3-$^{15}N_2$]-PHT [14]. Bailey et al. developed a capillary GC-NPD assay sensitive to 0.5 μg ml^{-1} for phenytoin quantitation in human plasma and a capillary GC-MS assay sensitive to 0.5 μg ml^{-1} for [$^2H_5$]-PHT in human plasma [15].

The pulse dose study of the stable isotope in the rat demonstrates the usefulness of this assay in studying single-dose kinetics in humans. Once the experimental rat was dosed to steady-state with PHT, the stable isotope analogue was given and its levels were followed for several half-lives. The plasma PHT levels increased after several h. of steady-state levels. The phenytoin plasma concentration in the rat may have reached the K_m, and addition of the stable isotope analogue resulted in saturation of the enzyme(s) responsible for pheny-

Table 3
Extraction recovery of PHT and [$^{13}C_3$]-PHT from human and rat plasma

Plasma Source	PHT [a]	PHT [b]	[$^{13}C_3$]-PHT [c]
Human	61.3 ± 11.2[d]	63.4 ± 8.5[d]	54.6 ± 5.1[d]
Rat	68.1 ± 12.0[d]	60.3 ± 10.3[d]	65.0 ± 19.2[d]
Rat (Freeze-Thawed)	65.6 ± 8.5[d]	65.9 ± 7.8[d]	59.9 ± 13.5[d]

[a] 20 μg ml^{-1} standard.
[b] 2.5 μg ml^{-1} standard.
[c] 2 μg ml^{-1} standard.
[d] Recovery (mean ± SD, $n = 3$) (%).

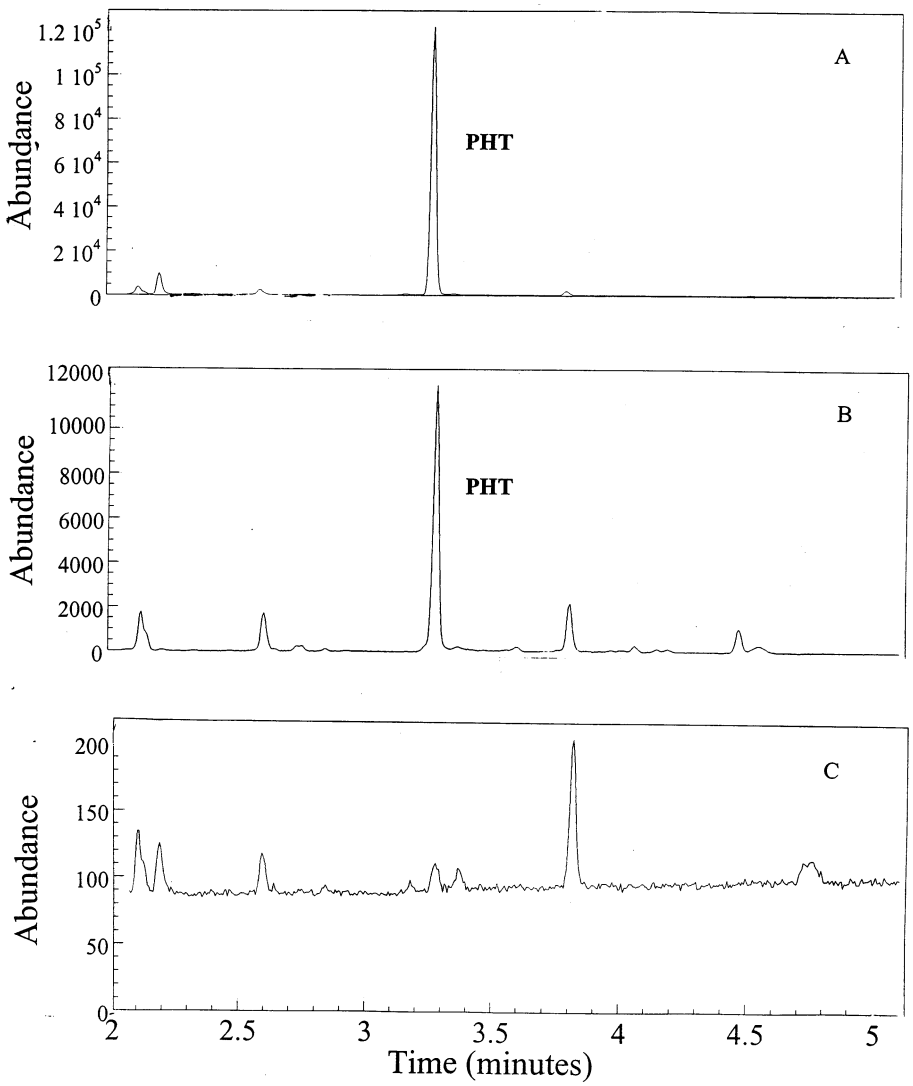


Fig. 5. Extracted-ion chromatogram for PHT ($m/z = 194$). (A) Extracted standard, (B) plasma sample, (C) blank plasma extract.

toin metabolism, leading to a decrease in clearance of the unlabelled phenytoin. The estimated K_m for PHT disappearance in rats has been reported to be 9–15 μg ml^{-1}, a value similar to the levels achieved in this animal [16]. Alternatively, this might be attributed to a loss of blood volume over time after repeated samples were drawn from the animal or to a loss of physiological function with time.

A major obstacle in determining single-dose kinetics of antiepileptic medications in epileptic patients is that often the patient cannot be withdrawn from therapy because of an increased seizure risk. Consequently, standard wash-out designs cannot be employed. In order to study single-dose kinetics of phenytoin in patients chronically treated with phenytoin, we have developed a GC-MS assay for the quantitation of both phenytoin and a stable-isotope analogue. The validation of this assay will allow determination of

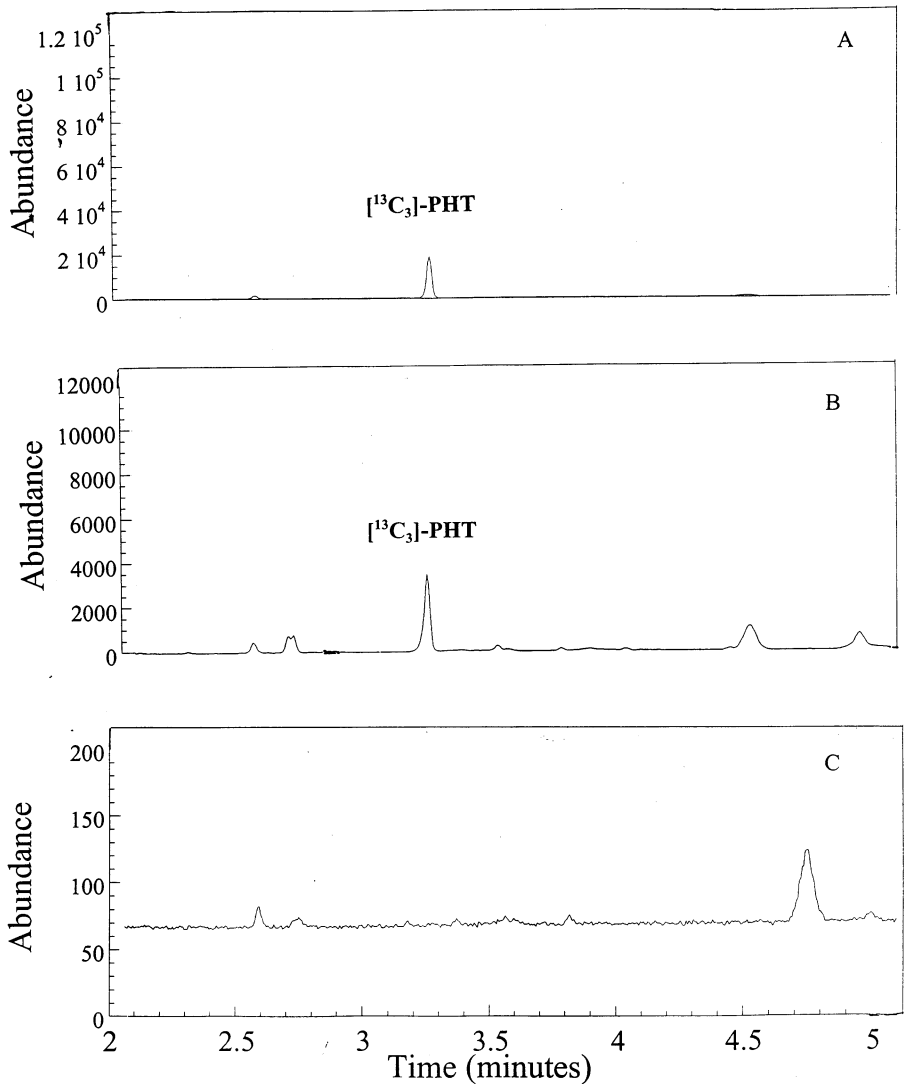


Fig. 6. Extracted-ion chromatogram for [$^{13}C_3$]-PHT ($m/z = 206$). (A) Extracted standard, (B) plasma sample, (C) blank plasma extract.

the single-dose kinetics of a stable isotope of phenytoin ([$^{13}C_3$]PHT) in elderly patients who are also at steady-state.

In conclusion, this work presents a precise, accurate capillary column GC-MS method for the simultaneous analysis of phenytoin and [$^{13}C_3$]-phenytoin from a small plasma sample. This method will be useful for future pharmacokinetic studies in elderly epilepsy patients.

Acknowledgements

We wish to thank Dr Richard Brundage for his assistance in performing surgical procedures on rats and Dr James C. Cloyd for his intellectual input on this project. We also wish to thank Dr Ron Sawchuk and Parke Davis for their generous gifts of fosphenytoin. This work was supported by P50-NS16308 (MHN, AKB and RPR) and a Melendy summer research fellowship (PJN).

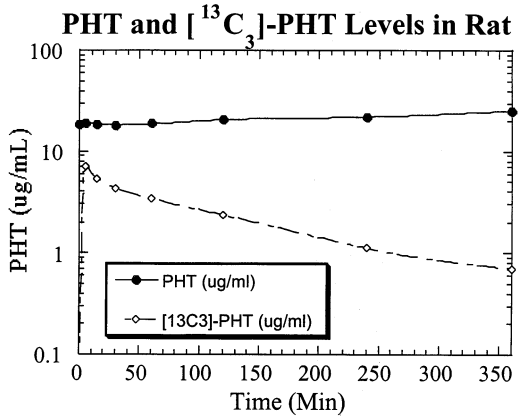
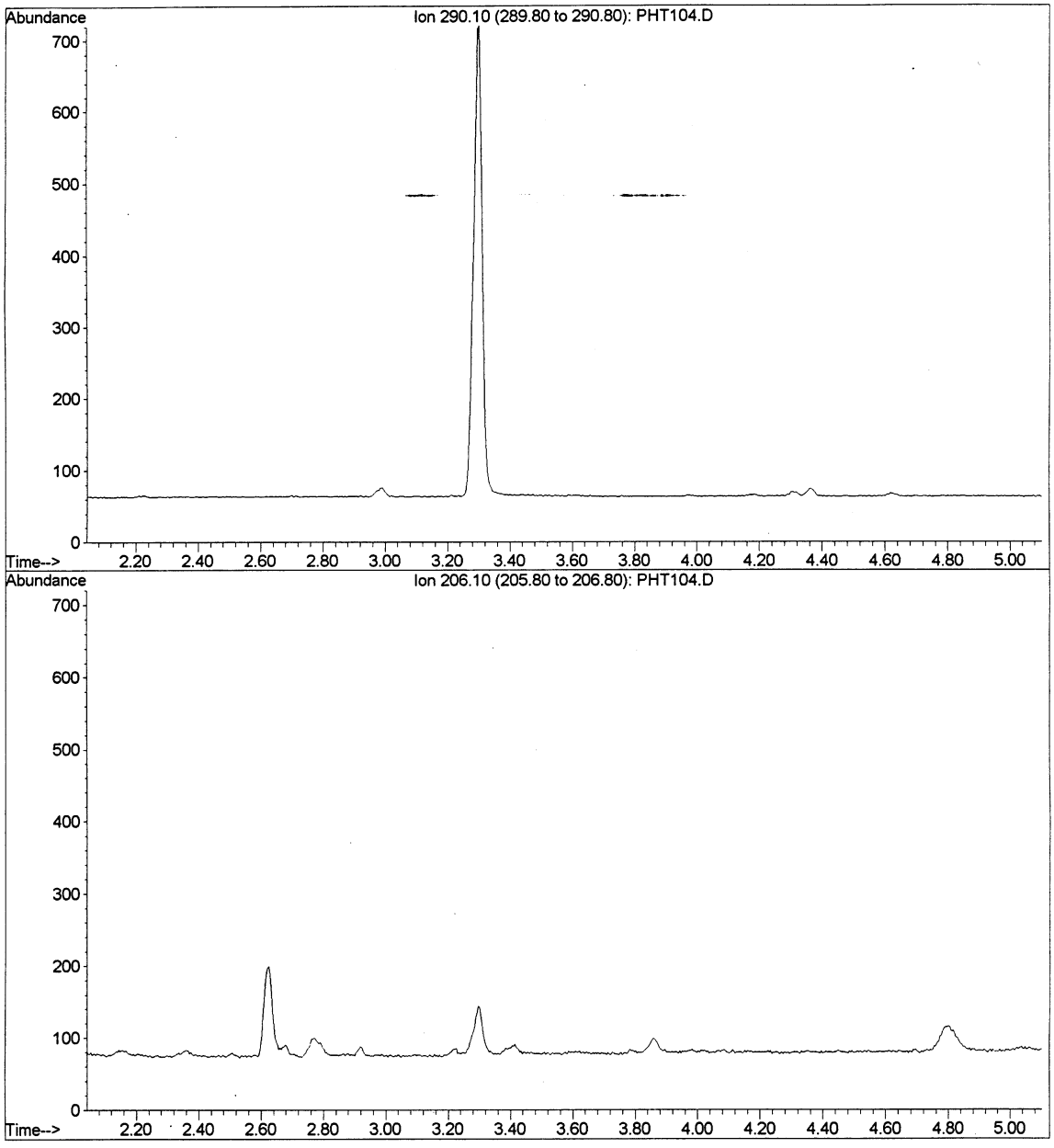


Fig. 7. Six hour plasma profile of PHT and [$^{13}C_3$]-PHT in a single Sprague–Dawley rat given a pulse dose of [$^{13}C_3$]-PHT after reaching PHT steady-state.

Appendix A

Validation run	Percent m/z 206 contributed by [$^2H_{10}$]-PHT
1	6.3 ± 0.2
2	6.5 ± 0.3
3	6.1 ± 0.3
4	6.7 ± 0.3
5	6.5 ± 0.2



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