Journal of Chromatography, 157 (1978) 416–420

CHROM. 11,165

Note

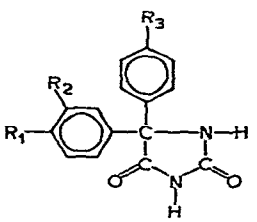
Identification and estimation of phenytoin and its major metabolite in rat brain following its administration by gas–liquid chromatography and gas–liquid chromatography–mass spectrometry

K. K. MIDHA*, C. CHARETTE, H. S. BUTTAR and I. DUPUIS

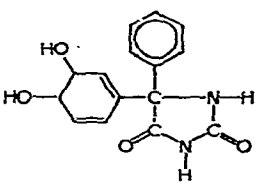
Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario K1A 0L2 (Canada)

(Received April 11th, 1978)

Phenytoin (I, DPH), a commonly prescribed anticonvulsant drug[1], undergoes biotransformation to several metabolites (II–VII) in various species. These meta-



I–VI, VIII



VII

Compound	R_1	R_2	R_3	*Name*
I	H	H	H	5,5-(diphenyl)-hydantoin
II	OH	H	H	5-(*p*-hydroxyphenyl)-5-phenylhydantoin
III	H	OH	H	5-(*m*-hydroxyphenyl)-5-phenylhydantoin
IV	OH	OH	H	5-(3,4-dihydroxyphenyl)-5-phenylhydantoin
V	OCH_3	OH	H	5-(4-methoxy-3-hydroxyphenyl)-5-phenylhydantoin
VI	OH	H	OH	5-5-bis-(4-hydroxyphenyl)-5-hydantoin
VII				5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin
VIII	CH_3	H	H	5-(*p*-methylphenyl)-5-phenylhydantoin

bolites have been identified in urine, plasma, bile and liver[2–8]. The presence of I in brain has been reported as an evidence for its central anticonvulsant action[9]. In this communication, we report identification and quantitation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (II, *p*-HPPH) and I in rat brain following intravenous administration of I by gas–liquid chromatography (GLC) and GLC–mass spectrometry (MS).

* To whom correspondence should be addressed.

EXPERIMENTAL

Instrumentation

For GLC–MS analysis, a Hewlett Packard 5985 GC–MS data system was used under the following conditions: 70 eV as the ionization beam energy and the source was maintained at 150°. The chromatographic column was a coiled-glass tubing 1.22 m × 2 mm I.D. packed with 3% OV-7 on Chromosorb W (80–100 mesh; Chromatographic Specialties, Brockville, Canada). The injection port, the column and the interphase temperatures were 300, 215 and 250°, respectively. The carrier gas was helium with a 26 ml/min flow-rate. Aliquots (2–4 μl) were chromatographed.

For GLC analysis a Tracor 550 gas chromatograph (Canlab, Montreal, Canada) with a flame-ionization detector was used. The 1.83 m × 4 mm I.D. coiled-glass column was packed with 5% OV-7 on Chromosorb W (100–120 mesh; Chromatographic Specialties). The column temperature was 240°, the injection port and the detector temperatures were 310°, and the carrier gas was nitrogen at a flow-rate of 50 ml/min. Hydrogen and air flow-rates were were adjusted for optimal sensitivity.

Standards and reagents

Stock solutions containing 400 μg/ml of I were prepared by dissolving appropriate amounts of the sodium salt (Parke, Davis and Co., Brockville, Canada) in distilled water. Stock solutions containing 100 μg/ml of the metabolite II (Aldrich, Milwaukee, Wisc., U.S.A.) were prepared by dissolving the appropriate amount in 0.01 *N* NaOH. Appropriate dilutions of the drug (20–100 μg/ml) and the metabolite (2–24 μg/ml) were prepared as required.

Aqueous solutions (400 μg/ml) of the internal standard VIII, 5-(*p*-methylphenyl)-5-phenylhydantoin (Aldrich) were prepared by dissolving an appropriate amount of the internal standard in 0.01 *N* NaOH and diluting to the appropriate concentration with distilled water before use. Methanolic trimethylanilinium hydroxide was synthesized according to the method of Barrett[10].

Diethylether (Mallinckrodt, Montreal, Canada) was glass-distilled prior to use. All the other chemicals were of analytical grade.

Animals

Female Wistar rats (184 ± 10 g) were treated with Dilantin (100 mg/kg; i.v.) and killed 5 h after treatment. The brains were immediately removed; a 10% homogenate solution was made with citrate buffer (pH 7.3) and then stored at −20° until assay.

Extraction procedure

A modified literature procedure[11] was employed. To 4-ml samples of rat brain homogenate in screw-capped centrifuge tubes (20 ml) were added 1 ml of the internal standard (52 μg/ml), 1 ml distilled water (for calibration curves, the distilled water contained I and II) and 3 ml of 1 *N* HCl. The samples were then extracted twice with 7-ml portions of diethyl ether by shaking (Roto-Rack, Fisher Scientific, Montreal, Canada) for 10 min at 30 rpm followed by centrifugation at 600 *g* for 10 min.

The organic extracts were transferred into a tube (20 ml) containing 5 ml of 0.2 *M* phosphate buffer pH 11.2. The tubes were mixed for 10 min followed by cen-

trifugation for 10 min, after which the organic extract was discarded. A 4-ml portion of the aqueous phosphate solution was transferred to another tube (20 ml), acidified with 2 ml of 2 *N* HCl and extracted twice with 5-ml portions of diethyl ether (mixed 10 min, centrifuged 10 min).

Four ml of the first extract and 5 ml of the second extract were transferred into an evaporating tube (Canlab) and the combined ether extracts were evaporated to dryness at 40° under a stream of dry nitrogen. The dried extracts were dissolved by mixing (Vortex Genie Mixer, Fisher Scientific) with 100 μl of methanolic trimethylanilinium hydroxide (0.2 *M*) and aliquots (2–4 μl) were injected into the GLC–MS system or the GLC.

Calibration curves

Peak height ratios were calculated by dividing the height of the peak from the drug or the metabolite by the height of the internal standard. Calibration curves were assembled from the results of spiked control brain homogenate by plotting the peak height ratios against the concentrations of the drug or the metabolite. The recovery of I and II from brain homogenate was similar to that reported earlier from this laboratory[11].

RESULTS AND DISCUSSION

Identification of phenytoin and its possible metabolites in rat brain was carried out by GLC–MS using the selected-ion-monitoring technique. It has been demonstrated[8,11] that flash-heater methylation with trimethylanilinium hydroxide converts compounds I–V into their respective di-, tri- or tetramethylated derivatives which are separated by GLC. These derivatives give molecular ions when subjected to MS analysis in the electron impact mode. Therefore, in the present study, aliquots of extracts of brain homogenates in trimethylanilinium hydroxide were injected and ions at *m/e* 280, 310 and 340 were monitored for the presence of I, II, III, IV and V.

Fig. 1 shows the presence of signals only at *m/e* 280 and 310 but not at *m/e* 340. The peak at *m/e* 280 had the same retention time as the methylated synthetic sample of I, and the peak at *m/e* 310 had the same retention time as the methylated synthetic sample of II (whereas methylated III had a shorter retention time).

The complete mass spectra (Fig. 2) of peaks eluting at retention times 3.16 and 7.9 min, respectively were identical to the mass spectra of methylated synthetic I and II, respectively. This established the presence of I and II in rat brain following the administration of I to rats. Quantitative estimation of I and II in rat brain was accomplished by GLC.

Blank and spiked rat brain homogenates were extracted and injected into the GLC as described previously. The typical chromatogram obtained by processing control blank brain homogenate showed extraneous peaks at 1.7 and 3.9 min, respectively. However, these peaks did not interfere with peaks due to the flash-heater methylated derivatives of I (retention time, t_R, 4.7 min), internal standard VIII (t_R 6.2 min) and II (t_R 10.0 min). The amounts of I and II in unknown samples were estimated from calibration curves assembled from the results of spiked control brain homogenates.

In the six female Wistar rats examined, the concentration of phenytoin (I) and

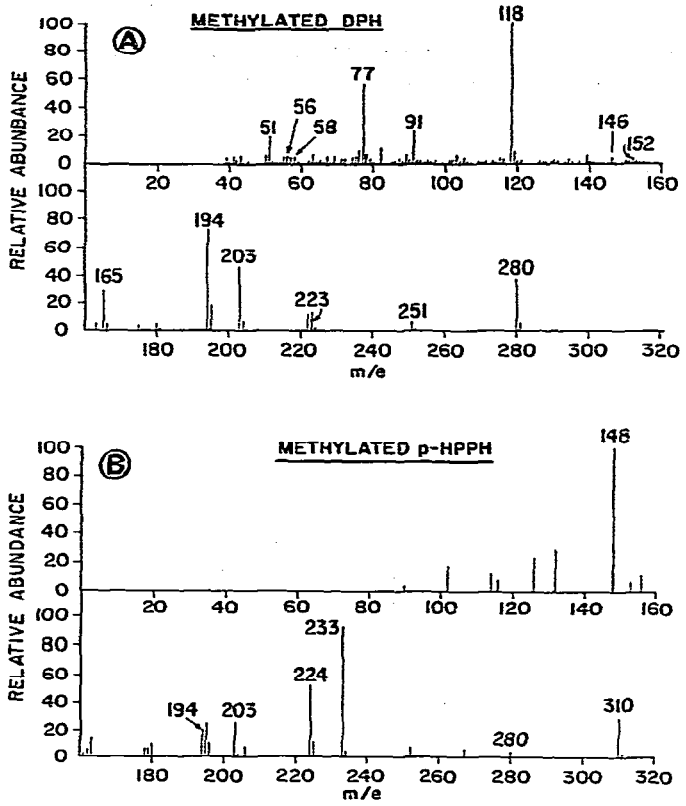


Fig. 1. Chromatogram (A) of a trace from selected-ion-monitoring technique and (B) of total ion from flash-heater methylated extract of rat brain homogenate injected into GC–MS.

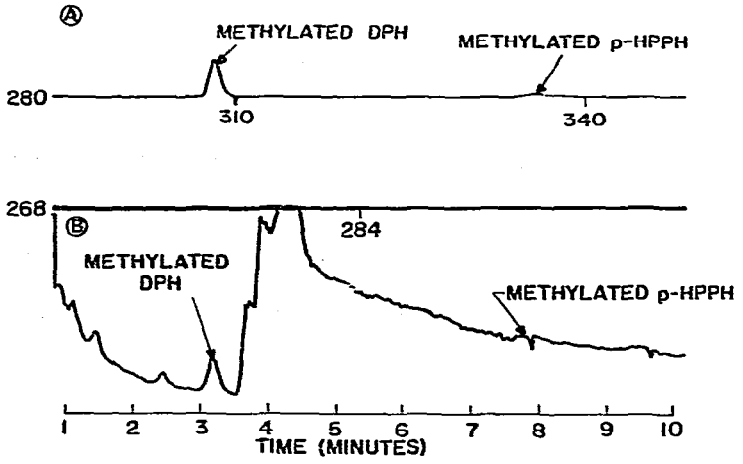


Fig. 2. Mass spectra of flash-heater methylated phenytoin I (A) and metabolite II (B) from rat brain homogenate extract.

TABLE I

CONCENTRATIONS OF PHENYTOIN I AND ITS METABOLITE 5-(*p*-HYDROXYPHENYL)-5-PHENYLHYDANTOIN II IN RAT BRAIN FOLLOWING INTRAVENOUS ADMINISTRATION OF DILANTIN 100 mg/kg

Animal	*Injected (mg)*	*Amount of I in brain (μg/g)*	*Amount of II in brain (μg/g)*
1	20.25	27.28	0.85
2	18.25	26.49	0.72
3	18.25	32.88	0.75
4	17.25	28.83	0.75
5	18.00	29.95	0.90
6	18.50	29.02	1.00
Mean	18.42	29.08	0.83
S.D.	±1.00	±2.25	±0.11
Coefficient of variation (%)	±5.41	±7.72	±13.10

the metabolite II in the brain were estimated to 29.08 ± 2.25 μg/g and 0.83 ± 0.11 μg/g (Table I) at 5 h following the administration of Dilantin at 100 mg/kg.

In summary, the presence of phenytoin and its metabolite II, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, in rat brain following administration of phenytoin has been demonstrated by GLC–MS using selected-ion-monitoring technique. A GLC procedure is described for estimation of phenytoin and its metabolite II in rat brain.

ACKNOWLEDGEMENT

The authors express their appreciation to Mr. J.-C. Ethier for running the mass spectra.

REFERENCES

1 W. S. Parker and R. J. Gumnit, *J. Neurol.*, 24 (1974) 1178.
2 M. G. Horning, C. Stratton, A. Wilson, E. C. Horning and R. M. Hill, *Anal. Lett.*, 4 (1971) 537.
3 O. Borgå, M. Garle and Marcela Gutova, *Pharmacology*, 7 (1972) 129.
4 R. M. Thompson, N. Gerber, R. A. Seibert and D. M. Desiderio, *Drug Metab. Dispos.*, 1 (1973) 489.
5 N. Gerber, R. A. Seibert and R. M. Thompson, *Res. Comm. Chem. Path. Pharmacol.*, 6 (1973) 499.
6 A. J. Glazko, *Epilepsia*, 16 (1975) 367.
7 R. M. Thompson, J. Beghin, W. K. Fife and N. Gerber, *Drug Metabol. Dispos.*, 4 (1976) 349.
8 K. K. Midha, K. W. Hindmarsh, I. J. McGilveray and J. K. Cooper, *J. Pharm. Sci.*, 66 (1977) 1596.
9 D. M. Woodbury and E. A. Sevinyard, *Antiepileptic Drugs*, Raven Press, New York, 1972, p. 113.
10 M. J. Barrett, *Clin. Chem. Newsl.*, 3 (1971) 16.
11 K. K. Midha, I. J. McGilveray and D. L. Wilson, *J. Pharm. Sci.*, 65 (1976) 1240.