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GAS CHROMATOGRAPHIC DETERMINATION OF *m*- AND *p*-HYDROXYPHENYTOIN IN THE URINE OF EPILEPTIC PATIENTS

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SUMMARY

An analytical method for determining phenytoin and its metabolites in the urine of epileptic patients is described. The analysis was performed for the *m*- and *p*-isomers of hydroxyphenytoin, the oxidative products of phenytoin, using gas chromatography. As an internal standard, 5-(4-methylphenyl)-5-phenylhydantoin was chosen; the extraction solvent from human urine was ether—chloroform (3:7).

Phenytoin and its hydroxy isomers were satisfactorily determined by the modified on-column methylation technique on a 2% OV-17 column using temperature programming from 180° C to 240° C at 5° C/min.

INTRODUCTION

The high performance liquid chromatographic determination of phenytoin and its hydroxy isomers in serum and human urine was discussed in our first [1] and second [2] reports. The analysis of phenytoin and its metabolites in rat urine was also tried in a previous investigation [3].

Phenytoin is converted to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HDPH) by biological metabolism, about half of the administered dose being excreted in the urine in unchanged form and/or as the conjugated glucuronide. In human urine, the hydantoin ring in the phenytoin molecule is cleaved to produce diphenylhydantoic acid to the extent of 1—5% of the original amount

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of drug, and also to aminodiphenylglycine at 25%. *m*-Hydoxyphenytoin, the isomer of *p*-HDPH, is also present in human urine at a low level [4–6].

The present report is concerned with the determination of human urinary *m*- and *p*-HDPH as well as phenytoin, which was also administered to epileptic patients. Solow et al. [7] reported the determination of phenytoin and phenobarbital in blood by a gas chromatographic (GC) method with an on-column methylation technique. For a simple, rapid and quantitative method, we have attempted to analyse the *m*- and *p*-HDPH isomer using a partially modified on-column methylation technique.

EXPERIMENTAL

Reagents and solutions

Phenytoin (DPH) was recrystallized from one normalized according to the Japanese Pharmacopoeia. *m*-HDPH was from Aldrich (Milwaukee, WI, U.S.A.) and *p*-HDPH was from Sigma (St. Louis, MO, U.S.A.). Diphenylhydantoic acid was prepared from diphenylglycine by the method of Connors et al. [8]. Diphenylglycine was a product from Aldrich. Carbamazepine was one normalized according to the Japanese Pharmacopoeia. Tetramethylammonium hydroxide was used as a 10% solution in methanol, obtained from Tokyo Kasei (Tokyo, Japan). 5-(4-Methylphenyl)-5-phenylhydantoin was purchased from Sigma.

All solvents and other reagents were of analytical grade and used without further purification. As a control urine, Tek-Chek® No. 1 was obtained from Ames (Elkhart, IN, U.S.A.).

DPH and *p*-HDPH (20 mg each) were independently dissolved in 2–6 ml of 0.1 *M* potassium hydroxide and made to a total volume of 100 ml with water. *m*-HDPH solution was obtained by dissolving 10 mg of the substance in 3 ml of 0.1 *M* alcoholic potassium hydroxide and made to 100 ml with water. Diphenylhydantoic acid (5 mg) was dissolved in a small volume of ethanol and made to 100 ml with water. Diphenylglycine (1 mg) was dissolved in a small volume of ethanol and made to 10 ml with water. Carbamazepine solution was prepared by dissolving 2 mg in a small volume of 0.1 *M* alcoholic potassium hydroxide and made to 10 ml with water. As a buffer solution, monobasic sodium phosphate (GR grade, Wako) was dissolved in water to a concentration of 0.3 *M*. As internal standard solution, 0.5 mg of 5-(4-methylphenyl)-5-phenylhydantoin was dissolved in 100 ml of an ether–toluene (3:7) mixture.

Instruments

GC analyses were performed by a JGC-20 KFP (JEOL, Tokyo, Japan) gas chromatograph on a glass column (2 m × 2 mm I.D.) with 2% OV-17 as stationary phase (80–100 mesh, Gas-Chrom Q) coupled with a Hitachi 834 type chromatoprocessor. The injection and flame ionization detector temperature was maintained at 280°C, and the column oven was temperature programmed from 180°C to 240°C at 5°C/min. The carrier gas was nitrogen with a flow-rate at 30 ml/min.

Calibration and recovery

To 1.0 ml of the urine, known amounts of DPH and *m*- and *p*-HDPH solutions were added. Then 1.0 ml of 0.3 *M* phosphate buffer solution (pH 6.8) and 5.0 ml of the internal standard solution were added. The mixture was vortex-mixed for 30 sec and centrifuged at 700 *g* for 30 min. The supernatant was transferred to another centrifuge tube and 0.5 g of anhydrous sodium sulphate was added. The mixture was also vortex-mixed for 30 sec and centrifuged again at 700 *g* for 5 min. To the supernatant thus obtained, 100 μl of tetramethylammonium hydroxide (TMH) were added and this mixture was vortex-mixed for 30 sec, then centrifuged at 1100 *g* for 5 min. After the upper layer was discarded, 50 μl of methanol were added to the TMH (lower) layer and mixed well; 2 μl of the mixture were injected into the gas chromatograph. The calibration and recovery tests were replicated four times for each substance. The recovered amounts of the substances were calculated from the ratio of the peak height of the samples to that of the internal standard.

Analytical procedure

When the analytical procedure was applied to patient's urine, acid hydrolysis was carried out to isolate the conjugated drug and its metabolites. To 1.0 ml of patient's urine, 1 ml of 12 *M* hydrochloric acid was added and heated at 90°C for 2 h. The hydrolysate thus obtained was neutralized with 20% sodium hydroxide, followed by the procedure as described above for calibration and recovery.

RESULTS AND DISCUSSION

Trimethylphenylammonium hydroxide and TMH have been widely utilized as on-column methylation agents for gas—liquid chromatographic (GLC) analysis. The former is useful for methyl esterification of straight-chain fatty acids. On the other hand, the latter has been used in the determination of

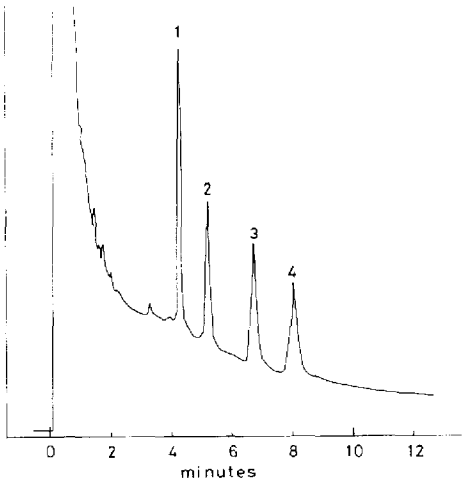

Fig. 1. Gas chromatogram of phenytoin and its hydroxy compounds. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin.

barbituric acids [9, 10] or phenytoin [9, 11]. The present report deals with a modification of the latter methylation technique.

An extraction solvent suitable for DPH and *m*- and *p*-HDPH in human urine was sought. Ethyl acetate or ether—chloroform mixed solvents gave undesirable extraction ratios for these substances in urine. Chloroform also makes the layer separation from the TMH layer difficult.

An ether—toluene mixture, which was used by Dorrity and Linnoila [12], was selected as the extraction solvent for DPH and other compounds from urine samples. The extraction was carried out using two solvent ratios: 50% ether and 50% toluene, and 30% ether and 70% toluene. The latter was more effective than the former for the extraction of DPH, and *m*- and *p*-HDPH and internal standard. Consequently, the ether—toluene (3:7) mixture was used as the extraction solvent for DPH and its metabolites; in addition, the internal standard was dissolved in the same solvent system.

The results of the experiment concerning the effect of phosphate buffer concentration on extraction showed that 3 *M* [12] phosphate buffer in 10% methanol was superior to 0.3 *M* [7].

In preliminary experiments using on-column methylation and a constant column temperature of 240°C, the chromatogram showed that DPH was well resolved from methanol used as a solvent for TMH. The improved resolution is required to analyse small amounts of DPH in urine. Solow et al. [7] used the following temperature programme for GLC: 1 h at 250°C, 4 h at 325°C, and overnight at 250°C. Interference from the methanol peak could be avoided by introducing linear temperature programming rising from 180°C to 240°C at a rate of 5°C/min by slightly modifying the conditions of Solow et al. DPH was completely resolved from methanol, as shown in Fig. 1.

The retention times for DPH, internal standard and *m*- and *p*-HDPH were 4.3, 5.2, 6.7 and 8.0 min, respectively. Quantitative measurements were attempted for DPH and *m*- and *p*-HDPH under these experimental conditions. Straight lines which passed through the origin were obtained between detector response and increasing amounts of standards added to the urine samples before extraction. As the detector response, the ratio of the peak height of samples to that of the internal standard, 5-(4-methylphenyl)-5-phenylhydantoin, was adopted. The regression lines between the amount (x) injected into the column and the peak height ratio (y) were $y = 0.06079x + 0.01430$ ($r = 0.997$) in the range 10—50 μg/ml for DPH, $y = 0.02727x - 0.02089$ ($r = 0.998$) in the range 15—50 μg/ml for *m*-HDPH, and $y = 0.02346x - 0.01650$ ($r = 1.000$) in the range 15—200 μg/ml for *p*-HDPH. Thus, it was confirmed that each result showed significant correlations.

Experiments on the recovery of DPH and *m*- and *p*-HDPH added to the control urine and healthy human urine were performed. To 1.0 ml of urine, 20—60 μg of DPH, 20—80 μg of *m*-HDPH, and 10—150 μg of *p*-HDPH were added, and DPH and its hydroxy metabolites were analysed using the on-column methylation technique. The recoveries of DPH were 94.4—99.9% for control urine and 93.9—99.9% for human urine, those of *m*-HDPH were 103.3—109.5% for control and 95.9—106.1% for human urine, and those of *p*-HDPH were 88.4—96.7% for control and 90.8—94.6% for human urine. The recovery percentages were all satisfactorily significant, as shown in Table I.

TABLE I

RECOVERY OF DPH AND *m*- AND *p*-HDPH ADDED TO CONTROL AND HEALTHY HUMAN URINE

	DPH			*m*-HDPH			*p*-HDPH		
	Added (μg/ml)	Recovery* (%)	C.V.** (%)	Added (μg/ml)	Recovery* (%)	C.V. (%)	Added (μg/ml)	Recovery* (%)	C.V. (%)
Control urine***	20	94.4 ± 2.15	2.28	20	103.3 ± 1.15	1.11	10	96.7 ± 3.45	3.57
	40	99.7 ± 0.65	0.65	40	109.5 ± 3.93	3.59	50	93.4 ± 1.05	1.12
	60	99.9 ± 1.73	1.73	80	103.7 ± 2.08	2.01	100	88.4 ± 1.40	1.58
Human urine	40	95.1 ± 1.70	1.79	25	106.1 ± 0.10	0.09	10	90.8 ± 0.65	0.72
	50	93.9 ± 0.85	0.91	50	95.9 ± 2.95	3.08	50	94.6 ± 0.65	0.69
	60	99.9 ± 0.80	0.80	60	103.1 ± 7.68	7.45	150	93.4 ± 0.00	0.00

*Mean ± S.D. (n = 4).
**Coefficient of variation.
***Tek-Chek No. 1 (Ames, Elkhart, IN, U.S.A.) was used as a control urine.

Consequently, DPH and *m*- and *p*-HDPH were quantitatively determined by GC through on-column methylation using TMH as N-methyl derivatization reagent. The partially modified on-column methylation technique allowed the simple and rapid determination of the substances added to the control and the human urine.

Subsequently, the influence of the coexistence of DPH metabolites and another combination drug on the measured value was examined. Carbamazepine, a combination drug, diphenylhydantoic acid and diphenylglycine as DPH metabolites except the hydroxy compound of DPH were selected for this purpose. On the gas chromatogram, carbamazepine, hydantoic acid and diphenylglycine appeared earlier than DPH and also overlapped at the solvent peak. Thus, DPH, and *m*- and *p*-HDPH can be quantitatively determined by GLC without the interference of carbamazepine or DPH metabolites, as shown in Table II.

As an application of the on-column methylation technique using the method described, urinary DPH and *m*- and *p*-HDPH in patients who were hospitalized at the Department of Psychiatry could be determined with a single injection

TABLE II

EFFECT OF CARBAMAZEPINE, HYDANTOIC ACID AND DIPHENYLGLYCINE ON RECOVERY OF DPH

DPH concentration was 20 μg/ml for each experiment.

	Added (μg/ml)	Recovery* (%)	C.V.** (%)
Carbamazepine	24	104.1 ± 1.48	1.4
	36	94.0 ± 1.34	1.4
	240	101.8 ± 1.77	1.4
Hydantoic acid	500	105.2 ± 0.64	0.6
Diphenylglycine	100	100.3 ± 4.3	4.3

*Mean ± S.D. (n = 4).
**Coefficient of variation.

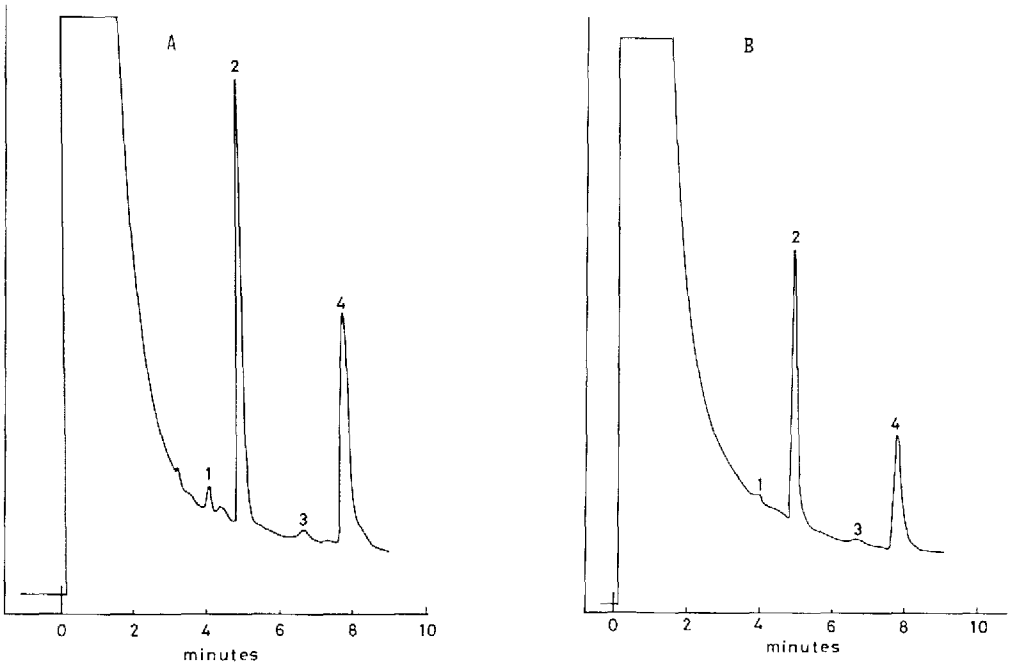

Fig. 2. (A) Gas chromatogram of urine from an epileptic patient after administration of 250 mg of DPH and 300 mg of carbamazepine per day. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin. The amounts of peaks 1, 3 and 4 corresponded to 2.23, 5.43 and 74.82 mg per one day's urine, respectively. (B) Gas chromatogram of urine from an epileptic patient after administration of 150 mg of phenytoin per day. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin. The amounts of peaks 1, 3 and 4 corresponded to 0.28, 5.30 and 56.48 mg per one day's urine, respectively.

TABLE III

AMOUNT OF DPH AND *m*- AND *p*-HDPH IN PATIENTS' URINE AFTER ADMINISTRATION OF DPH

Name	Age	Sex	Administered (mg)		Found (mg)		
			DPH	Carbamazepine	DPH	*m*-HDPH	*p*-HDPH
R.K.	25	F*	250	300	2.23	5.43	74.82
F.S.	68	F	150	—	0.28	5.30	56.48

*F = female.

of the urine extract. Chromatograms for the extracts of patients' urine are shown in Fig. 2A and B.

Fig. 2A shows a chromatogram from the urine of patients to whom 250 mg of DPH and 300 mg of carbamazepine were administered daily, while Fig. 2B shows that obtained when 250 mg of DPH only were administered to patients. These results are summarized in Table III. From these experimental data it can be seen that only a small amount of DPH, less than 1% of the initial amount, exists as unchanged form, most (30—40%) of the substance being

converted to its oxidative product, *p*-HDPH. Kozelka and Hine [13] reported that DPH is formed through the ring closure of diphenylhydantoic acid by heating with acid. Furthermore, there is an increase in the amount of DPH over that found in untreated urine. These results were all in good agreement with data of Chang et al. [4].

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