Europ.J.clin.Pharmacol. 9, 161-168 (1975)

Quantitation of Phenobarbital and Its Main Metabolites in Human Urine

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Received: March 11, 1975, and in revised form: July 7, 1975

Summary. A method for the quantitative determination of phenobarbital and free and conjugated *p*-hydroxyphenobarbital in urine samples is described. The method includes initial extraction, purification on a small chromatographic column and finally determination by gas chromatography. The barbituric acids are methylated by trimethylanilinium hydroxide which serves as a "flash heater" methylating agent. The conjugate of *p*-hydroxyphenobarbital, which appears to be a glucuronide, is hydrolysed with hydrochloric acid.

Key words: Conjugate-cleavage, drug metabolism, gas chromatography, phenobarbital, urinary excretion.

With the aim of investigating the pharmacokinetics of phenobarbital (I, Fig. 1) a gas chromatographic method for its assay in plasma had previously been developed and employed in this laboratory (13, 15). More detailed investigation of metabolism and renal elimination required quantitative determination of the excretion of the drug and its metabolites in urine.

Numerous reports have been published about the metabolism of phenobarbital. The principal metabolic route common to all species including man is aromatic hydroxylation to *p*-hydroxyphenobarbital (II, Fig. 1) (4, 8, 10, 11, 17). In man, about half the hydroxylated compound is reported to be conjugated, "presumably with sulphate" (IIa, Fig. 1) (4), whilst in the dog and the horse almost all of the metabolite is conjugated with glucuronic acid (IIb, Fig. 1) (4, 17). In the rat, the most extensively studied species, beside the main metabolite *p*-hydroxyphenobarbital, certain minor metabolites have been identified as 5-(3,4-dihydroxyphenyl)-5-ethyl-barbituric acid (V, Fig. 1), 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-ethyl-barbituric acid (VI, Fig. 1) and 5-(1-hydroxyethyl)-5-phenylbarbituric acid (VII, Fig. 1) (10). The metabolites VI and VII have also been found in guinea pig (10).

The *m*-hydroxylated compound (IV, Fig. 1) is believed to be formed in the rat (11) and the *o*-hydroxylated compound (III, Fig. 1) has also been suggested as a metabolite in this species (8). The same paper reported an unidentified compound amounting to as much as 20 per cent of the total metabolites excreted. Metabolite VI (Fig. 1) has also been found in man (10). Phenobarbital is also partly excreted unchanged (4).

To separate phenobarbital from its metabolites countercurrent methods (4) and chromatographic methods, such as paper chromatography (1, 17) and gas chromatography (10, 11, 20) have been used. In the present report, the method published previously for analysis of phenobarbital in blood (15) has been further developed to permit determination of urinary metabolites.

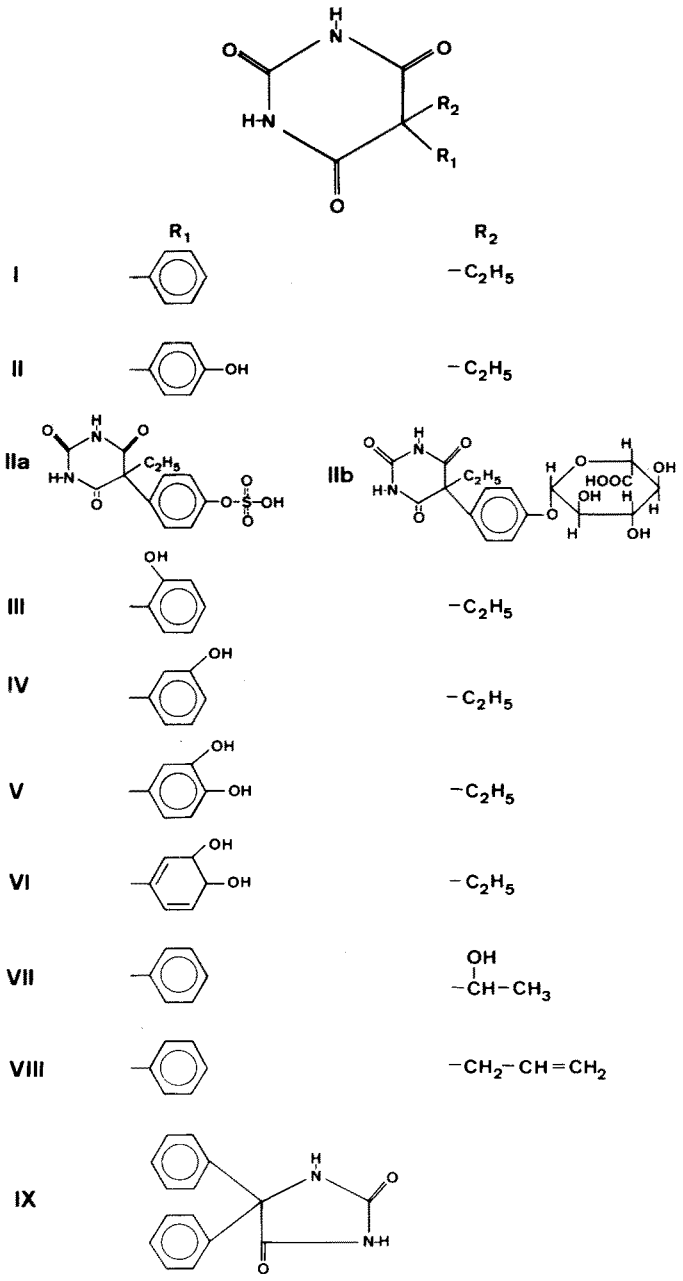

Fig. 1. Structures of the compounds in the text

MATERIAL AND METHODS

Instruments and Reagents

A Varian Aerograph Model 1400 Gas Chromatograph with flame ionization detector and Goertz Servogor recorder was used. The steel column (6 ft., 3 mm i.d.) was packed with 3% SE-30 on Varaport 30 100/120 mesh (Varian Co.). The following flow rates were used: N_2 35 ml/min, H_2 20 ml/min and O_2 250 ml/min. Temperatures were 218° C in the injector, 190° C in the column and 230° C in the detector.

In the studies of partition ratios, the concentration measurements were performed spectrophotometrically using a Zeiss PMQ II spectrophotometer with 1 cm quartz cells. The pH-values were determined by use of a Radiometer pH-meter 4 equipped with glass and calomel electrodes.

The following reagents were used: anhydrous diethyl ether (Mallinckrodt) saturated with water ; cyclohexane p.a. (Merck); amyl alcohol p.a. (Fisher); conc. acetic acid (Ph. Nord.); acid-washed diatomaceous earth, Celite 535.. Trimethylanilinium hydroxide (TMAH) was prepared according to Brochmann-Hanssen & Oke (3). Phenobarbital (I, Fig. 1), 5-allyl-5-phenylbarbituric acid (VIII, Fig. 1) and diphenylhydantoin (IX, Fig. 1) were obtained from commercial sources.

For thin layer chromatographic (TLC) analysis, precoated TLC Plates Silica Gel 60 F-254 (Merck) were used.

The enzymes used were: sulphatase from limpets (Type III) and β-glucuronidase from limpets (soluble Type L-1; Sigma Chemical Company). Activity of the enzymes was checked using *p*-nitrocatechol sulphate and phenolphthalein glucuronide as substrates, respectively (Sigma Chemical Company).

The synthesis of *p*- and *m*-hydroxyphenobarbital was performed according to references 4 and 19, with minor modifications.

Patients

Urinary samples were obtained from three pediatric patients: A.K. (boy), aged 8 years 10 months, given phenobarbital 5.0 mg/kg daily; P.F. (girl), 1 year 8 months given 4.3 mg/kg daily; and P.C. (boy), 5 months, given 7.0 mg/kg daily.

Extraction Procedures

Principle. Phenobarbital and *p*-hydroxyphenobarbital were extracted from an acidified urine sample into a diethyl ether phase. The extract was purified by column chromatography prior to re-extraction of the barbiturates into a small volume of an alkaline aqueous methylating agent. Part of the extract was injected into a gas chromatograph (GC), where flash methylation (3) improves the chromatographic properties of the barbituric acids.

Phenobarbital, p- and m-Hydroxyphenobarbital. To 1.00 ml of urine in a glass stoppered tube was added:
a. 25 µl internal standard solution (5-allyl-5-phenylbarbituric acid 800 µg/ml);

b. 500 µl of a saturated solution of sodium chloride;
c. 50 µl of phosphate buffer pH 5.5 (4 M sodium dihydrogen phosphate).

The mixture was extracted with water-saturated diethyl ether (2 x 1.5 ml). The combined ether extracts were evaporated to about 1 ml under a stream of nitrogen and passed through a small purification column which was washed twice with water-saturated diethyl ether (1.5 ml).

The purification column was prepared in the following way: Celite 535 and phosphate buffer pH 7.7 was thoroughly mixed in the proportion 2:1. A Pasteur pipette (160 x 7 mm) was two thirds filled with the moist mixture.

The combined column eluates were re-extracted into 50 µl of a freshly prepared mixture of equal volumes of 0.6 M trimethylanilinium hydroxide (TMAH) in methanol and water. One µl of the lower aqueous phase was injected into the gas chromatograph.

Conjugated Metabolites. Conjugated metabolites were hydrolysed with hydrochloric acid prior to extraction as described above.

1.00 ml urine and 1 ml 12 M hydrochloric acid were sealed in a glass tube and placed in a water bath at 100°C for 30 min. After cooling the mixture was neutralized with 12 M sodium hydroxide 1 ml.

The internal standard was added and the mixture buffered to pH 5.5. Extraction and derivatization was then carried out as before.

In order to determine both free and conjugated *p*-hydroxyphenobarbital, the urine samples were analysed before and after hydrolysis. The amount of conjugated *p*-hydroxyphenobarbital was then calculated from the difference between the total amount of *p*-hydroxyphenobarbital in the hydrolysed sample and the amount in the unhydrolysed sample.

Studies of Urinary Metabolites

Hydrolysis of Conjugates. Urine samples from three patients were analysed for phenobarbital, *p*-hydroxyphenobarbital and conjugated *p*-hydroxyphenobarbital according to the above procedures. Urine samples from these patients were also hydrolysed both with sulphatase and β-glucuronidase: 1.00 ml aliquots of urine from each patient were incubated with three different amounts of sulphatase in 1 ml citrate buffer pH 5.0. Similarly, 1.00 ml aliquots of each urine were hydrolysed with β-glucuronidase in 1 ml buffer pH 3.8 that also contained phosphate (0.1 M) in order to inhibit sulphatase activity. Control tubes were set up without the enzyme. The eight urine samples from each patient were incubated with agitation at 37° C for 24 hours, and then extracted and analysed according to the above procedure. The experiment was performed twice (see Table 2).

Hydroxy-metabolites of Phenobarbital. Urine (140 ml) from patient A.K. contained a compound which on GC-analysis gave a peak at the retention time for *m*-hydroxyphenobarbital (Table 2). The sample was treated in the following manner: the urine sample was buffered to pH 3.8 with citrate buffer, also containing phosphate. β-Glucuronidase (340 mg) was added to the sample, which was cautiously agitated in a water bath at 37° C for 24 hours. The mixture was passed through a XAD-2 column (20 x 1.8 cm; BDH Chemicals, Ltd, Poole, England) onto which phenobarbital and metabolites are adsorbed. After washing the column with distilled water (one bed volume), phenobarbital and metabolites were eluted with methanol (two bed volumes), which was evaporated to about 5 ml. After addition of buffer to pH 5.5, the mixture was shaken five times with an equal volume of toluene, which mainly extracted phenobarbital, and then five times with an equal volume of diethyl ether to extract the hydroxyphenobarbital (4, 15). The toluene and diethyl ether fractions were evaporated to small volumes and chromatographed on TLC-plates (20 x 20 cm, 0.5 mm thickness).

Table 1. Acid constants of barbituric acids determined in the system diethyl ether/water and partition coefficients in the same system, without and with added sodium chloride

	Phenobarbital	*m*-Hydroxy-phenobarbital	*p*-Hydroxy-phenobarbital	5-Allyl-5-phenylbarbituric acid
Acid constant (pK'_{a_1})	7.1	7.2	7.5	7.2
Partition coefficient (log k_d)				
Without sodium chloride	1.86	1.09	0.83	1.81
With sodium chloride	1.85	1.24	1.11	

Table 2. Concentration of barbituric acids (in µg/ml) in urine from three patients. 1.00 ml aliquots of each urine sample were treated in different ways

	Phenobarbital			*p*-Hydroxyphenobarbital			Compound at a retention time of *m*-hydroxyphenobarbital		
Patient	A.K.	P.F.	P.C.	A.K.	P.F.	P.C.	A.K.	P.F.	P.C.
Urine sample:									
Untreated	52.0	21.0	12.9	4.9	15.6	8.0	-	-	-
Sulphatase blank	54.0	21.0	11.1	5.9	18.3	7.7	-	-	-
Sulphatase[a] I (0,06 mg/ml)	52.0	20.3	12.1	6.3	18.9	9.5	-	-	-
Sulphatase[a] II (0,6 mg/ml)	51.8	19.8	11.2	5.3	21.8	7.8	-	-	-
Sulphatase[a] III (1,4 mg/ml)	55.0	21.0	12.4	6.0	17.9	10.4	-	-	-
β-Glucuronidase blank	53.0	20.3	12.1	6.3	18.9	9.5	-	-	-
β-Glucuronidase[b] I (0,25 mg/ml)	53.4	21.1	11.3	8.5	28.0	16.9	1.4	+	+
β-Glucuronidase[b] II (2,5 mg/ml)	52.5	19.8	10.8	14.8	36.5	17.5	2.1	1.9	1.0
β-Glucuronidase[b] III (4,8 mg/ml)	50.7	19.1	12.1	16.7	43.0	21.9	2.3	1.2	+
Acid hydrolysis	52.0	21.0	12.9	18.2	46.0	21.7	3.1	5.5	2.3

[a] Also containing β-glucuronidase as impurity

[b] Also containing sulphatase, which was inhibited by phosphate 0,1 M

\+ Traces (less than 1.0 µg/ml)

\- Not detectable

Table 3. R_f-values for barbituric acids in two TLC-systems

	System A	System B
	Solvent A, nontreated plate	Solvent B, treated plate[a]
Standards		
Phenobarbital	0.62	0.59
m-Hydroxyphenobarbital	0.48	0.52
p-Hydroxyphenobarbital	0.41	0.52
Samples from preparative TLC (patient A.K.):		
"Phenobarbital"-zone	0.60	0.58
"*m*-Hydroxyphenobarbital"-zone	0.45	No spot in the neighbourhood of 0.52
"*p*-Hydroxyphenobarbital"-zone	0.42	0.55

Solvent A = Cyclohexane, Diethyl ether, Amyl alcohol, Acetic acid (40:20:6:5)

Solvent B = Cyclohexane, Amyl alcohol, Acetic acid (50:50:5)

[a] The plates were pre-treated by running them in a TLC-tank containing a solution of 10% formamide in ethanol

The plates were run with system A (Table 3) and the spots localized under UV-light and with 0.2 per cent potassium permanganate, which gave very clear spots for the hydroxy compounds. The zones for each metabolite were scraped off, eluted with methanol and the eluates examined by TLC (see Table 3) and GC.

RESULTS AND DISCUSSION

Extraction Studies

Basis of the Method. An evaluation of partition ratio and the efficacy of extraction can be based on partition coefficients and acid constants. These have been determined for phenobarbital (I, Fig.1), 5-allyl-5-phenylbarbituric acid (VIII, Fig. 1), *m*-hydroxyphenobarbital (IV, Fig. 1) and *p*-hydroxyphenobarbital (II, Fig. 1) in the system diethyl ether/water using the method described by Modin & Tilly (16). The results are shown in Table 1. Diethyl ether has previously been found to be useful in the extraction of both phenobarbital from plasma (15) and *p*-hydroxyphenobarbital from urine (4).

To improve the partition properties of the hydrophilic hydroxylated molecules, a saturated solution of sodium chloride was added and the salting-out effect (17) evaluated. The partition properties of phenobarbital, *m*-hydroxyphenobarbital and *p*-hydroxyphenobarbital were determined both in the system diethyl ether / water and diethyl ether / sodium chloride solution of the same concentration as in the actual extraction of the urine samples (Table 1).

As seen in Table 1, the addition of sodium chloride had no effect on the extraction of phenobarbital. For the *p*-hydroxylated compound, the partition to diethyl ether was almost doubled, whilst the effect on the *m*-hydroxylated compound was less pronounced.

Based on these constants, the percentage of each drug present in the organic phase at equilibrium was calculated. At equal phase volumes, as in the extraction procedure, 98.6 per cent of phenobarbital, 98.5 per cent of 5-allyl-5-phenylbarbituric acid, 94.4 per cent of *m*-hydroxyphenobarbital and 92.9 per cent of *p*-hydroxyphenobarbital can be assumed to appear in the organic layer. Since the extraction was repeated once, a virtually quantitative extraction was achieved.

Procedure. Purification of the crude ether extract of urine with a Celite column was necessary in order to remove co-extracted substances that would consume the methylating reagent and might disturb the chromatogram. The column removed interfering acidic compounds, whilst the barbituric acids passed through the column without delay and no loss was observed.

The re-extraction of phenobarbital to the alkaline aqueous phase had previously been examined (15) and found to be 94 per cent under the present conditions. Re-extraction into an aqueous phase is more advantageous for the more hydrophilic hydroxylated compounds than for non-hydroxylated compounds.

The use of 0.1 M TMAH (15) in this procedure gave rise to memory effects from the column at a subsequent injection, suggesting adsorption of incompletely derivatised *p*-hydroxyphenobarbital. Using 0.6 M TMAH, the memory effects were eliminated.

To hydrolyse the conjugates, the urine sample was heated at 100° C for 30 minutes in 6 M hydrochloric acid (4, 6). With this treatment the conjugates of phenobarbital were rapidly hydrolysed (Fig. 2a). The *m*-and *p*-hydroxylated phenobarbitals appeared to be stable, while phenobarbital itself showed a decline of about 10 per cent during the hydrolysis procedure (Fig. 2b).

Quantitative Determination

Standard curves were prepared by submitting urine samples containing known amounts of phenobarbital, its *m*- and *p*-hydroxylated derivatives and the internal standard, to the procedure described. As internal standard 5-allyl-5-phenylbarbituric acid was used, but when disturbing peaks appeared around this standard in the chromatograms, making quantitation uncertain, diphenylhydantoin (IX) was used instead, which has a retention time longer than that of *p*-hydroxyphenobarbital. Peak areas were determined by multiplying the peak height by the width at half height. The peak area ratio was then plotted against the amount of standard added (Fig. 3). The standard curve was linear over the range tested (1 - 20 µg/ml urine). The lower limit of determination was about 1 µg of each barbiturate per ml urine, which was of the sensitivity required for clinical application. If necessary, the sensitivity of the method may be increased by using larger samples or by reducing the volume in the re-extraction step to 25 µl.

The precision of the method is demonstrated by the values given in the standard curve (Fig. 3).

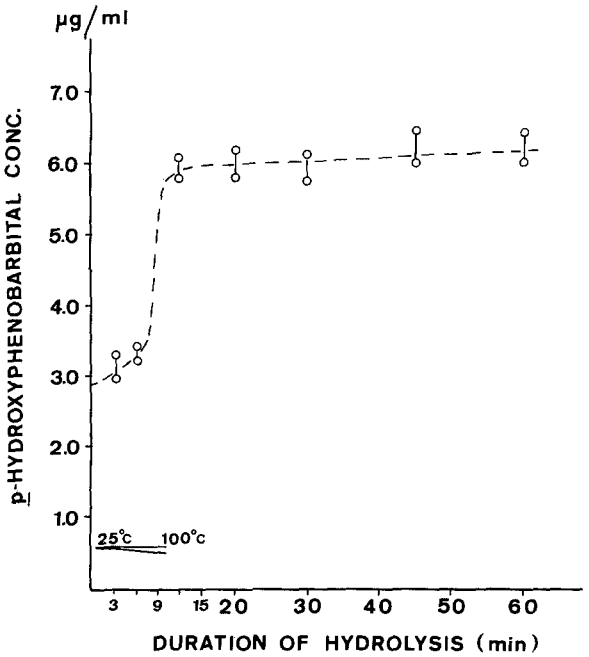

Fig. 2a. The rate of hydrolysis of conjugate in 6 M hydrochloric acid. (The bars represent the range of duplicate determinations of the same sample)

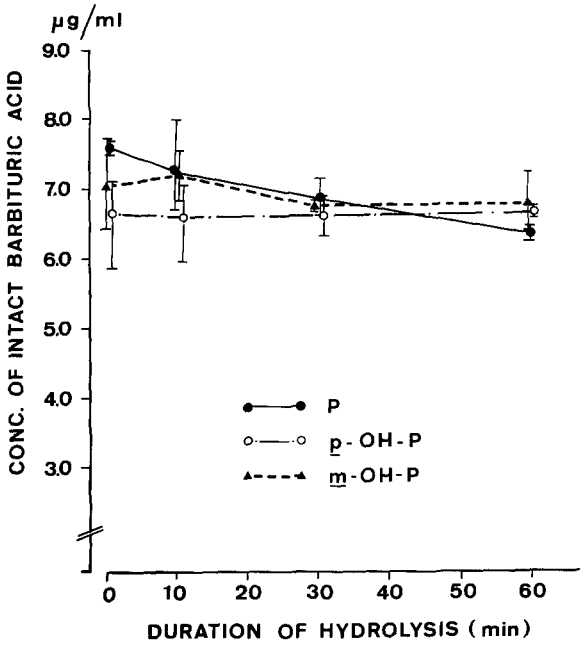

Fig. 2b. The stability of phenobarbital (P), *p*-hydroxyphenobarbital (*p*-OH-P) and *m*-hydroxyphenobarbital (*m*-OH-P) during acid hydrolysis. (The bars represent ranges of multiple determinations of the same sample)

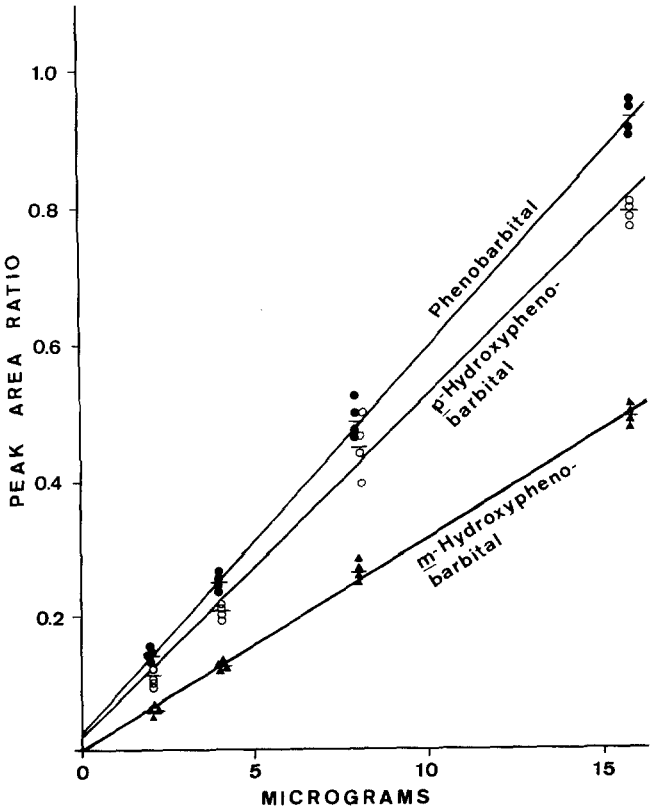

Fig. 3. Standard curve for determination of phenobarbital, *p*-hydroxyphenobarbital and *m*-hydroxyphenobarbital. Internal standard: 5-allyl-5-phenylbarbituric acid. All individual values and the mean for each point are given

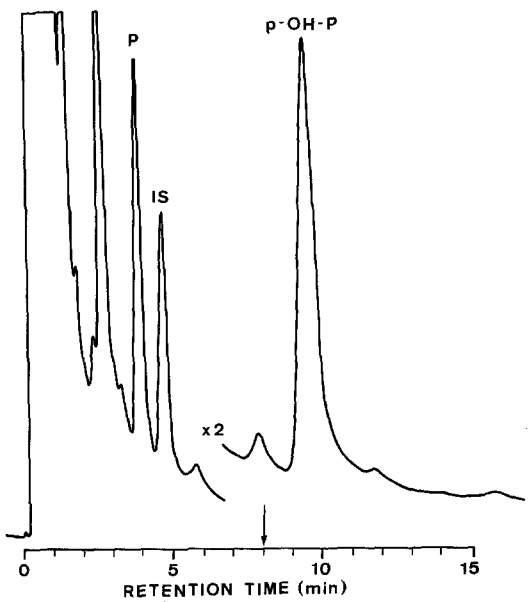

Fig. 4. Gas chromatogram of urine from patient A.K. showing phenobarbital (P), internal standard (5-allyl-5-phenylbarbituric acid (IS)) and *p*-hydroxyphenobarbital (*p*-OH-P). The retention time for *m*-hydroxyphenobarbital is indicated by an arrow. Acid-hydrolysed sample

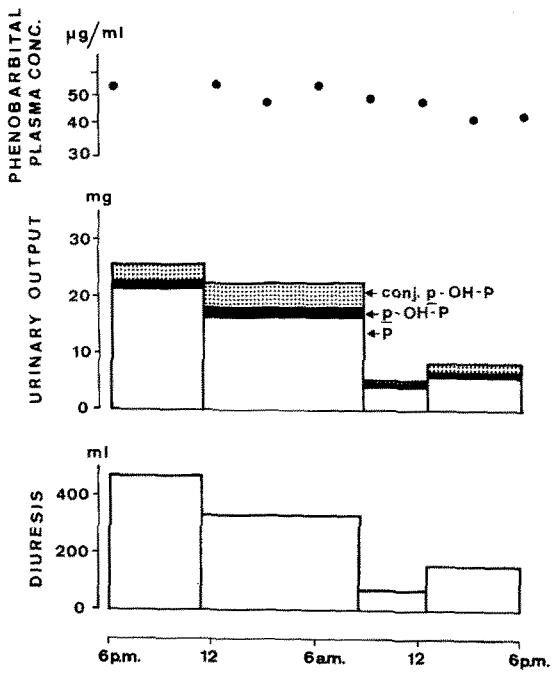

Fig. 5. The plasma concentration of phenobarbital and the urinary output of phenobarbital (P), *p*-hydroxyphenobarbital (*p*-OH-P) and conjugated *p*-hydroxyphenobarbital (conj. *p*-OH-P) in patient A.K. during 24 hours. The daily dose of 125 mg was given at 6 p.m.; almost 60 mg was found in the urine as unchanged phenobarbital and metabolites

Identification of Conjugate

As shown in Table 2, β-glucuronidase treatment of urine samples from three phenobarbital-treated patients gave a considerable increase in the height of the *p*-hydroxyphenobarbital peak. Comparison with the results of acid hydrolysis led to the conclusion that *p*-hydroxyphenobarbital was conjugated mainly with glucuronic acid.

Metabolic hydroxylation of an aromatic ring may involve an epoxide intermediate (9, 12, 18) which can then form a dihydrodiol and result in the formation of phenolic metabolites. Thus, the principal metabolites of phenobarbital and the related compound diphenylhydantoin are the *p*-hydroxylated compounds (4, 5). In the case of phenobarbital, diphenylhydantoin and methsuximide the different dihydrodiols are known as metabolites (10, 7, 12) in man; for methsuximide the dihydrodiol is the major metabolite (12). The possible formation of *m*-hydroxylated metabolites of these different anticonvulsants must also be taken into account. Small amounts of a metabolite tentatively identified as *m*-hydroxyphenobarbital have been found in the rat (11).

Treatment of a urine sample with hydrochloric acid in order to liberate phenolic metabolites from their conjugates may transform any dihydrodiol present to *m*- and *p*-hydroxylated metabolites (7). Therefore, metabolic studies of the compounds in question must involve specific enzymatic cleavage of the conjugate (2).

In the GC-analysis of urine samples from patients, there was a small peak with a retention time corresponding to *m*-hydroxyphenobarbital. This substance, assumed to be *m*-hydroxyphenobarbital, may have been formed during the analysis (hydrochloric acid treatment, GC) or by the patients as a metabolite. To reveal any metabolically formed *m*-hydroxyphenobarbital, urine samples were treated enzymatically with β-glucuronidase, as described above. The results presented in Table 2 do not exclude the occurrence of the *m*-hydroxylated compound. However, the treatment in the GC-system made it difficult to conclude that *m*-hydroxyphenobarbital was originally present. Enzymatic treatment of a urine sample from patient A.K. and qualitative analysis by use of TLC in two systems (Table 3) and with GC gave no evidence of the presence of *m*-hydroxyphenobarbital in the sample.

As mentioned above, after acid hydrolysis the peak measured as *p*-hydroxyphenobarbital probably also contained *p*-hydroxyphenobarbital emanating from the dihydrodiol. However, the amount of this dihydrodiol metabolite in the rat, guinea pig and in man is known to be small (10).

CLINICAL APPLICATION

Phenobarbital is extensively used for the treatment and prophylaxis of convulsions in infants and has also been introduced recently as a prophylactic for neonatal hyperbilirubinaemia. The rate of its disappearance from plasma has been shown to vary with age (14), but variation in its metabolism with age has not been examined. This problem is now under investigation.

Application of the present method is illustrated in Figs. 4 and 5. A gas chromatogram of urine from a nine-year-old boy receiving phenobarbital daily as a prophylactic anticonvulsant is shown in Fig. 4. His urinary output of the drug and its main metabolites during a 24-hour-period is represented graphically in Fig. 5.

Acknowledgements. This study was supported by grants from the Arméns läkemedelsanslag (N.K.) and from Föreningen Margarethahemmet, Sigtuna (N.K., S.A., B.J., L.O.B.), the Swedish Medical Research Council (L.O.B.) and the Foundation for Child Health, New York (L.O.B.).

We are deeply indebted to Dr. Bengt-Arne Persson for his most valuable suggestions. The skilful assistance of Miss Margit Berg and Mrs. Siv Leander is very much appreciated.

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