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MICROASSAY FOR PRIMIDONE AND ITS METABOLITES PHENYLETHYLMALONDIAMIDE, PHENOBARBITAL AND *p*-HYDROXYPHENOBARBITAL IN HUMAN SERUM, SALIVA, BREAST MILK AND TISSUES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING SELECTED ION MONITORING

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SUMMARY

A method for the quantitative determination of primidone and its metabolites phenobarbital, phenylethylmalondiamide (PEMA) and hydroxyphenobarbital (free and conjugated) in serum, urine, saliva, breast milk and tissue has been developed. Following the addition of the methyl analogues of primidone, phenobarbital and PEMA as internal standards and of saturated ammonium sulphate, the samples (5—100 μl) were extracted twice with ethyl acetate—benzene (20:80). The extracts were divided into two equal portions; one portion was ethylated by Greeley's method for the analysis of primidone, phenobarbital and hydroxyphenobarbital, while the other was trimethylsilylated for the analysis of primidone and PEMA. A gas chromatographic—mass spectrometric system was used for the analysis of the derivatized extracts. Linear calibration curves were obtained in the concentration range studied (between 100 ng/ml and 30 μg/ml). The recoveries of the drugs were between 80 and 93%. The relative standard deviations were between 3.2 and 5.9% (100-μl serum samples containing 1 μg/ml of the drugs). The lower detection limits were found to be between 1.4 and 3.7 ng/ml using serum samples of 100 μl.

These methods have been applied to the study of the placental transfer and neonatal disposition of primidone and its metabolites in the human.

INTRODUCTION

Pharmacokinetic studies of placental transfer and neonatal metabolism (for recent reviews cf. ref. 1), particularly if low levels of metabolites are to be determined, require extremely sensitive methods of analysis, since only small amounts of tissue and body fluids are available. The methods presently available for pharmacokinetic studies of primidone are either directed only to the

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analysis of the parent drug for the purpose of anticonvulsant monitoring [2—12], or are too insensitive and require relatively large amounts of sample [13—17].

We have therefore developed methods for the assay of primidone and its metabolites (see Fig. 1) phenobarbital, phenylethylmalondiamide (PEMA) and hydroxyphenobarbital (free and conjugated) in small volumes (5—100 μl) of human serum, saliva, urine, breast milk and fetal tissues. Analysis was performed by a gas chromatograph—mass spectrometer—computer system (GC—MS—computer) operated in the selected ion monitoring mode (mass fragmentography). The sensitivity and selectivity of the GC—MS methods enabled us to use very simple and rapid sample handling procedures.

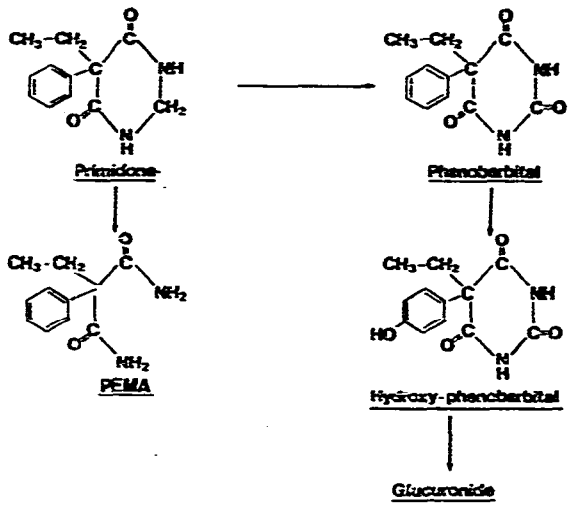

Fig. 1. Metabolic scheme of primidone.

MATERIALS AND METHODS

Chemicals and reagents

The internal standards (i.s.) used were: i.s. 1, 4-methylprimidone, for primidone; i.s. 2, 5-ethyl-5-(*p*-tolyl)barbituric acid, for phenobarbital, and i.s. 3, 2-ethyl-2-(*p*-tolyl)malonic acid diamide for PEMA, all obtained from EGA-Chemie, Steinheim, G.F.R.

These compounds were dissolved in methanol (1 mg/ml) and the methanolic solutions were added to benzene—ethyl acetate (both "Nanograde" from Promochem, Wesel, G.F.R.) (80:20, v/v) to a final concentration of 300 ng/ml each. Pyridine ("getrocknet"), N,N-dimethylacetamide and ethyl iodide were obtained from Merck (Darmstadt, G.F.R.), β-glucuronidase/aryl sulfatase from Boehringer (Mannheim, G.F.R.). Regisil RC-2, bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane from Regis (Hedinger, Stuttgart, G.F.R.), and tetramethylammonium hydroxide (20% in methanol) from EGA-Chemie.

Hydrolysis of conjugated metabolites

To 5—100 μl serum or urine were added an equal volume of 1 *N* sodium acetate buffer (pH 5.0) and 10 μl (per 100 μl sample volume) of β-glucuronidase/aryl sulfatase (5 U/ml, Boehringer). This mixture was slowly agitated at 37° for 16—20 h and then processed as described below.

Extraction procedure

A sample (5—100 μl) of serum, urine, saliva, breast milk or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf reaction vessel, diluted to 100 μl and 100 μl of saturated ammonium sulfate and 1 ml of benzene—ethyl acetate (80:20, v/v) containing the internal standards were added. The tube was shaken for 15 min and then centrifuged for 2 min in a 5012 Eppendorf centrifuge. One 400-μl portion of the supernatant organic phase was transferred to another 1.5-ml reaction vessel, and a further 400-μl portion to a 1.5-ml glass serum vial. The extraction was repeated using 1 ml of solvent mixture without internal standards. The combined extracts were evaporated at 40° under a stream of nitrogen.

The samples in the conical Eppendorf reaction vessels were ethylated according to the procedure of Greeley [18] by adding 40 μl dimethylacetamide, 5 μl 20% tetramethylammonium hydroxide and, after vortexing, 10 μl ethyl iodide. The samples were centrifuged for 2 min and 2 μl of the clear supernatant were injected into the GC—MS system. The samples in the glass vials were trimethylsilylated with a mixture of 20 μl pyridine and 30 μl Regisil RC-2 at 60° for 1 h. A 2-μl aliquot of the clear solution was injected into the GC—MS system.

GC—MS—computer analysis

A Perkin-Elmer F-22 gas chromatograph was coupled via a Watson-Biemann separator to a Varian MAT CH-7A mass spectrometer. A 2-m glass column (6 mm O.D. and 2.5 mm I.D.) packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q was used (Applied Science Labs., Serva, Heidelberg, G.F.R.). The ethylated samples were injected at 220°, and the trimethylsilylated samples at 190°. After an initial period of 1 min the column temperature was raised 30° at a rate of 10°/min. During this period the derivatives to be measured eluted from the GC column. The temperature was then raised quickly (20°/min) to 270° to elute the accompanying substances. During this time, the selected ion records were plotted and the peak height ratios calculated. After the GC column had cooled down to the initial temperature, the next sample was injected. Six to eight samples were analyzed in this way within one hour.

The mass spectrometer was controlled by the SS-100 Varian data system and operated in the selected ion monitoring mode. The following ions were selected for the ethylated samples: *m/e* 246 (primidone), 260 (phenobarbital and i.s. 1 for primidone), 274 (i.s. 2 for phenobarbital and hydroxyphenobarbital) and 275 (hydroxyphenobarbital). For the trimethylsilylated samples: *m/e* 232 (primidone), 235 (PEMA), 246 (i.s. 1 for primidone), and 249 (i.s. 3 for PEMA). The results were displayed on a Textronix 4010 oscilloscope and plotted on a Textronix hardcopy unit.

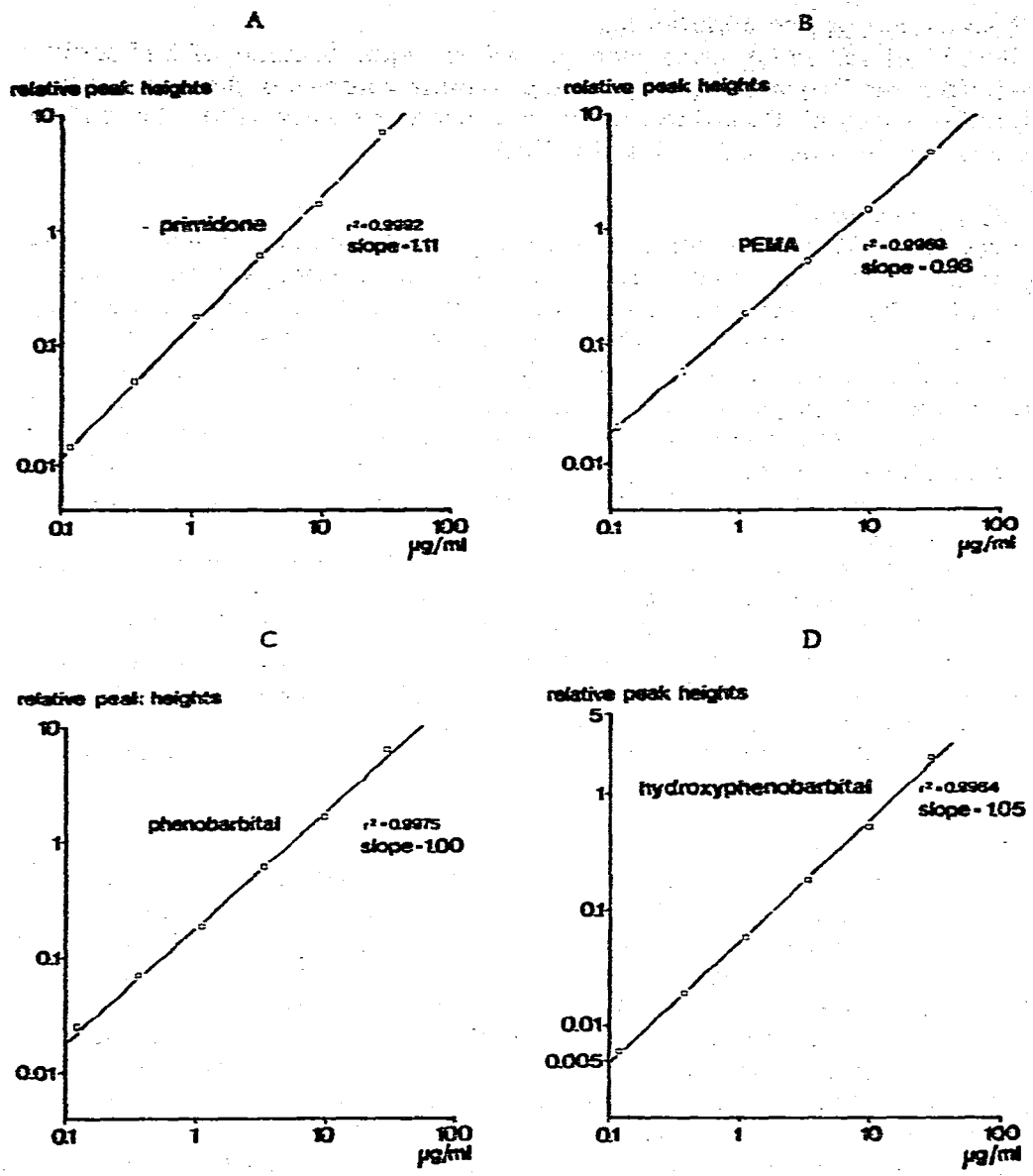

Fig. 2. Double logarithmic plots of the peak heights of the selected ions from derivatives of primidone and its metabolites (relative to the corresponding internal standards used; see Experimental) vs. amounts present in 100-µl serum samples. (A) *m/e* 232, the M−130 of the bis-trimethylsilylated primidone derivative; (B) *m/e* 235, the M−115 of the bis-trimethylsilylated PEMA derivative; (C) *m/e* 260, the M−28 ion of the diethylated phenobarbital derivative; and (D) *m/e* 275, the M−57 ion of the triethylated hydroxyphenobarbital derivative. The calibration graph for the ethylated primidone, monitoring the ion *m/e* 246 (M−28) of the diethylated derivative, is not shown.

Quantitation

Standard calibration graphs were obtained by the analysis of 100-μl portions of drug-free human serum, to which were added known amounts of primidone, phenobarbital, hydroxyphenobarbital and PEMA (Fig. 2). These samples were processed as described above. The stored samples were kept frozen at −30° and the concentrations of primidone and phenobarbital present were periodically checked by comparative analysis of antiepileptic drug calibrator serum standards (Emit-aed., Merck).

RESULTS AND DISCUSSION

We have evaluated a number of solvents for the extraction of the drugs, for example chloroform, ethyl acetate, benzene, methylene chloride and some of their mixtures. A mixture of benzene—ethyl acetate (80:20) and saturated ammonium sulfate for "salting out" [19,20] led to good yields (Table I). Furthermore, this mixture is less dense than water which simplified the transfer of the organic phase. Most importantly, low baselines and clean selected ion records (mass fragmentograms) were obtained even after conjugated substances had been released by glucuronidase/arylsulfatase.

TABLE I

RECOVERIES, RELATIVE STANDARD DEVIATIONS AND LOWER DETECTION LIMITS

Compound	Derivatization*	Recovery of isolation procedure (%)	Lower detection limit** (ng/ml)	Relative standard deviation*** (%)
Primidone	Ethyl.	89	2.5	3.9
	TMS		2.2	5.9
Phenobarbital	Ethyl.	89	1.4	3.2
p-Hydroxyphenobarbital	Ethyl.	80	3.7	4.1
PEMA	TMS	93	1.7	3.9

*Ethyl., ethylation according to Greeley [18]; TMS, trimethylsilylation.
**Signal-to-noise ratio = 2 using 100-μl serum samples.
***Twelve samples (100 μl serum) containing 1 μg/ml of each compound were analyzed as described.

Primidone, phenobarbital and hydroxyphenobarbital were ethylated, PEMA and primidone were trimethylsilylated prior to analysis by GC—MS. Similar results were obtained for primidone with either method. The derivatization reactions were performed by simply adding the reagents. After an appropriate reaction time the mixture could be directly injected into the GC—MS system. The derivatives were found to be stable: similar results were obtained by analyzing the same samples on two consecutive days.

The mass spectra generated by electron impact are shown in Fig. 3. All intense ions above *m/e* 200 were tried but those indicated in the legend of Fig. 2 were selected in the final method by the criteria of favourable baselines and

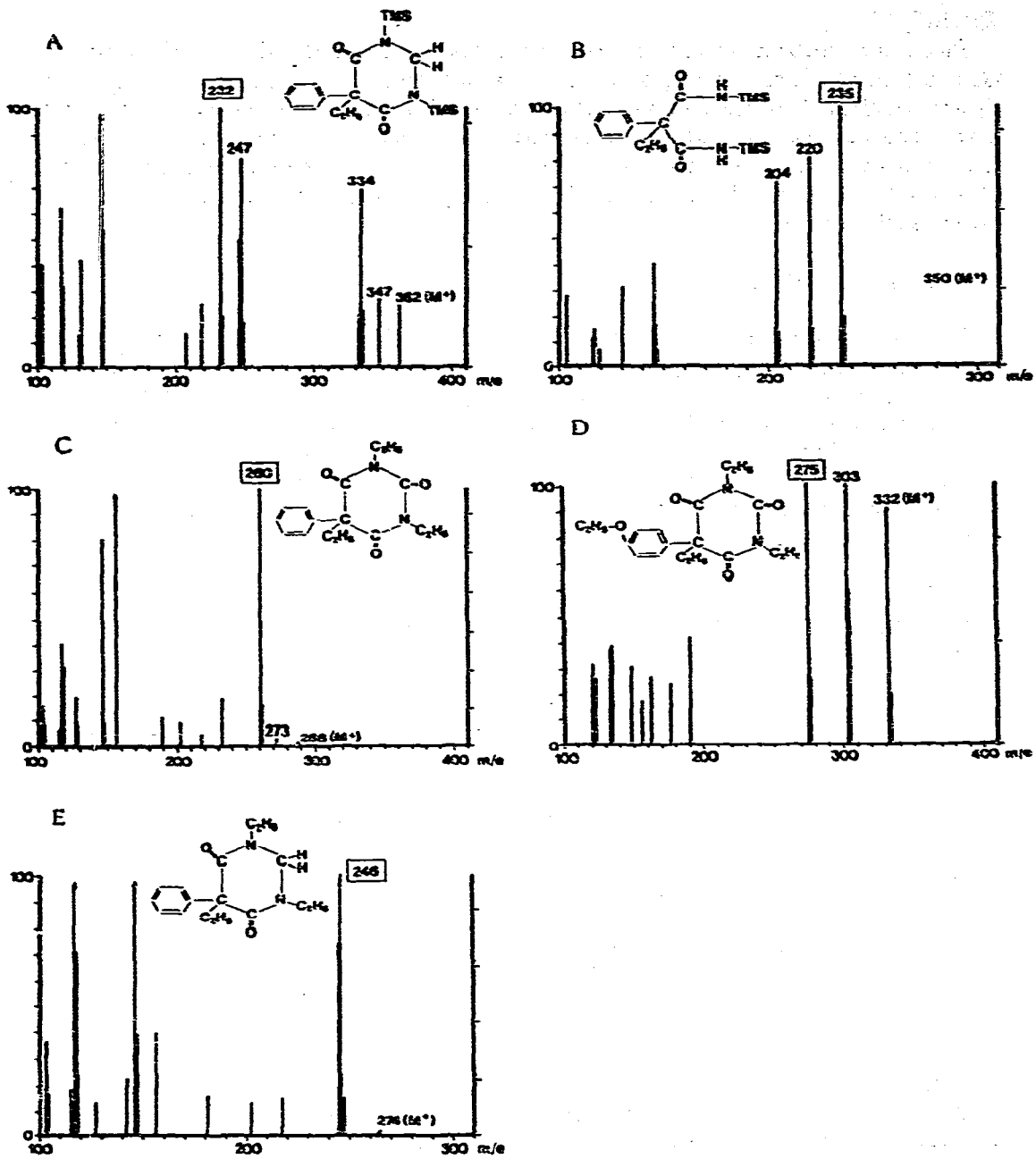

Fig. 3. Electron impact mass spectra of the trimethylsilylated derivatives of primidone (A) and PEMA (B) as well as the ethylated derivatives of phenobarbital (C), hydroxyphenobarbital (D), and primidone (E).

lower detection limits. The ions selected for the internal standards were 14 mass units higher than those of the corresponding drugs.

The reproducibility of the method was evaluated by the analysis of 12

100-μl serum samples which contained 1 μg/ml of the substances studied. The relative standard deviations are presented in Table I. The lower detection limits were found to be in the low ng/ml range using 100-μl sample volumes (Table I). Linear dependence of the peak height ratios vs. amounts of drug and metabolites added was found throughout the calibration range studied (between 100 ng/ml and 30 μg/ml corresponding to 10 ng per sample and 3 μg per sample, the square of the correlation coefficient r^2 exceeded 0.99 (Fig. 2).

The methods developed for the assay of primidone and its metabolites were well suited for the analysis of small samples of saliva (Fig. 4), serum (Fig. 5), urine, breast milk and tissue homogenates. Owing to the high sensitivity of the

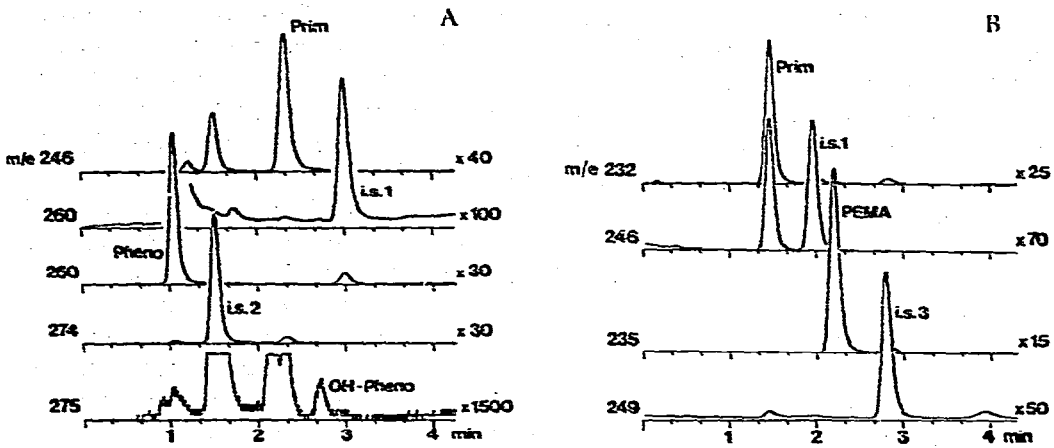

Fig. 4. Selected ion records of a 100-μl saliva sample of an epileptic woman treated with primidone (daily dose: 1125 mg). (A) Ethylated portion of the sample extract and (B) trimethylsilylated portion of the sample extract. Concentrations found: primidone (Prim), 4.9 μg/ml; phenobarbital (Pheno), 8.6 μg/ml; PEMA, 8.0 μg/ml; hydroxyphenobarbital (OH-Pheno), 50 ng/ml. For the description of the internal standards (i.s. 1—3) see Experimental.

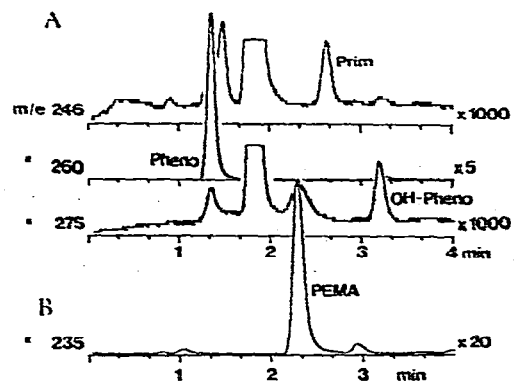

Fig. 5. Selected ion records of a 50-μl serum sample from a human neonate two days after birth. Last maternal dose: 500 mg primidone 4 h before delivery. (A) Ethylated portion of serum extract and (B) trimethylsilylated portion of serum extract. Concentrations found: primidone (Prim), 0.14 μg/ml; phenobarbital (Pheno), 22.4 μg/ml; PEMA, 8.5 μg/ml; hydroxyphenobarbital (OH-Pheno), 0.25 μg/ml.

method, low levels of metabolites such as hydroxyphenobarbital could be determined (Figs. 4 and 5). The highly specific and sensitive GC—MS method allowed us to use simple and rapid sample handling procedures (see Experimental).

We have applied these methods to the study of the placental transfer of primidone and its metabolites during early pregnancy and at term. Analysis of human fetal tissues obtained following interruption of pregnancy during the first trimester indicated that the compounds analyzed were present in comparable amounts in fetal tissues and maternal serum [21]. At term, the levels of primidone and metabolites in cord blood samples also approached those present in the corresponding blood samples of the epileptic mothers who had been treated by primidone for seizure control [22]. Thus, all compounds studied readily passed the placental membranes, both during early pregnancy and at term. Interestingly, the conjugated *p*-hydroxyphenobarbital was already present in neonatal blood at birth. The concentration ratio of total hydroxyphenobarbital (free and conjugated) to unconjugated hydroxyphenobarbital in the neonatal blood was found to be approximately 2.

The biological half-lives of primidone during the first five postnatal days of four neonates studied were found to vary widely between 6 and 60 h [22]. This range overlapped with that of normal adults (6—12 h) [23,24]. The concentrations of PEMA decreased only slightly during the first five postnatal days, while those of phenobarbital even showed small but significant increases. Apparently PEMA and phenobarbital were continuously formed by neonatal metabolism of primidone. Since one or both of these metabolites may be biologically active, the determination of their levels in the neonate is of great importance.

The analysis of saliva samples from epileptic patients treated with primidone confirmed earlier reports on the usefulness of monitoring the concentrations of primidone and phenobarbital present in this fluid [25—27]. In addition, we have also measured the saliva concentrations of PEMA; the saliva:plasma concentration ratios found indicated very low plasma protein binding for this metabolite [28]. Also hydroxyphenobarbital was found in saliva in low concentrations (Fig. 4). Monitoring of metabolites in saliva may be useful for the study of drug metabolism in man [28].

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