Journal of Chromatography, 115 (1975) 493–500
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CHROM. 8586

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF BARBITURATES AND PHENYTOIN IN SERUM AND BLOOD

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(Received July 1st, 1975)

SUMMARY

A method is described for the measurement of blood, serum and/or plasma levels of hexobarbital, phenobarbital, cyclobarbital and phenytoin by ultraviolet reflectance photometry on thin-layer chromatograms. The lowest concentrations measured were 0.3–0.7 μg/ml. The accuracy was similar to that of gas chromatographic procedures. For phenytoin determinations 5-(*p*-methylphenyl)-5-phenylhydantoin may be used as internal standard. The method has been applied to clinico-pharmacological assays, to the measurement of cyclobarbital elimination in man following a therapeutic dose, and to the study of phenobarbital kinetics in rats using serial blood samples.

INTRODUCTION

Direct densitometry of thin-layer chromatograms is increasingly used as an alternative to gas–liquid chromatography (GLC) for the determination of xenobiotics. In the field of barbiturate and phenytoin analysis in biological fluids, the majority of recently published methods involve GLC, though attempts have been made to make use of the good resolving power of thin-layer chromatography (TLC) for these substances. In the procedures described up to now, TLC was followed by removal of the compounds from the plate and quantitation by photometry in solution[1,2] or GLC[3,4]. Alternatively, the plates were stained and the quantities contained in the samples estimated by visual comparison with reference spots[5–7].

Since instruments are available for reflectance photometry on thin-layer plates in the ultraviolet region, a possibility exists for the measurement of barbiturates and phenytoin (diphenylhydantoin, DPH) by direct scanning of the chromatograms. In fact, appropriate procedures have been described for the measurement of barbiturates, but they were only designed and recommended for the analysis of pharmaceutical preparations[8,9]. The use of a similar method in a pharmacokinetic study has been reported for primidone[10] and in clinico-pharmacological investigations for carbamazepine[11].

The present report will describe a procedure utilizing photometry of TLC

plates by which therapeutic concentrations of DPH and various barbiturates can be measured in blood, plasma or serum. The method has been applied to pharmacokinetic studies including those with multiple blood sampling from rats.

EXPERIMENTAL

Chemicals

Barbiturates and phenytoin in the forms of their salts and also partly in the free acid forms were obtained as commercial preparations. Where the free acids were not available, they were librated with potassium dihydrogen orthophosphate solution and crystallized from water (hexobarbital) or benzene (cyclobarbital). 5-(*p*-Methylphenyl)-5-phenylhydantoin (MPPH) (EGA Chemie, Steinheim, G.F.R.) was donated by Dr. Brügmann (Universitäts-Kinderklinik, Tübingen, G.F.R.).

Standard solutions for TLC were prepared by dissolving the free acids to a concentration of 0.1 or 0.2 mg/ml in 1,2-dichloroethane.

The MPPH internal standard was prepared as follows: 0.5 ml of a MPPH solution in methanol (5 mg/ml) was mixed with 12.5 ml serum and made up to 25 ml with 0.9% NaCl. The concentration then was 0.1 mg/ml.

Organic solvents were of ordinary grade purity and were distilled before use.

Biological samples

From epileptic patients under oral treatment with DPH, phenobarbital and/or primidone blood was drawn usually before administration of the morning dose. The serum was stored at 4° for not more than two weeks or at −20° for not more than eight weeks.

A healthy female volunteer (52 kg) was administered a tablet of 0.2 g cyclobarbital calcium (Phanodorm®, Bayer, Leverkusen, G.F.R.) on three successive days at 11 p.m. Blood samples were drawn on the following days between 8 a.m. and 8 p.m. and heparinized. The plasma was stored at −20°.

Female rats (200–230 g) were treated for several days with phenobarbital (for details see under Results). Under light ether anaesthesia 0.1–0.5 g of blood was obtained from the retrobulbar venous plexus with the aid of a heparinized capillary[12]. Collection was made in a pre-weighed tube containing 0.01 ml heparinized saline (5 mg/ml), the quantity being determined by weighing. It was immediately haemolyzed by the addition of 1 ml water.

Procedure

Extraction of hexobarbital, phenobarbital and DPH. Serum (1 ml) or haemolyzed blood was mixed with 1 *M* potassium dihydrogen orthophosphate solution (0.1 ml) and 0.4 g of ammonium sulphate and extracted twice with 1.5 ml of diethyl ether. Phase separation was achieved by centrifugation at 300 × *g* for 6 min. The combined ether phases were extracted with 1 ml of 0.02 *M* sodium hydroxide solution and removed as completely as possible. The aqueous layer was acidified with 0.1 ml of 1 *M* potassium dihydrogen orthophosphate solution and extracted twice with 1 ml of ethyl acetate. The extract was evaporated at 30–35° with a stream of nitrogen in a 3-ml tube with a ground-glass neck and a conical tip. The extracted material was concentrated in the tip by rinsing the sides with 0.2 ml ethyl acetate and evaporating

igain. In some instances, 2 ml of serum were analyzed with doubling of the 1 *M* potassium dihydrogen orthophosphate solution and the ammonium sulphate used in he first extraction.

For the analysis of DPH, MPPH internal standard (0.1 ml, corresponding to 10 μg) was added per milliliter of serum. Where sufficient serum was not available, 0.5 ml was mixed with 0.05 ml of internal standard and worked up in the same manner.

Extraction of cyclobarbital. To plasma (2 ml) was added 1 *M* potassium dihydrogen orthophosphate solution (0.2 ml) and 0.8 g of ammonium sulphate, and extraction was carried out with two 1.5-ml portions of diisopropyl ether. The organic phases were processed as described above for diethyl ether.

TLC. Glass plates (20 × 20 cm) pre-coated with non-fluorescent silica gel 60 (Merck, Darmstadt, G.F.R.) were washed to the upper edge with the solvent given below, dried and stored in a closed tank. Before use they were activated for 10 min at 103°. Samples were spotted on a line 1.5 cm above the lower edge 2 cm apart. Serum, plasma or blood extracts were dissolved in a defined volume (usually 40, 50 or 100 μl) of dichloroethane and aliquots were spotted with the aid of capillary pipettes (Brand, Wertheim, G.F.R.). The size of the aliquot was chosen in such a way that quantities of 1–2 μg of the substances could be expected to be present. Except in DPH analyses including an internal standard, samples and standard solutions were spotted in alternating fashion. The quantities of standard compounds mostly varied between 0.4 and 4 μg.

The solvent was chloroform–isopropanol (5:1) and the atmosphere was saturated with ammonia vapour by placing into the tank a flat trough (16 × 2 cm) which contained 3 ml of 25% ammonia. Pre-equilibration of the system was performed for 1 h. The plates were developed to a height of 12 cm above the origin and dried for 30 min. The R_F values obtained in this system are given in Table I.

Photometry. Light remission was measured with a Chromatogramm-Spektralphotometer (Zeiss, Oberkochen, G.F.R.) coupled to a potentiometric recorder (Servogor S; Metrawatt, Nürnberg, G.F.R.). The wavelength was 240 nm for barbiturates and 230 nm for DPH. The plate was moved at a speed of 120 mm/min under a slit of 14 × 2 mm. The chart paper speed was set at 120 mm/min. Peak areas were obtained as the products of height and width at 50% height. DPH in samples spiked with MPPH was determined from the peak area ratio using a calibration curve (Fig. 2). In analyses of barbiturates and DPH without an internal standard, individual calibration curves were constructed from the standard values run on each plate.

Recovery experiments. Aqueous solutions of the drug salts were added to the appropriate blank material, and the samples were processed as described above. In the same way a calibration curve (Fig. 2) for the DPH determination using MPPH as internal standard was obtained.

RESULTS

Separation

From the data in Table I and Fig. 1 it can be seen that MPPH introduced as internal standard for DPH determination by GLC[13,14] was also separated from DPH in TLC and can therefore be used here, too, as a standard. The five barbiturates investigated were separated from one another except for cyclobarbital and barbital.

TABLE I

R_F VALUES IN TLC ON SILICA GEL WITH CHLOROFORM–ISOPROPANOL (5:1) IN A CHAMBER SATURATED WITH AMMONIA VAPOUR

Compound	*R_F value*
Hexobarbital	0.78
Pentobarbital	0.57
Cyclobarbital	0.40
Barbital	0.34
Phenobarbital	0.20
DPH	0.52
MPPH	0.61

Control experiments

Extracts from blank blood or plasma produced a peak at the origin and a high absorption at the front of thin-layer chromatograms, but no peaks interfering with the substances to be analyzed (Fig. 1). The same was true for human plasma when the individual had been treated with nitrazepam or primidone, except for the presence of phenobarbital in the latter case. Small peaks with R_F values above 0.7 were seen following ingestion of carbamazepine or caffeine.

The sensitivity and reproducibility of the method can be derived from the results of recovery experiments (Table II). Down to 0.2 μg of hexobarbital and phenobarbital and 0.8 μg of cyclobarbital have been analyzed without a loss in accuracy. The smallest quantity producing a measurable peak was 0.1 μg.

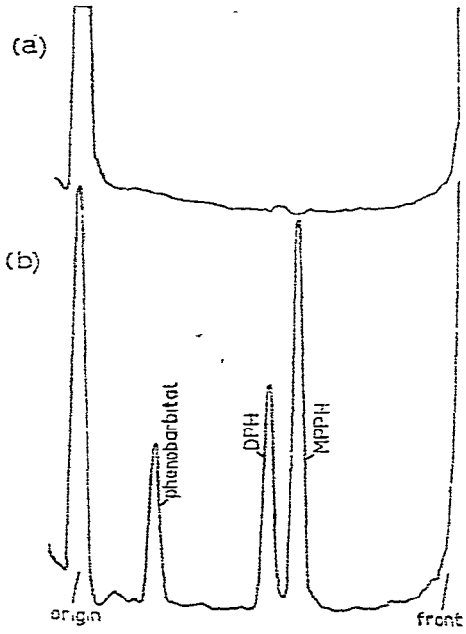

Fig. 1. Records obtained upon reflectance photometry at 230 nm of thin-layer chromatograms of (a) the extract of a blank serum corresponding to 0.4 ml; (b) a serum extract corresponding to 0.2 ml from a patient being treated with DPH and phenobarbital. MPPH was added as internal standard.

TABLE II

RECOVERIES OF BARBITURATES AND DPH (WITHOUT INTERNAL STANDARD) FROM RAT BLOOD OR HUMAN SERUM OR PLASMA

Compound	Material	Concentration (μg/ml)	Sample volume (ml)	Solvent	Recovery (Mean ± S.D.)	n
Hexobarbital	blood	2–34	0.1	diethyl ether	94 ± 5	8
	blood	0.4–77	0.5	diethyl ether	81 ± 8	10
Phenobarbital	blood	2–25	0.1	diethyl ether	100 ± 4	8
	blood	0.8–70	0.5	diethyl ether	81 ± 6	12
	serum	13–51	1	diethyl ether	84 ± 4	4
	serum	6.5–25	2	diethyl ether	74 ± 3	3
	serum	0.3–3	2	diisopropyl ether	73 ± 5	6
Cyclobarbital	plasma	0.4–4	2	diisopropyl ether	78 ± 1	6
DPH	serum	0.7–34	2	diethyl ether	72 ± 5	13

Due to evaporation of the ammonia from the plate, the barbiturate peak heights decreased with time, since free acids exhibiting lower absorbances were liberated. However, during the time of scanning, *i.e.* from 30–45 min after development, the decrease amounted to not more than 4%.

The very good reproducibility of DPH measurements with MPPH as internal standard is reflected by the calibration curve (Fig. 2). Duplicate analyses on patient serum containing 3–30 μg/ml resulted in an average deviation of 3.7% ($n = 26$). In five cases, the results of analyses on 0.5- and 1-ml serum samples were compared and found to deviate on an average by 4.3%. Five duplicate determinations of cyclobarbital in plasma (0.5–2 μg/ml) gave an average deviation of 5.4%.

The results of DPH and phenobarbital measurements were in good agreement with those obtained by a GLC method[14] on the same samples[15].

Pharmacokinetic studies

As an example for the application of the method, plasma levels of cyclobarbital

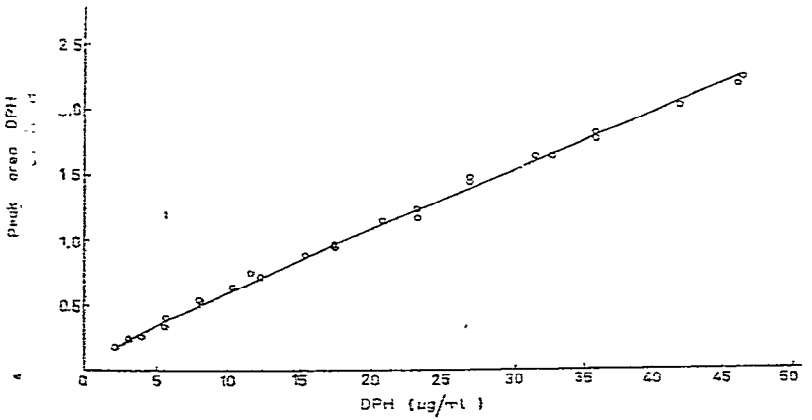

Fig. 2. Calibration curve for the determination by TLC of serum DPH using MPPH as internal standard.

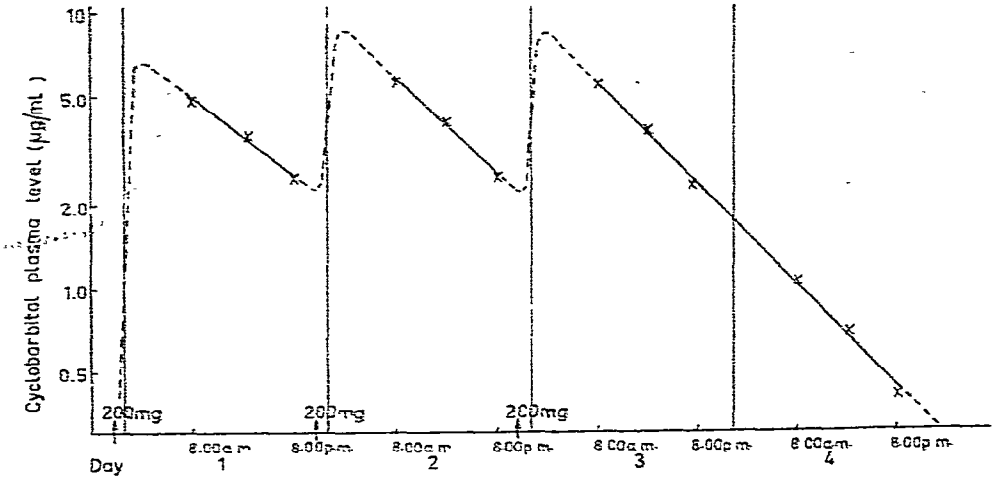

Fig. 3. Cyclobarbital kinetics in the plasma of a volunteer who ingested 200 mg of cyclobarbital calcium at 11.00 p.m. on days 0, 1 and 2. Plasma concentrations are depicted on a logarithmic scale.

during and following repeated ingestion of therapeutic doses are shown in Fig. 3. A strictly exponential decline was observed, the half-life after ingestion of the last dose being 10.0 h. Accordingly, a slight cumulation only was apparent from day 1 to days 2 and 3.

Phenobarbital concentrations in rat blood were compared in the steady-state during treatment via the drinking water or by once-daily gavage. Both treatment

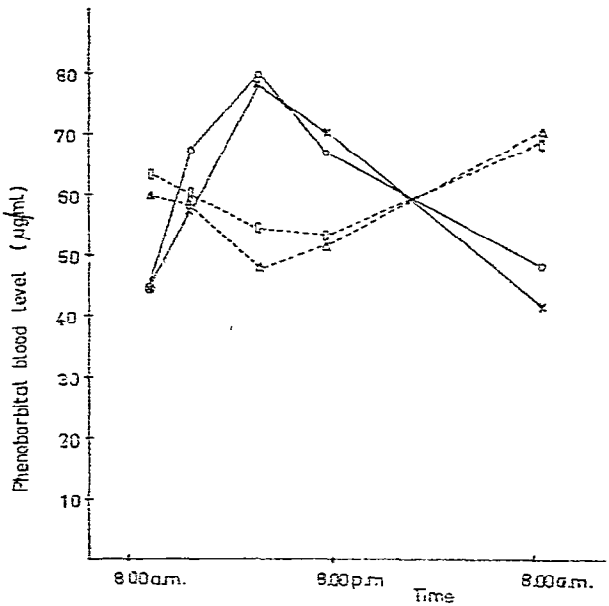

Fig. 4. Whole blood phenobarbital in female rats being treated for four days either with 0.1% phenobarbital in the drinking water (– – – –) or with 50 mg/kg phenobarbital by gavage each day a 9.30 a.m. (———). ○, ×, △ and □ refer to four individual rats.

chedules produced comparable blood levels, but the variations during the day were omewhat larger in the animals treated by gavage (Fig. 4). In the same way, hexobarbital blood levels could be determined at short intervals after intraperitoneal injection of 90 mg/kg into female rats.

DISCUSSION

The experiments described show that TLC followed by direct UV photometry provides a method of sufficient sensitivity and accuracy for clinico-pharmacological and pharmacokinetic investigations on barbiturates and DPH. The chromatography was preceded by an extraction procedure in which smaller volumes of solvent were used than in the majority of existing methods. In studies where the presence of additional drugs can be excluded, the procedure can be further simplified by omission of the back-extraction into sodium hydroxide solution, and in barbiturate analyses a second barbiturate may be added as an internal standard[16]. The sensitivity of the photometric recording was increased by a factor of about 2 when the barbiturates were converted to their salts by spraying with potassium hydroxide solution[9]. When sufficient material was available, this step was avoided, since it tended to produce uneven base lines. Exposure of the plates to ammonia vapour shortly before scanning[8] did not prove advantageous in our case, since the large peaks produced faded rapidly. Under the conditions used here, the process of fading was close to completion and the decline during the time required for scanning did not influence the results appreciably.

Concerning sensitivity and reproducibility, the reported method is comparable to the best available GLC procedures[13,17–22], though the quantity of material required is higher than in methods specially designed for the use in pediatrics[23,24].

The DPH analyses using an internal standard have been carried out to a lower limit of 2 μg/ml. Smaller concentrations down to 0.5 μg/ml would be measurable upon addition of smaller quantities of the internal standard.

That the cyclobarbital plasma level could be followed up to 44 h corresponding to more than four half-lives after ingestion of a therapeutic dose, seems to prove the potential of quantitative TLC as a tool in the establishment of barbiturate pharmacokinetics.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. G. Brügmann, Universitäts-Kinderklinik Tübingen, for valuable advice and Dr. W. Dünges, Pharmakologisches Institut, Universität Mainz, for a helpful discussion. Thanks are due to Bayer AG, Leverkusen, for supplying cyclobarbital calcium. The work was supported by the Deutsche Forschungsgemeinschaft.

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