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Method for the determination of 5,5-diphenylbarbituric acid and its separation from 1,3-dimethoxymethyl-5,5-diphenylbarbituric acid in plasma by high-performance liquid chromatography

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Abstract

A method is described for the measurement of 5,5-diphenylbarbituric acid in plasma using high-performance liquid chromatography with UV detection. Briefly, the compounds are separated on a C_{18} reversed-phase column using a mobile phase of 50 m*M* sodium acetate (pH 4.5) and methanol. The flow-rate is 1.0 ml/min and 25 μl are injected and detected at 215 nm. The method is specific and sensitive in the range of concentrations tested, with a limit of quantification of 0.25 μg/ml. The calibration curves are linear for concentrations between 0.25 and 10 μg/ml. Intra-day and inter-day coefficients of variation are less than 8.5 and 10.5%, respectively, over the linear range. Intra-day and inter-day bias are less than 7.0 and 8.0%, respectively. A pharmacokinetic study conducted in male Beagle dogs administered 10 mg/kg of 1,3-dimethoxymethyl-5,5-diphenylbarbituric acid or 8 mg/kg of 5,5-diphenylbarbituric acid intravenously demonstrates the utility of this method. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: 5,5-Diphenylbarbituric acid; 1,3-Dimethoxymethyl-5,5-diphenylbarbituric acid

1. Introduction

5,5-Diphenylbarbituric acid (DPB) was initially prepared as a potential sedative in the 1930s [1,2] but, unlike many of the barbiturates synthesized previously or subsequently, the compound lacked sedative hypnotic properties and, hence, was not developed for such purposes. However, subsequent evaluations of DPB in experimentally induced seizures demonstrated that DPB was an anticonvulsant in cats [3,4], rabbits [5], mice [6,7] and rats [8]. Electrophysiological studies have shown that the drug suppresses high frequency repetitive neural discharges on cat motor nerve terminals [9] in a manner similar to that of phenytoin [10]. Thus, DPB, which may be viewed as being chemically derived from both phenytoin and phenobarbital (see Fig. 1), exhibits anticonvulsant properties with seemingly little neurotoxicity and may be a promising agent for the treatment of epilepsy.

We have recently examined 1,3-dimethoxymethyl-5,5-diphenylbarbituric acid (DMMDPB) as an anticonvulsant in the rat. Our work indicates that orally administered DMMDPB is converted to DPB in the rat and the dog and that it therefore has potential as a long-acting, non-sedating, anti-epileptic in humans

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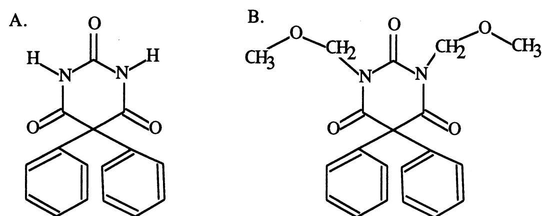

Fig. 1. Structures of diphenylbarbituric acid (A) and dimethoxymethyl diphenylbarbituric acid (B).

[11]. We present here a high-performance liquid chromatography (HPLC) method for the assay of DPB in plasma, which effectively separates DPB from DMMDPB. We have tested the utility of this assay by analyzing plasma concentrations in dogs administered DPB and DMMDPB by the intravenous route.

2. Experimental

2.1. Chemicals and reagents

Pure reference standards of 5,5-diphenylbarbituric acid and 1,3-dimethoxymethyl-5,5-diphenylbarbituric acid were obtained from Taro Pharmaceuticals (Hawthorne, NY, USA). The sodium salt of 5,5-diphenylhydantoin (phenytoin) was obtained from Sigma (St. Louis, MO, USA). Solid-phase extraction (SPE), Bakerbond 1 ml C_{18} SPE columns, were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of HPLC grade.

2.2. Stock solutions

Standard solutions of DPB were prepared by dissolving 5 mg of DPB in 1 ml of dimethylsulfoxide (DMSO). Sequential dilutions to 100, 50, 25, 10, 5 and 2.5 μg/ml were made in 50:50 (v/v) DMSO–H_2O. The internal standard was prepared by dissolving 10 mg of the sodium salt of 5,5-diphenyl-hydantoin in 10 ml of water and then diluting to 10 μg/ml with water. The standards were prepared by adding a 10× solution of DPB in 50:50 DMSO–H_2O to human plasma for all standards.

2.3. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Model 600 dual-piston multi-solvent delivery system, a Waters WISP 717 auto-sampler and a Waters Model 490 ultraviolet detector (Waters, Milford, MA, USA). The data were collected on the Waters Millenium Chromatography Software Manager. The separation system consisted of a 150×4.6 mm I.D. Waters Spherisorb ODS-2 (3 μm particle size) stainless-steel column supplied by Alltech (Avondale, PA, USA) and a Waters Nova-Pak C_{18} guard column (4 μm particle size, 4×6 mm I.D.) at room temperature. The mobile phase was prepared by mixing 7.5 ml of 3 *M* sodium acetate in 442.5 ml of nanopure water and adjusting the pH to 4.5 with glacial acetic acid, before adding 550 ml of methanol. After stirring, this mobile phase was filtered through a 0.45-μm membrane filter and degassed with a helium sparge for 5 min. The flow-rate was 1.0 ml/min. The detector was set to measure at a wavelength of 215 nm. The injection volume was 25 μl.

2.4. Extraction procedure

Plasma calibration curves were prepared from 250 μl of spiked plasma containing 0.25, 0.5, 1.0, 2.5, 5.0 and 10 μg/ml of DPB. Blank plasma samples were extracted with each curve to verify the absence of interfering peaks. The SPE columns were conditioned with 1 ml of pure methanol on a J.T. Baker extraction block by applying a vacuum to the block to draw the solvent into and through the column. Then the SPE columns were conditioned with 1 ml of 10 m*M* potassium phosphate monobasic, pH 7.0, as described above. Internal standard (200 μl of 10 μg/ml phenytoin) was added to each column, followed by 250 μl of sample or standard. Each sample was then adsorbed onto the column by again applying a vacuum, then washing with 1 ml of 10 m*M* potassium phosphate, pH 7.0. The columns were eluted with 2 ml of methanol and the eluent was dried under vacuum in a Savant speed-vac system (Farmingdale, NY, USA). The resulting residue was resuspended in 100 μl of 50:50 (v/v) methanol–water and 25 μl were injected onto the HPLC system.

2.5. Pharmacokinetic studies

2.5.1. DMMDPB

A total dose of 10 mg/kg DMMDPB was dissolved in 1.0 ml of DMSO and infused at 0.1 ml/min through a 0.2-μm bacterial filter into the cephalic vein of each of five male Beagle dogs (mean weight, 12.6 kg) that had been fasted for 12 h. After the total volume of drug was infused, it was followed by a 2-ml saline flush. Blood samples were collected from the jugular vein into sterile glass tubes prior to, and at 1, 2, 4, 6, 8, 12, 16, 20, 24 and 28 h after the start of infusion. Plasma was separated by centrifugation at approximately 200 *g* in a bench-top centrifuge for 20 min, and was then transferred to cryovials and stored at −20°C pending analysis. Pharmacokinetic parameters, including C_{max}, T_{max} and the area under the plasma concentration–time curve extrapolated to infinity (AUC_{inf}), were calculated using Prism 2.0 (Graphpad Software, San Diego, CA, USA).

2.5.2. DPB

A total dose of 8 mg/kg DPB was dissolved in 10 ml of saline solution (with the addition of a minimal volume of 0.01 *M* NaOH to effect dissolution of the drug). This was then infused at 1 ml/min through a 0.2-μm bacterial filter into the cephalic vein of each of five male Beagle dogs (mean weight, 12.72 kg) that had been fasted for 12 h. After the total volume of drug was infused, it was followed by a 2-ml saline flush. Blood samples were collected from the jugular vein into sterile glass tubes prior to, and at 0.5, 1, 2, 4, 6, 8, 12, 16, 20, 24 and 30 h after the start of infusion. Plasma was separated by centrifugation at approximately 200 *g* in a bench-top centrifuge for 20 min, and was then transferred to cryovials and stored at −20°C pending analysis. The pharmacokinetic parameters, including C_{max}, T_{max} and the area under the plasma concentration–time curve extrapolated to infinity (AUC_{inf}) were calculated using Prism 2.0 (Graphpad Software). The clearance of DPB was calculated by dividing the dose by the AUC_{inf}. The terminal elimination rate constant, β, was calculated by fitting the data to a monoexponential decay equation, $y=A\ e^{-\beta x}$. The terminal elimination half life ($t_{1/2}$) was calculated by dividing 0.693 by β. The volume of distribution (V_d) was calculated by the area method by dividing the clearance by β, the terminal elimination rate constant.

3. Results

Chromatography of DPB, DMMDPB and the internal standard, phenytoin, under the conditions described, is illustrated in Fig. 2. The chromatograms demonstrate the separation of drugs and the lack of endogenous interference in blank plasma. The retention times were 4.7, 5.4, 7.8 and 13.5 min for DPB, phenytoin, the intermediate metabolite and DMMDPB, respectively. The total run time was 15 min. Phenobarbital eluted at approximately 3.7 min.

The recovery of DPB during the extraction from plasma was established by comparing the response of extracted standards with that of direct injection of standard solutions. As demonstrated in Table 1, the mean recovery of DPB from plasma across the concentration range tested (0.25–10 μg/ml) was consistently greater than 82%.

To determine the precision and accuracy of the method, a set of standards were run in triplicate on three different days. From these analyses, the intra-day (Table 2) and inter-day (Table 2) relative standard deviation (R.S.D.; %) and bias (%) were calculated. The intra-day R.S.D. and bias were less than 8.5 and 7.0%, respectively, over the concentration range of 0.25–10 μg/ml. The inter-day R.S.D. and bias were less than 10.5 and 8.0%, respectively, over the concentration range of 0.25–10 μg/ml.

A linear response of the DPB to internal standard peak-area ratio was observed over the concentration range 0.25–10.0 μg/ml. Weighted linear regression ($1/Y^2$) analysis using least-squares fit was performed using Prism 2.0 (Graphpad Software). The square of the correlation coefficients (r^2) were all greater than or equal to 0.9994 ($n=3$). The limit of quantification of the method was 0.25 μg/ml, and this concentration was used as the lowest standard in all subsequent assays. The limit of detection, determined as a concentration that resulted in a response with a signal-to-noise ratio of three or greater, was 0.05 μg/ml.

To determine the specificity of the method, plasma was collected from healthy human donors, rats and

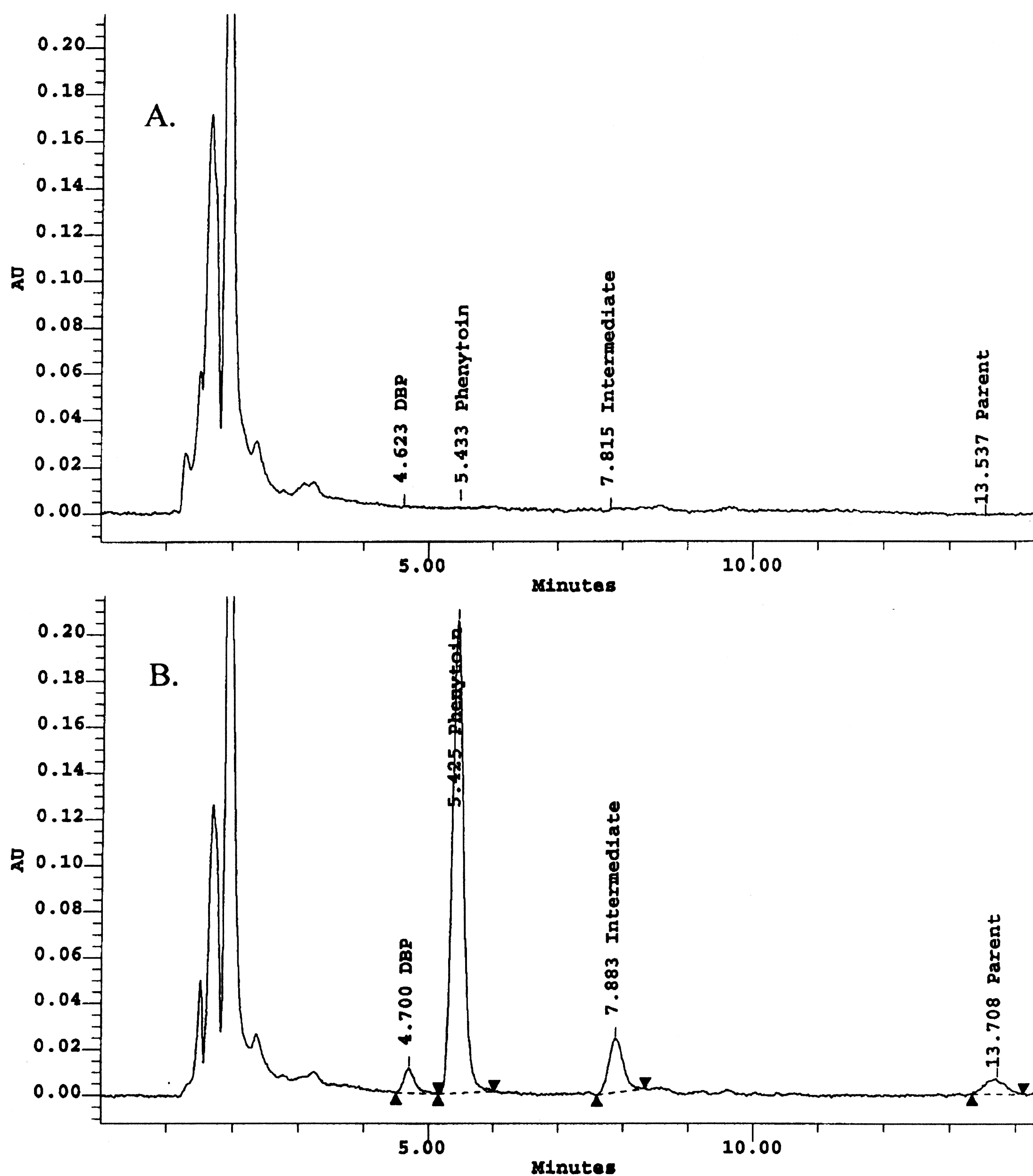

Fig. 2. Chromatograms of extracted samples. (A) Blank sample extracted from dog plasma and (B) a sample taken from a dog 0.5 h after an intravenous dose of 10 mg/kg of DMMDPB (Parent=DMMDPB).

dogs, and assayed for interference at the retention times of DPB, DMMDPB, internal standard and an intermediate metabolite that appeared after administration of DMMDPB. No interfering peaks were observed in any of the human, rat or dog plasma samples tested. Interference resulting from phenytoin and phenobarbital was tested for and none was found.

Table 1
Percentage recovery of diphenylbarbituric acid from human plasma

[DPB] (μg/ml)	Recovery (%)			
	Day 1	Day 2	Day 3	Mean
10	83.6	82.6	81.0	82.4
5	83.5	81.3	83.5	82.8
2.5	79.4	86.5	84.0	83.3
1	81.7	82.0	84.6	82.8
0.5	90.0	98.7	107.0	98.6
0.25	92.2	67.5	90.6	83.4

3.1. Plasma concentrations in dogs

To determine the utility of the method in a clinical setting, either DMMDPB parent drug or DPB was administered intravenously to male Beagle dogs, and the plasma obtained was assayed. Although the lack of availability of dog plasma precluded extensive comparison or validation with this matrix, useful clinical data were obtained. Fig. 3 shows the relationship between time and the DPB plasma concentrations after intravenous administration of DMMDPB. These data demonstrate a mean C_{max} of 1.33±0.46 μg/ml 6 h after administration of drug. The terminal elimination half life of DPB was 3.03 h. Fig. 4 shows the relationship between time and DPB plasma concentrations after intravenous administration of DPB. The pharmacokinetic parameters described in Table 3 were calculated from these data.

4. Discussion

We present a reproducible and sensitive assay method for DPB in plasma that is appropriate for use in clinical studies. The method has a limit of quantification of 0.25 μg/ml, which is adequate for the analysis of plasma samples after the administration of DPB, or its parent drug, DMMDPB, to dogs. Given the similarity between the chemical structures of DPB and known anticonvulsants, such as phenytoin and phenobarbital, and the documented effective plasma concentrations of the drugs in the 1 to 20

Table 2
Variability of diphenylbarbituric acid determined in human plasma

[DPB] (μg/ml)	Concentration (μg/ml)			Mean concentration	R.S.D. (%)	Bias (%)
	A	B	C			
Intra-day						
10	10.610	10.566	10.517	10.564	0.44	5.64
5	5.041	5.306	5.100	5.149	2.70	2.98
2.5	2.426	2.357	2.543	2.442	3.86	2.32
1	0.963	0.939	0.934	0.945	1.67	5.45
0.5	0.517	0.448	0.463	0.476	7.60	4.79
0.25	0.262	0.291	0.247	0.267	8.43	6.67
r^2	0.9991	0.9992	0.9998	0.9996	0.04	
Inter-day						
10	10.564	9.851	9.976	10.130	3.76	1.30
5	5.149	4.776	5.015	4.980	3.80	0.40
2.5	2.442	2.581	2.512	2.512	2.76	0.46
1	0.945	0.971	0.945	0.954	1.53	4.62
0.5	0.476	0.582	0.559	0.539	10.31	7.76
0.25	0.267	0.237	0.239	0.248	6.67	0.94
r^2	0.9996	0.9994	0.9999	0.9999	0.02	

DPB, diphenylbarbituric acid.
R.S.D. (%), percentage relative standard deviation=standard deviation/mean.
Bias (%), |(calculated value−theoretical value)|/theoretical value.

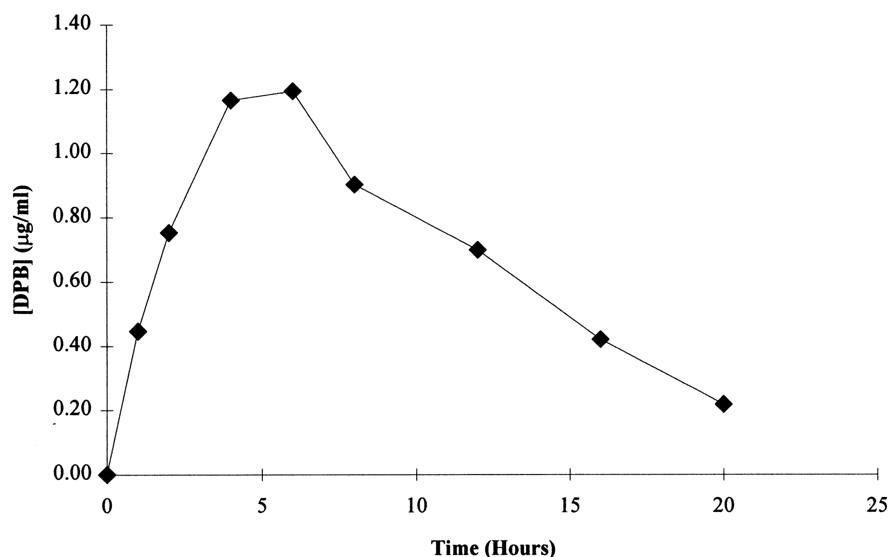

Fig. 3. Average DPB plasma concentration vs. time curve after a 10.0-mg/kg intravenous dose of DMMDPB to five dogs.

μg/ml range [6–11], it is likely that this method will also be applicable to studies in humans. The concentration of DPB achieved in dogs suggests that the clinical activity of DPB is sufficient to suppress artificially induced seizures in animals [3–8].

We have demonstrated that this method can be

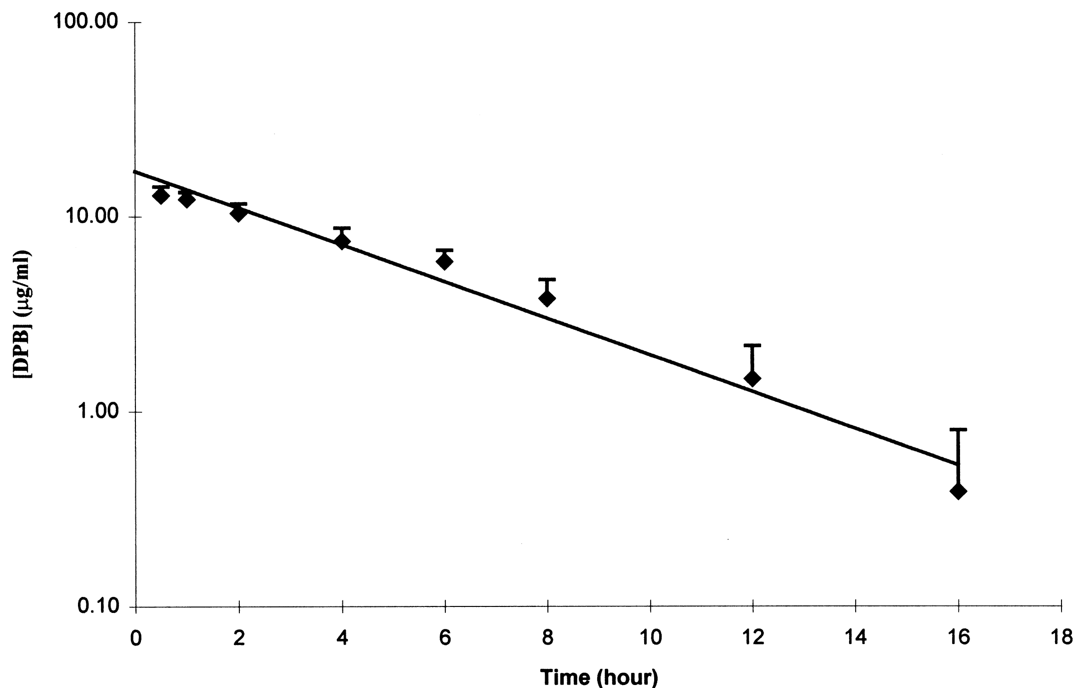

Fig. 4. Average DPB plasma concentration vs. time curve after an 8.0-mg/kg intravenous dose of DPB to five dogs.

Table 3
Average pharmacokinetic parameters for an 8.0 mg/kg I.V. dose of diphenylbarbituric acid administered to five male Beagle dogs

Parameter	Mean
Weight (kg)	12.72±0.87
T_{max} (h)	0.60±0.22
C_{max} (μg/ml)	12.95±1.32
AUC_{inf} (h μg ml^{-1})	77.08±10.38
V_d (ml/kg)	457.1±69.57
Clearance (ml h^{-1} kg^{-1})	105.38±14.61
$T_{1/2}$ (h)	3.03±0.37

used to separate and measure DPB in clinical plasma samples, and that there is no interference from endogenous elements in human plasma, the parent drug or exogenous phenytoin or phenobarbital. These data form the analytical basis for future pharmacokinetic studies of diphenylbarbituric acid in human plasma after administration of the parent dimethoxymethyl derivative.

Acknowledgements

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