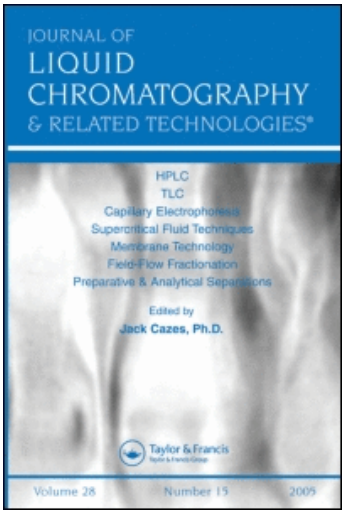

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ABSTRACT

A liquid chromatographic procedure for the determination of pentobarbital in urine and plasma samples is described. The proposed system uses a Spherisorb octadecyl-silane ODS-2 C_{18} analytical column, a guard column of similar characteristics, and a 0.02 M CTAB-15% 1-propanol at pH 7.5 mobile phase.

The UV detector was set at 250 nm. Pentobarbital was isolated from urine and plasma samples by using a single solid phase extraction procedure with LMS cartridges. Mephobarbital was used as internal standard. Limits of detection were 0.53 μg/mL and 0.60 μg/mL in urine and plasma samples respectively. In both cases the coefficients of variation were lower than 6.5%, and the recoveries ranged between 73-105%.

INTRODUCTION

Barbiturates are capable of producing all levels of CNS depression, from mild sedation to hypnosis to deep coma to death. The degree of depression depends upon dosage, route of administration, and pharmacokinetics of the particular barbiturate. In addition, the patient's age, physical or emotional state, and/or the concurrent use of other drugs may alter the response. Barbiturates have a low therapeutic index and are therefore prone to cause poisoning. Monitoring their concentrations in body fluids is therefore essential to optimise pharmacotherapy.[1] Determination of barbiturates in body fluids is also important for the investigation of intoxication, and pharmacokinetic and metabolic studies.

Pentobarbital is a barbiturate used principally as a hypnotic in the short-term treatment of insomnia, and preoperatively to relieve anxiety and provide sedation. Occasionally, pentobarbital is used to control status epilepticus or acute seizure episodes resulting from meningitis, poisons, eclampsia, alcohol withdrawal, tetanus, or chorea.[2] Pentobarbital shares the toxic potentials of the barbiturates and the usual precautions of barbiturate administration should be taken. In fact, dosage of pentobarbital must be individualised for each patient. Oral hypnotic dose of pentobarbital for adults is 100-200 mg; following oral administration, peak plasma concentrations are usually reached in 30-60 min. Plasma pentobarbital concentrations of 1-5μg/mL generally produce sedation, and plasma concentrations of 5-15 μg/mL produce sleep in most patients. However, plasma concentrations of greater than 15μg/mL may produce deep coma, and those in excess of 30 μg/mL are potentially lethal. Pentobarbital is metabolised by the liver; approximately 40-50% of an oral hypnotic dose of pentobarbital is excreted in urine as hydroxypentobarbital. Less than 1 % of an oral dose of pentobarbital is excreted in urine unchanged.

Different procedures have been developed in order to determine pentobarbital in biological samples which use chromatographic techniques as gas-liquid chromatography,[3] high-performance liquid chromatography (HPLC),[4-11] gas chromatography/mass spectrometry (GC/MS),[12-16] micellar electrokinetic capillary chromatography (MECC),[17,18] capillary electrophoresis,[19,20] and immunological techniques.[21-24] The procedures reported require labour sample pre-treatments that included protein precipitation[6,20] or solvent extraction.[4,7,9]

The use of solid phase extraction as clean-up procedure has been also reported.[8,13-15] In these procedures, C_{18} solid-phase columns, CH_2Cl_2, $CHCl_3$, ethyl acetate, hexane, or mixtures of them as eluents; solvent evaporation and reconstitution of solid extract are generally the conditions used in order to obtain an adequate recovery of the analyte and elimination of sample proteins.

This paper describes a new, simple, and rapid RPLC procedure for determining pentobarbital in plasma and urine samples by using micellar mobile phases and solid phase extraction. Mephobarbital was used as internal standard. The use of polimeric solid-phase cartridges and propanol as eluent provide adequate clean up and pentobarbital recovery of plasma samples. For urine samples better results were obtained using ethyl acetate as eluent.

EXPERIMENTAL

Apparatus

A Hewlett-Packard HP 1100 chromatograph with an isocratic pump, and UV-visible detector was used (Palo Alto, CA, USA). Data acquisition and processing were performed on an HP Vectra XM computer (Amsterdam, The Netherlands) equipped with HP-ChemStation software from Hewlett-Packard (1996 version, Waldbronn, Germany).

The solutions were injected into the chromatograph through a Rheodyne valve (Cotati, CA, USA) with a 20 μL loop. A Spherisorb octadecyl-silane ODS-2 C_{18} column (5 μm, 120 x 4.0 mm i.d.) and a guard column of similar characteristics (35 x 4.0 mm i.d.) (Scharlau, Barcelona, Spain) were used. The mobile phase flow rate was 1.5 mL min^{-1}. Detection was performed in UV at 250 nm, which allows adequate sensitivity for detecting pentobarbital and mephobarbital in the selected mobile phase.

All the assays were carried out at room temperature. The dead time value (average t_m = 0.83 min) was determined for each injection as the first perturbation in the chromatogram. A solid phase extraction vacuum station Vac Elut 20 (Varian Sample Preparation products, Harbor City, CA, USA) and bond-Elut LMS polymeric cartridges (1cc/ 25 mg, Varian Sample Preparation products, Harbor City, CA, USA) were used.

Reagents and Standards

Mobile phases were prepared by mixing aqueous solutions of cetyl methyl ammonium bromide (CTAB, Acros Chimica, Geel, Belgium) and the corresponding amount of 1-propanol (analytical-reagent grade, Scharlau, Barcelona, Spain). The pH was adjusted to 7.5 before the addition of 1-propanol with a 0.05 M phosphate buffer, prepared with disodium hydrogen phosphate and sodium dihydrogen phosphate (analytical reagent, Panreac, Barcelona, Spain). Mephobarbital (Sigma, St. Louis, Missouri, USA) was used as internal standard.

Stock standard solutions of mephobarbital and pentobarbital (donated by B. Braun Medical, S.A.) were prepared by dissolving 10 mg of the compound in 10 mL of 0.02 M CTAB, and stored in the dark at 4°C. Working solutions were prepared by dilution of the stock standard solutions in CTAB 0.02 M.

Barnstead E-pure, deionized water (Sybron, Boston, MA) was used throughout. The mobile phases and the solutions injected into the chromatograph were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westborough, MA, USA).

Sample Preparation

Plasma and urine samples were kept in the refrigerator at 4°C until use. Extraction of pentobarbital and mephobarbital (internal standard) from plasma and urine samples was achieved using Bond-Elut LMS polymeric cartridges and the Vac-Elut set-up. The cartridges were conditioned immediately prior to use by passing sequentially 1 mL of methanol and an equal volume of 0.5 M phosphate buffer (pH 6) prepared in CTAB 0.02 M through the columns. The columns were loaded by application of a mixture of 500 μL of plasma or urine conditioned by addition of 500 μL of 0.5 M phosphate buffer in CTAB 0.02 M (pH 6). The columns were cleaned with 1 mL of the phosphate buffer and dried under full vacuum. For plasma samples, elution was effected with 500 μL of 1-propanol and the eluate was directly injected into the chromatographic system. For urine samples the compounds were eluted with 0.5 mL of ethyl acetate into a test-tube before evaporation to dryness under vacuum at 70°C. The residue was dissolved in 250 μL of mobile phase and injected into the chromatographic system. In these conditions an enrichment factor of 2 was achieved.

RESULTS

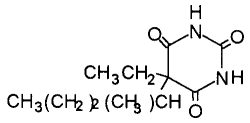
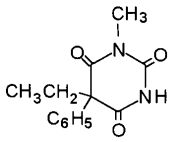
Chromatographic Conditions

A study to select the nature and composition of the micellar mobile phase that allows the determination of pentobarbital in biological samples was performed. Mephobarbital, which has similar characteristics (hydrophobicity and ionization constant) to pentobarbital, was selected and used as internal standard. Table 1 shows the structure, protonation constants, and the partition coefficients octanol-water of the compounds.

Firstly, the nature of surfactant was optimised. For this purpose, different mobile phases of SDS (0.05, 0.10 and 0.15 M), Brij35 (0.02, 0.04 and 0.06M) and CTAB (0.02 and 0.05M) at pH 7.5 were assayed. In these conditions, the retention

Table 1

Structure, Protonation Constants, and Octanol-Water Partition Coefficients of Pentobarbital and Mephobarbital

Compound	Structure	Log K	Log P
Pentobarbital		8.0	2.07
Mephobarbital		7.8	1.85

factors of pentobarbital were 29.1, 17.2, and 16.6 for SDS; 52.7, 27.9, and 19.4 for Brij35; and 86 and 36.7 for CTAB, respectively (Table 2). As can be observed, in all cases large changes in the retention were obtained upon increasing the surfactant concentration in the mobile phase. However, the efficiency values obtained using SDS and Brij35 were very low (N< 100). The use of CTAB provides larger N values.

The decrease of the mobile phase pH (pH 3.5) modified the retention factor of pentobarbital in the different micellar mobile phases assayed (Table 2).

These modifications can be explained, taking into account, the existence of electrostatic interactions between the surfactant adsorbed in the stationary phase and the barbiturates. However the decrease of the mobile phase pH did not improve the efficiency values, so a pH of 7.5 and CTAB was selected to prepare the micellar mobile phase.

In order to reduce the retention of pentobarbital and mephobarbital and improve the efficiency values, different amounts of 1-propanol were added to the mobile phase. Table 2 shows the retention factors of compounds and the selectivity values obtained in the experimental conditions assayed.

A mobile phase containing 0.02 M CTAB, 15% 1-propanol at pH 7.5 mobile phase was chosen. In these conditions the retention factors of pentobarbital and mephobarbital (k = 13.2 and 5, respectively) and the efficiency values were adequate (N > 1000).

Table 2

Retention Factors and Selectivity of Pentobarbital (k_1) and Mephobarbital (k_2) Obtained Using Different Mobile Phases

Surfactant	Mobile Phase Composition	k_1	k_2	α
SDS	0.05 M SDS, pH 7.5	29.1	35.8	1.23
	0.10 M SDS, pH 7.5	17.17	21.7	1.26
	0.15 M SDS, pH 7.5	16.6	18.2	1.10
	0.15 M SDS, pH 3.5	28.5	22.2	1.28
Brij35	0.02 M Brij35, pH 7.5	52.8	26.4	2.00
	0.04 M Brij35, pH 7.5	27.9	15.7	1.77
	0.06 M Brij35, pH 7.5	19.4	11.7	1.66
	0.02 M Brij35, pH 3.5	52.8	17.21	3.07
CTAB	0.02 M CTAB, pH 7.5	86	60.4	1.42
	0.05 M CTAB, pH 7.5	36.7	24.3	1.51
	0.06 M CTAB, pH 7.5	25.7	19.2	1.34
	0.05 M CTAB, pH 3.5	16.0	17.21	1.08
	0.02 M CTAB, pH 7.5 + 3% 1-propanol	60.4	41	1.47
	0.04 M CTAB, pH 7.5 + 1.5% 1-propanol	35.2	24.9	1.41
	0.06 M CTAB, pH 7.5 + 3% 1-propanol	19.5	13.7	1.42
	0.02 M CTAB, pH 7.5 + 15% 1-propanol	13.2	5.0	2.64

Determination of Pentobarbital in Biological Samples

Using the experimental chromatographic conditions selected, a study was performed in order to optimise procedures for determining pentobarbital in plasma and urine samples taking into account its therapeutic and toxic concentrations in these samples. For this purpose spiked plasma and urine samples were prepared by adding different amounts of compounds to the samples.

The possibility of performing direct injection of samples using CTAB as micellar mobile phase was investigated. As can be observed in Figure 1a, direct injection of urine led to a chromatogram with a strong matrix band at the beginning of the chromatogram and peaks of endogenous compounds from the urine overlapping the barbiturate peaks.

On the other hand, the direct injection of plasma produces a high noisy background that impedes the measure of barbiturate peaks. These results indicates that a sample pre-treatment is required in order to obtain adequate clean-up and/or preconcentration of analyte in biological samples.

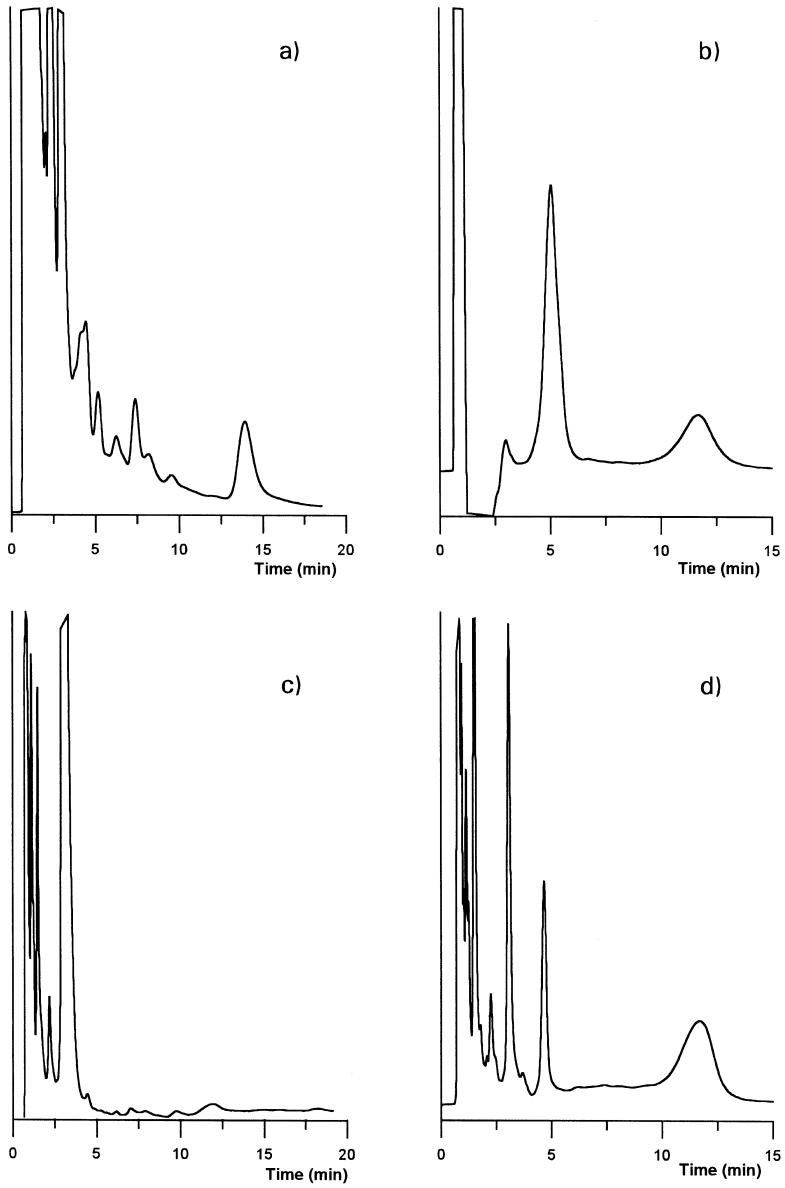


Figure 1. Experimental chromatograms corresponding to: (a) direct injection of a urine sample (b) Eluate obtained from a plasma sample spiked with 5 μg/mL of pentobarbital and 8 μg/mL of mephobarbital (i.s.). (c) eluate from a urine sample (d) eluate from a urine sample spiked with 15 μg/mL of pentobarbital and 8 μg/mL mephobarbital.

The ability of the Bond Elut LMS cartridges to retain quantitatively pentobarbital and the composition of the eluent was studied. The cartridges were activated using methanol and conditioned using 0.5 M phosphate buffer (pH 6) prepared in CTAB 0.02 M. The same buffer solution was used for conditioning samples. The presence of CTAB in the conditioning buffer provided better results than similar solutions prepared in the absence of surfactant. This may be due to the fact that pentobarbital is bound to sample proteins (i.e. for plasma 35-45%) and the addition of CTAB produces the liberation of bounded compound. Columns were rinsed using the phosphate buffer solution.

Different eluents were assayed, acetonitrile, methanol and 1-propanol. The use of 1-propanol provided adequate recoveries of pentobarbital and mephobarbital; while using acetonitrile and methanol as eluents lower recoveries were achieved. Figure 1b shows the chromatogram corresponding to an eluate obtained from a spiked plasma sample.

For urine samples, the use of acetonitrile, methanol, and 1-propanol as eluents was also assayed. As in the plasma samples, the use of methanol and acetonitrile did not provide an adequate elution of barbiturates. Although the use of 1-propanol produced the elution of pentobarbital, several urine endogenous compounds which overlapped with the barbiturates peaks were also eluted. The use of ethyl acetate was assayed. The injection of ethyl acetate eluates produced an important background in the chromatogram. However, when the eluent was evaporated and the solid extract was reconstituted with the mobile phase an adequate clean up of sample and a good recovery of pentobarbital was achieved. Using this sample treatment an enrichment factor of 2 was achieved, which is favourable taking into account that the urinary levels of pentobarbital are lower than the corresponding to plasma. Figure 1c and 2d show chromatograms corresponding to eluates obtained from a non-spiked and spiked urine samples, respectively.

The recovery of pentobarbital in plasma and urine after the clean-up and preconcentration step was evaluated. Recovery was obtained by comparing the peak areas of pentobarbital corresponding to three independent analysis, with those obtained by direct injection of aqueous standard solutions of pentobarbital containing the same concentration. In these conditions the recovery values obtained were 98 ± 20 % for 20 μg/mL pentobarbital plasma concentration level and 85 ± 17 and 103 ± 5% for 8 and 30 μg/mL pentobarbital urine concentration levels.

In spite of the mean recovery obtained being considered adequate, the reproducibility of the extraction was slightly low. In order to improve the reproducibility of the procedure, mephobarbital (internal standard) was added before the pre-treatment of samples and the ratio peak (pentobarbital /mephobarbital) was used as dependent variables.

Table 3

Regression Statistics for the Calibration Graphs of Pentobarbital in Plasma and Urine Samples

Parameter[a]	Plasma	Urine
Slope	0.77 ± 0.005	0.0411 ± 0.0016
C.I. slope	0.065, 0.089	0.0371, 0.0451
Intercept	0.05 ± 0.111	-0.16 ± 0.17
C.I. intercept	-0.24, 0.33	-0.35, 0.02
Standard error	0.11	0.10
r	0.99_1	0.99_5
N	7	8

[a] C.I. = confidence interval (95%); S.E. = standard error; r = correlation coefficient; N = number of points.

Analytical Data

The calibration curves of pentobarbital in urine and plasma were obtained by injection of spiked samples containing a varying concentration of the analyte in the 8-40 μg/mL and 6-37 μg/mL range for urine and plasma samples, respectively and a constant amount, 8 μg/mL, of mephobarbital. Samples were pre-treated as indicated in experimental section and relative peak areas were used as dependent variables. Table 3 shows the regression statistics for the calibration curves of pentobarbital.

In both cases, the calibration curves showed adequate regression coefficients over the working interval. The observation of intercepts statistically equal to zero indicates the absence of systematic errors.

The limits of detection (LODs) were calculated from the standard deviation of the relative peak areas corresponding to five independent samples spiked with 1 μg/mL solution of pentobarbital. The LODs obtained were 0.53 μg/mL and 0.60 μg/mL for urine and plasma respectively.

The uncertainty of the method was evaluated at two different pentobarbital concentration levels, 1 and 20 μg/mL for urine samples and 1.5 and 25 μg/mL for plasma samples. In all cases series of five independent samples were analysed. For urine samples, the precision of the proposed method, expressed as the relative standard deviation (%), was 5.6% for both concentration levels. For plasma samples the precision was 6.3% and 2.7% for each concentration level, respectively.

The proposed method to determine pentobarbital in plasma and urine samples is rapid and simple and the limits of detection enable pentobarbital to be monitored in plasma and urine at the therapeutic and toxicological levels. The other barbiturates, which are less hydrophobic than pentobarbital, i.e. phenobarbital, did not interfere in the determination because, in these experimental conditions, they would be eluted at the beginning of the chromatogram.

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