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ANALYSIS OF BARBITURATES IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay for the identification and quantification of barbiturates in blood at therapeutic levels has been developed. An ODS-silica column is used with an eluent of 40% methanol at pH 8.5. The barbiturates are detected at 240 nm. The sample preparation procedure involves extraction of unfractionated blood (100 μl) with hexane—diethyl ether (50:50, v/v) and is very rapid. Talbutal is used as an internal standard.

The method has been applied to the determination of five barbiturates (amylobarbitone, butobarbitone, cyclobarbitone, pentobarbitone and quinalbarbitone) in blood after therapeutic doses of the drugs. An application of the HPLC assay to forensic casework is demonstrated.

INTRODUCTION

Our recent studies [1] have concluded that chromatographic systems using lipophilic stationary phases are the most effective for the separation of barbiturates, e.g. gas—liquid chromatography (GLC) using SE-30, high-performance liquid chromatography (HPLC) using ODS-silica. GLC is presently the most widely used method for the analysis of these drugs in biological fluids. Nevertheless, HPLC using ODS-silica can achieve several separations which are difficult by GLC [1] and could prove valuable for the identification of barbiturates in biological matrices. Furthermore, the GLC methods often involve lengthy extraction or derivatization steps while HPLC has the potential of requiring less complex sample preparation procedures which could save time in forensic and clinical analysis.

Some HPLC procedures for the analysis of barbiturates in biological fluids

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have been published [2—16]. These often deal with specific compounds and few procedures are suitable for the identification and quantification of a wide range of barbiturates. Furthermore the published procedures for blood analysis generally involve initial fractionation to either plasma or serum. This may be appropriate in clinical and pharmacokinetic laboratories where blood may be stored under ideal conditions from the time of collection but is of little value for haemolysed samples often encountered in forensic casework. A recent paper by Sprague and Poklis [17] gives a procedure for the analysis of barbiturates in post mortem blood by HPLC and, although whole blood is used, the method has limited sensitivity, particularly with decomposed specimens.

The analysis of barbiturates in biological fluids by HPLC requires a detection system capable of observing the compounds at low levels without interference from endogenous compounds. Derivatives of barbiturates suitable for enhancing both sensitivity and selectivity of detection in HPLC have been reported [18—20], however, these advantages are offset by the increase in sample preparation time. The purpose of the present work was to provide a rapid, simple, sensitive and high-resolution HPLC procedure without derivatization suitable for the identification and quantification of barbiturates in small volumes of blood including haemolysed samples.

EXPERIMENTAL

Materials

The extraction solvent was prepared from hexane (HPLC grade; Fisons, Loughborough, Great Britain) and diethyl ether (reagent grade, BDH, Poole, Great Britain), in the ratio 50:50, v/v. The ether was freshly distilled before use. All other chemicals used were of analytical grade.

Chromatography

The liquid chromatograph consisted of a pump (Waters M6000), an injection valve (Rheodyne 7120) fitted with a 1-ml loop and a variable-wavelength UV detector (Pye-Unicam LC-UV or Perkin-Elmer LC-75) operated at 240 nm. The column (10 cm × 5 mm I.D. stainless steel) was packed with 5 μm ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) using isopropanol as the slurry medium and hexane as the pressurising solvent. The eluent consisted of aqueous sodium dihydrogen phosphate (0.1 *M*)—methanol (60:40, v/v) adjusted to pH 8.5 with concentrated sodium hydroxide solution and was degassed under vacuum before use. A flow-rate of 2 ml/min was used with an operating pressure of 1700 p.s.i. The column was washed with methanol—water (50:50, v/v) at the end of each working day.

Extraction procedure

Blood (100 μl) was transferred to a pyrex graduated test tube (10 ml, Exelo Permagold, Scientific Supplies, London, Great Britain) fitted with a polythene stopper (E-MIL, Scientific Supplies) using a safety pipetter (P-7000 Sampler, Oxford Laboratories, Athy, Ireland) and the internal standard solution (talbutal in ethanol 20 μl, 25.16 μg/ml) added with a syringe (SGE, London, Great Britain). The volumes of the blood and internal standard solution were mea-

sured accurately, whilst all further additions and transfers were made using the graduations on the test tubes. Phosphate buffer (0.1 *M*, pH 7.5) was then added to give a total volume of 1 ml. The extraction solvent (5 ml) was then added and the phases mixed by repeated inversion of the tube for approximately 1 min. A portion of the organic layer (4 ml) was decanted into a second tube and evaporated to dryness by a stream of nitrogen. The residue was dissolved in the HPLC eluent (500 μl) and 200 μl injected on to the column. The detector was operated at a range of 0.01—0.02 A full-scale.

Calibration solutions were prepared by dissolving the appropriate barbiturate and the internal standard (talbutal, 5-allyl-5-*sec.*-butylbarbituric acid) in aqueous methanol (40%, v/v) at known concentrations. The injection of each blood extract was followed by an injection of the appropriate calibration solution (200 μl). The concentration of barbiturate in the blood was calculated using peak height ratio measurements.

Oral ingestion of barbiturates

Experiments were carried out with laboratory staff with the permission of the Medical Ethics Committee, Chemical Defence Establishment, Porton Down. Five volunteers each took an oral dose of one barbiturate (see Table III) in the morning (approximately 09.00 h). Venous blood was collected before and about 2 h and about 8 h after ingestion of the drug. The blood was stored in plastic screw-top vials (2.5 ml) containing EDTA (Sterilin, Teddington, Great Britain) at 4°C.

RESULTS AND DISCUSSION

The HPLC eluent used in this study (40% methanol, pH 8.5) was chosen as that capable of separating the most commonly abused barbiturates: amylobarbitone, butobarbitone, cyclobarbitone, pentobarbitone and quinalbarbitone [1]. Analytical procedures for these five barbiturates have been examined in detail; however, a wide range of barbiturates can be analysed using this eluent. Capacity factors (k') for 30 barbiturates are given in Table I. Several separations can be achieved with the present eluent which are not possible under acidic or neutral conditions [1].

Most of the published procedures for the analysis of barbiturates in biological fluids by reversed-phase HPLC have used acidic (or neutral) eluents with detection at short wavelengths (< 220 nm) where the detection of barbiturates is not very specific. The drugs show no specific UV absorption under acidic conditions while at pH 10 they show an absorption maximum at 240 nm. Clark and Chan [21] have suggested the post-column mixing of the HPLC eluent with pH 10 buffer to enhance the detectability of barbiturates. Solutions of barbiturates in the present HPLC eluent do show an absorption maximum at 240 nm and experiments revealed that a change from pH 8.5 to 10 gave only a small increase (< 25%) in absorption. This increase would be further reduced by the dilution resulting from the post-column addition of buffer. Furthermore, this procedure would involve an increase in complexity of the HPLC equipment and consequently the present assay was developed using direct detection at 240 nm.

TABLE I

HPLC RETENTION DATA FOR 30 BARBITURATES ON ODS-SILICA

Eluent: 40% methanol at pH 8.5.

Barbiturate	Capacity factor (k')	Barbiturate	Capacity factor (k')
Barbitone	0.63	Methylphenobarbitone	3.84
Phenylmethylbarbituric acid	0.94	Talbutal	4.67
Phenobarbitone	1.23	Idobutal	4.77
Allobarbitone	1.33	Heptabarbitone	4.93
Probarbitone	1.57	Hexobarbitone	5.67
Brallobarbitone	1.72	Nealbarbitone	6.19
Metharbitone	1.99	Thialbarbitone	6.78
Aprobarbitone	2.22	Enallylpropymal	6.96
Vinbarbitone	2.32	Amylobarbitone	7.05
Ibomal	2.58	Pentobarbitone	8.07
Cyclobarbitone	2.61	Thiopentone	9.20
Secbutobarbitone	3.32	Quinalbarbitone	11.47
Butobarbitone	3.42	Sigmodal	12.37
Butalbital	3.48	Hexethal	20.39
Cyclopentobarbitone	3.84	Methohexitone	20.48

The assay was designed to employ the smallest volume of blood (100 μl) consistent with the need to detect barbiturates at therapeutic levels. Initial experiments were conducted using drug-free blood spiked with the appropriate barbiturates. Talbutal was chosen as an internal standard as it could be separated from the five commonly abused barbiturates (see Fig. 1). It can be seen that all these barbiturates can be analysed in less than 9 min. The use of an internal standard means that only the blood volume (100 μl) and the volume of internal standard solution (20 μl) need to be measured accurately so that the use of graduated test tubes for all further volume measurements makes the extraction procedure very rapid (see Experimental).

Barbiturates have pK_a values in the range 7.6—8.8 and their unionized forms are lipid soluble. They can be extracted with hexane—diethyl ether (50:50,

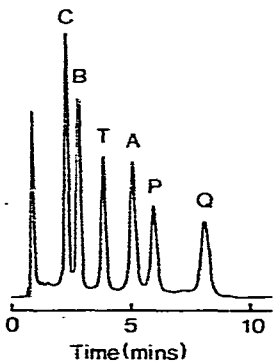

Fig. 1. HPLC of extract of spiked blood on ODS-silica. Peaks: C = cyclobarbitone; B = butobarbitone; T = talbutal; A = amylobarbitone; P = pentobarbitone; Q = quinalbarbitone (each about 5 μg/ml).

TABLE II

RECOVERY OF BARBITURATES ADDED TO BLOOD

Barbiturate	Blood concentration (μg/ml)	Recovery (%)
Cyclobarbitone	12.9	96.2
Butobarbitone	7.5	99.2
Talbutal	5.0	99.1
Amylobarbitone	8.4	99.2
Pentobarbitone	10.1	94.9
Quinalbarbitone	8.2	94.2

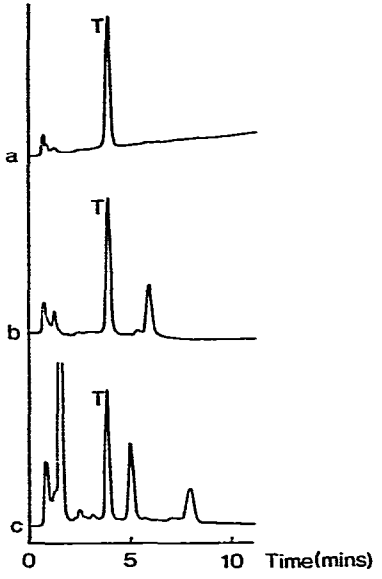

Fig. 2. Analysis of blood by HPLC: (a) drug-free blood spiked with internal standard (talbutal, T, 5 μg/ml); (b) sample from subject about 2 h after an oral dose of pentobarbitone (182 mg), drug peak at about 6 min; (c) sample from car driver suspected of taking barbiturates; peaks at about 5 and 8 min correspond to amylobarbitone and quinalbarbitone, respectively.

v/v) at pH 7.5 with recoveries of > 94% (Table II). Extraction at pH 7.5 gives little interference from endogenous materials over most of the chromatogram which allows the procedure to be applied to a wide range of barbiturates. Fig. 2a shows a typical chromatogram from a blank blood with an internal standard added at a level of 5 μg/ml. The amount of interfering material extracted with the barbiturates was increased by lowering the extraction pH or by raising the polarity of the extracting solvent. Low backgrounds were also obtained with blood from storage vials used for road traffic offences which contain preservatives and even with haemolysed samples. The detection limit for a barbiturate above the background of a blank blood was typically less than 1 μg/ml.

Quantification was performed using peak height ratio measurements with reductions of detector sensitivity for large peaks. The change in peak height ratio of quinalbarbitone to talbutal (internal standard, 5 μg/ml in blood) was

shown to be linear up to a blood concentration of 200 μg/ml. The high precision of the method was demonstrated by repeating the analysis of two blood samples containing quinalbarbitone. The samples gave mean values of 4.52 and 47.4 μg/ml with coefficients of variation of 2.9% (n = 6) and 1.5% (n = 6), respectively.

Most of the published procedures for the analysis of barbiturates involve adjustment of the pH of the body fluid followed by extraction with an organic solvent which is usually evaporated to dryness and the residue dissolved in the HPLC eluent for injection. However, in some assays complex back-extraction procedures have been employed to isolate further the barbiturates from interfering material (e.g. ref. 15). An alternative sample preparation procedure involves protein precipitation with acetonitrile or ethanol and then injection of the supernatant (e.g. refs. 4, 10, 13, 17). A further method involves the adsorption of the barbiturate from serum on to charcoal [3]. The most rapid procedures involve either a single-step solvent extraction or protein precipitation. Our initial experiments showed that protein precipitation with methanol did not remove enough endogenous material from whole blood to allow barbiturates to be detected at therapeutic levels and consequently the present assay was developed using a simple solvent extraction procedure.

Blood samples containing barbiturates after the oral ingestion of therapeutic doses of the drugs were also examined. The blank samples taken before ingestion of the drugs showed no interfering peaks on the chromatograms. Fig. 2b shows the chromatogram for a blood sample from one subject about 2 h after taking pentobarbitone. The results of all assays are given in Table III and plotted in Fig. 3. It can be seen that the levels of amylobarbitone, butobarbitone, pentobarbitone and quinalbarbitone have started to decrease by the end of the 8-h period while the level of cyclobarbitone has not reached a maximum, reflecting slow absorption of the drug by the subject.

The HPLC assay has also been applied to a forensic case where barbiturate abuse was involved in a driving accident. Fig. 2c shows the chromatogram obtained for the analysis of the blood sample by the present HPLC procedure. The two unknown peaks were found to correspond to amylobarbitone and

TABLE III

BARBITURATE BLOOD LEVELS FOR FIVE VOLUNTEERS AFTER ORAL DOSES OF THE DRUGS

Approx. time (h) after oral dose	Barbiturate blood level (μg/ml)				
	Subject 1, cyclobarbitone 185 mg*	Subject 2, butobarbitone 200 mg	Subject 3, amylobarbitone 182 mg**	Subject 4, pentobarbitone 182 mg**	Subject 5, quinalbarbitone 183 mg**
2	0.86	3.32	2.29	3.15	1.99
8	2.95	2.66	1.75	2.26	1.57

*Given as the calcium salt.

**Given as the sodium salt.

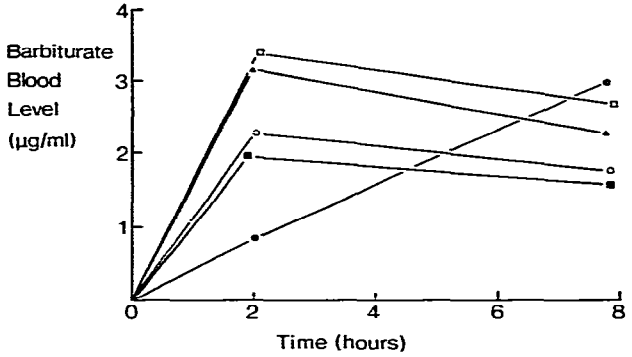

Fig. 3. Changes in barbiturate blood levels determined by HPLC over an 8-h period for five subjects each taking an oral dose of one barbiturate (see Table III). (●) Cyclobarbitone; (□) butobarbitone; (▲) pentobarbitone; (○) amylobarbitone; (■) quinalbarbitone.

quinalbarbitone (5.8 and 3.9 μg/ml, respectively) by co-injection of authentic samples.

In conclusion, the present HPLC assay provides a rapid method for the identification and/or quantification of therapeutic levels of barbiturates in blood which should prove useful in forensic and clinical analysis.

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