Journal of Chromatography, 343 (1985) 437—442
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2704

Note

Determination of thiopental and pentobarbital in plasma using high-performance liquid chromatography

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(First received February 18th, 1985; revised manuscript received May 6th, 1985)

High doses of intravenous barbiturates are effective in controlling persistant intracranial hypertension in patients suffering from severe head injuries [1—4], and improve the quality of survival. Initially, pentobarbital was used for this monitoring [1, 3, 4]. More recently, the effectiveness of thiopental, an ultra-short-acting barbiturate used since 1935 to induce anaesthesia, has been established [5, 6]. The correlation between the plasma levels of thiopental and its therapeutic effects suggests that measurement of plasma levels may provide valuable information for improving the clinical management of patients with head injuries [7] and to prevent toxic effects.

For the determination of thiopental in plasma or serum, gas chromatographic [8—11] and high-performance liquid chromatographic (HPLC) [12—19] methods have been reported. The HPLC method described in this paper, which requires an extractive pre-treatment of the plasma sample, has the advantage of being rapid and simple and also allows the quantitation of pentobarbital, an active metabolite of thiopental, obtained by desulphuration [7].

EXPERIMENTAL

Chemicals

Methanol and methylene dichloride (Merck, Darmstadt, F.R.G.) were of analytical-reagent grade. Stock solutions of thiopental (Laboratoires Specia, Paris, France) and pentobarbital (Société Francaise des Laboratoires Abbott, Paris, France) of concentration 50 mg/l in methanol were prepared. 5-Ethyl-5-

0378-4347/85/$03.30

p-tolylbarbituric acid (Aldrich-Europe, Beerse, Belgium) was used as an internal standard as a 25 mg/l solution in methanol.

Apparatus and conditions

A Model 1080 liquid chromatograph (Hewlett-Packard, Orsay, France), equipped with a variable-volume injector, an automatic sampling system and a detector allowing automatic change of wavelength setting, was used throughout.

Analysis was performed on a 5-μm μBondapak C_{18} column (30 cm × 4 mm I.D.) from Waters Assoc. (Milford, MA, U.S.A.) operating at ambient temperature. The analytical column was protected by a small pre-column (5 cm × 4 mm I.D.) packed with μBondapak C_{18} Corasil B (Waters Assoc.).

The mobile phase was methanol—0.01 *M* potassium phosphate (1:1) adjusted to pH 4.40 ± 0.05 with 0.15 *M* phosphoric acid. The flow-rate was 1.7 ml/min.

Sample preparation

To prevent losses due to adsorption on glass, all glassware was acid-washed before use and finally rinsed with methanol.

A 50-μl volume of the internal standard, 5-ethyl-5-*p*-tolylbarbituric acid, solution (25 μg/ml) was transferred into a screw-capped centrifuge tube (150 × 10 mm) and dried under a stream of nitrogen. To the dry residue were added 500 μl of plasma and 5 ml of methylene dichloride. The tubes were mixed on a rotary mixer for 5 min. After brief centrifugation, the organic layer was transferred into another tube and evaporated to dryness under nitrogen. The residue was dissolved in 500 μl of mobile phase and a 40-μl aliquot or less was injected.

Quantitation

Quantitation was carried out using the peak area ratio method with 5-ethyl-5-*p*-tolylbarbituric acid as the internal standard. Calibration graphs were obtained first using various amounts of stock thiopental and pentobarbital solutions (2.5, 5, 10, 25, 50 and 100 mg/l) and a constant amount of internal standard (25 mg/l). Calibration graphs were also obtained by spiking control plasma with the same amounts of stock thiopental and pentobarbital solutions (2.5, 5, 10, 50 and 100 mg/l) and the same amount of internal standard (25 mg/l).

The within-run variation was determined by analysing ten-fold two plasma samples spiked with thiopental and pentobarbital.

Interferences

Twenty drug-free plasma samples were extracted and analysed for possible interferences by endogenous constituents. The retention times of barbiturates and commonly used drugs such as phenacetin, phenytoin, carbamazepine, theophylline, theobromine and caffeine were also determined in order to avoid possible interferences.

Stability

The stability of thiopental and pentobarbital in plasma was tested by using

fresh control plasma spiked with thiopental and pentobarbital at concentrations of 25 and 100 mg/l. Aliquots of these plasma standards or controls were frozen at −10°C and were found to be stable for at least one year.

RESULTS

Chromatography

On the basis of absorbance scans, 212 nm was selected as the wavelength for the detection of 5-ethyl-5-*p*-tolylbarbituric acid and pentobarbital and 284 nm for the detection of thiopental.

The total chromatographic time for an analysis of thiopental and its active metabolite pentobarbital was less than 10 min with reproducible retention times. The retention times were 4.62, 6.25 and 8.90 min for 5-ethyl-5-*p*-tolylbarbituric acid, pentobarbital and thiopental, respectively, as shown on the chromatogram of plasma from a patient on thiopental therapy (Fig. 1b). Thiopental could be also monitored at 212 nm with lower sensitivity (Fig. 1c). Initially, after the first methylene dichloride extraction, the aqueous (upper) phase was re-extracted with 5 ml of methylene dichloride but this did not increase the recovery of the drug and was therefore omitted.

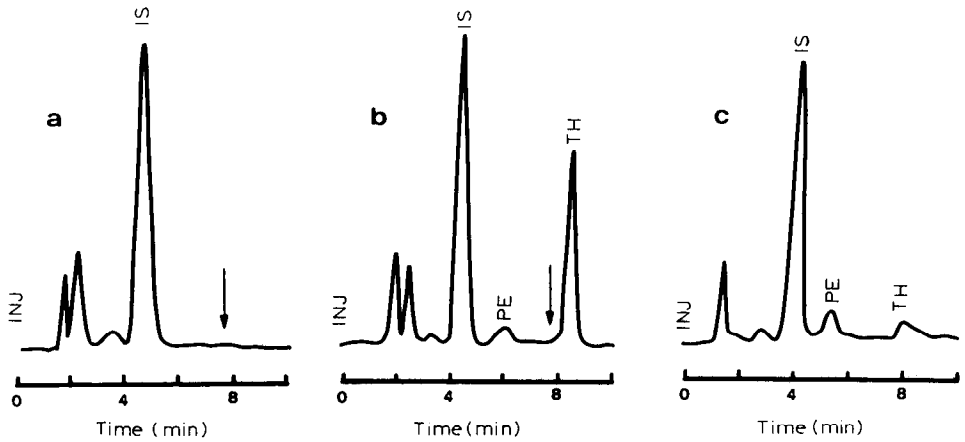

Fig. 1. Chromatograms obtained following plasma extraction and injection of 40-μl aliquots. (a) Drug-free plasma supplemented with internal standard (IS); (b) and (c) patient plasma samples containing 1 mg/l pentobarbital (Pe) and 24 mg/l of thiopental (Th). In a and b, automatic change of wavelength setting is indicated by arrows; in c, elution of the three compounds was monitored at 212 nm.

Precision and accuracy

Linearity. The linearity of the method was studied for each drug in methanolic solution and in spiked plasma samples. Regression lines were obtained by plotting the peak area ratio of each drug to that of the internal standard, 5-ethyl-5-*p*-tolylbarbituric acid, against their concentrations in the spiked plasma samples. The relationship was linear for the drugs in the range 1—100 mg/l. The response factors were fairly constant for the drugs at the given concentrations, indicating good linearity. No significant differences were observed between standards prepared in methanol and spiked plasma samples.

The regression lines for the drugs in methanol are given by the following equations: for thiopental: $y = 0.0352x - 0.077$ ($r = 0.9998$); for pentobarbital: $y = 0.0106x - 0.02$ ($r = 0.9998$).

These equations were similar to those obtained for the drugs in spiked plasma: for thiopental: $y = 0.0313x - 0.093$ ($r = 0.9999$); for pentobarbital: $y = 0.0072x - 0.0214$ ($r = 0.9996$).

Recovery. The analytical recoveries of pentobarbital and thiopental were measured at five different concentrations ranging from 5 to 100 mg/l. The extraction efficiencies were comparable for the two drugs and the relative recoveries of thiopental and pentobarbital were in the range 97—103%.

Reproducibility. The precision within each run and from day to day was established at concentrations within the limits of the therapeutic ranges for thiopental and pentobarbital. The within-day precision was 0.2 mg/l (± 1 S.D.) for thiopental ($n = 10$, $x = 26.61$ mg/l; C.V. = 0.69%) and for pentobarbital 0.17 mg/l (± 1 S.D.) ($n = 10$, $x = 29.50$ mg/l; C.V. = 0.55%). The between-day precision was 0.71 mg/l ($n = 10$, $x = 23.24$ mg/l; C.V. = 3.09%) for thiopental and 0.4 mg/l ($n = 10$, $x = 19.0$ mg/l; C.V. = 2.1%) for pentobarbital. Plasma thiopental and pentobarbital are stable for at least 24 h at room temperature and no appreciable degradation was observed on spiked plasma samples at −20°C between one week and six months.

Interferences. First, twenty drug-free plasma samples were extracted and analysed for possible interferences by endogenous constituents. As shown in Fig. 1a, no background interference was observed. So far plasma samples from more than 200 patients have been analysed and no interferences have been encountered.

Other barbiturates that might be extracted in the same plasma extract were tested for possible interferences (Table I). Only butobarbital is likely to interfere with the internal standard, but this drug is not used in the monitoring of head injuries. Commonly used drugs such as phenacetin, phenytoin, theophylline, theobromine and caffeine did not interfere with the analysis (Table I). The retention time of carbamazepine, a common antiepileptic drug, is close to that of pentobarbital and could interfere in the determination of this metabolite.

Sensitivity and detection limit. The extraction procedure yields a relatively clean extract under the conditions of the assay; a detection limit of 0.5 mg/l

TABLE I

RETENTION TIMES FOR OTHER COMMON DRUGS

Drug	Retention time (min)	Drug	Retention time (min)
Theobromine	1.90	5-Ethyl-5-*p*-tolylbarbituric acid	4.62
Theophylline	2.12	Phenytoin	4.86
Barbital	2.30	Butalbital	5.10
Caffeine	2.43	Carbamazepine	6.20
Phenobarbital	3.11	Pentobarbital	6.25
Acetaminophen	3.31	Amobarbital	7.00
Phenacetin	3.61	Secobarbital	7.82
Vinbarbital	4.03	Thiopental	8.90
Butobarbital	4.30		

can be achieved for each drug, using 212 nm for pentobarbital and 284 nm for thiopental. A sensitivity of 0.3 mg/l may be obtained by injecting a larger sample volume (a sensitivity of only 10 mg/l can be achieved for thiopental when its detection is carried out at 212 nm). The sensitivity of the method is more than adequate for therapeutic monitoring.

DISCUSSION

Numerous methods for the analysis for thiopental and pentobarbital have been described. Oroszlan and Maengwyn-Davies [20] reported a spectrophotometric assay for the determination of the two compounds, involving three different wavelengths, and with a sensitivity of 1 mg/l. Scoppa [21] described a spectrofluorimetric determination of pentobarbital with a sensitivity of 0.5 mg/l. Neither method is specific for thiopental or pentobarbital and interferences can be numerous.

Several methods requiring extraction followed by gas–liquid chromatography have been used to separate and quantify barbiturates [8–11, 22]. Because of the recent utilization of nitrogen-specific detectors [14], these methods are the most sensitive but they require lengthy derivatization procedures.

Liquid chromatography, in addition to the simplicity of the instrumentation and preparation, has the advantage of allowing the separation of the components at room temperature. Blackman et al. [12] developed an HPLC method for thiopental without extraction but did not report the recoveries of the drug from plasma. Christensen and Andreasen [13] also described a procedure for assaying thiopental without extraction; the chromatographic patterns showed an important "serum peak" that could rapidly contaminate the columns.

Gupta et al. [23] described a simple determination of pentobarbital in plasma using a resin column and an alkaline mobile phase. Freeman [14] also described a convenient monitoring of serum thiopental by liquid chromatography. These last two techniques have been applied to either pentobarbital or thiopental alone. Shiu and Nemoto [18] reported a simple, rapid and sensitive HPLC method for the determination of thiopental and pentobarbital in plasma and brain tissue. This method could not be applied to the therapeutic monitoring of thiopental; pentobarbital, used as an internal standard, is a metabolite of thiopental [7]. Recently, Premel-Cabic et al. [17] described a micro-scale method for the determination of thiopental in human plasma by HPLC using as an internal standard the drug flunitrazepam and were not able to detect the active metabolite by this method. Kelner and Bailey [19] analysed the two compounds by HPLC after extraction and without evaporation, using two drugs, thiamylal and barbital, as internal standards. This method allows concentrations of 1.0 mg/l for thiopental and 2.0 mg/l for pentobarbital to be measured.

HPLC methods described by Björkman and Idvall [16] and Sharman and Ahern [15] for the determination of thiopental are unable to detect the active metabolite pentobarbital and use the drugs hexobarbital and flufenamic acid, respectively, as internal standards.

In our procedure, the use of a pre-column allows the removal of serum proteins and particles. Other barbiturates and numerous commonly used drugs do not interfere. The interference due to carbamazepine is not a great problem because this antiepileptic drug is normally not associated with thiopental therapy. Even though the method is less sensitive than gas chromatographic determinations [10, 11], it has sufficient sensitivity to follow blood thiopental and pentobarital levels in the therapeutic range (8–25 mg/l) [12] and is useful in adjusting the therapy of patients with head injuries. When thiopental is detected at 212 nm, the sensitivity is lower and is just sufficient to follow the blood levels of the drug in the therapeutic range.

The method is linear up to 100 mg/l, allows the determination of both thiopental and its active metabolite, pentobarbital, does not require a drug as an internal standard and can be applied to serum samples.

ACKNOWLEDGEMENTS

The authors gratefully thank Dr. S. Blond (Department of Neurosurgery, Centre Hospitalier Régional de Lille, France) for providing blood samples. This investigation was supported by La Fondation pour la Recherche Médicale Française.

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