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High-performance liquid chromatographic determination of thiopentone enantiomers in sheep plasma

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Abstract

An HPLC method was developed to determine the plasma concentrations of *R*(+)- and *S*(−)-thiopentone for pharmacokinetic studies in sheep. The method required separation of the thiopentone enantiomers from the corresponding pentobarbitone enantiomers which are usually present as metabolites of thiopentone. Phenylbutazone was used as an internal standard. After acidification, the plasma samples were extracted with a mixture of ether and hexane (2:8). The solvent was evaporated to dryness and the residues were reconstituted with sodium hydroxide solution (pH 10). The samples were chromatographed on a 100 mm × 4 mm I.D. Chiral AGP-CSP column. The mobile phase was 4.5% 2-propanol in 0.1 *M* phosphate buffer (pH 6.2) with a flow-rate of 0.9 ml/min. This gave *k'* values of 1.92, 2.92, 5.71, 9.30 and 11.98 for *R*(+)-pentobarbitone, *S*(−)-pentobarbitone, *R*(+)-thiopentone, *S*(−)-thiopentone, and phenylbutazone, respectively. At detection wavelength of 287 nm, the limit of quantitation was 5 ng/ml for *R*(+)-thiopentone and 6 ng/ml for *S*(−)-thiopentone. The inter-day coefficients of variation at concentrations of 0.02, 0.1 and 8 μg/ml were, respectively, 4.8, 4.4 and 3.5% for *R*(+)-thiopentone and, respectively, 5.0, 4.3 and 3.9% for *S*(−)-thiopentone ($n = 6$ each enantiomer). At the same concentrations, the intra-day coefficients of variation from six sets of replicates (measured over six days) were, respectively, 8.0, 8.0 and 8.8% for *R*(+)-thiopentone and 8.8, 7.4 and 9.6% for *S*(−)-thiopentone. Linearity over the standard range, 0.01–40 μg/ml, was shown by correlation coefficients > 0.998. This method has proven suitable for pharmacokinetic studies of thiopentone enantiomers after administration of *rac*-thiopentone in human plasma also and would be suitable for pharmacokinetic studies of the pentobarbitone enantiomers.

1. Introduction

Thiopentone remains an important intravenous anaesthetic agent 60 years after its introduction into clinical medicine. Over the years, it has been investigated progressively as to its pharmacokinetics and pharmacodynamics which have been summarised recently [1]. Thiopentone, however, is made and used as a racemic mixture of the enantiomers, *R*(+) and *S*(−)-thiopentone, but these have not received much attention despite evidence that *rac*-thiopentone, *R*(+)-thiopentone and *S*(−)-thiopentone have different pharmacodynamic profiles in mice [2]. It is

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not known whether these different profiles derive from the thiopentone enantiomers having different pharmacokinetics.

Although a number of contemporary methods, viz. gas–liquid chromatography [3–5], high-performance liquid chromatography [6–9] and capillary electrophoresis [10], have been used for the assay of thiopentone in biological fluids, in none of these has there been any attempt to separate the enantiomers.

We have decided to study the pharmacodynamics of the thiopentone enantiomers and to determine whether any differences between them could be attributed to differences in their pharmacokinetics. The first requirement of this study, therefore, was to develop a simple, selective, sensitive and precise HPLC assay for the thiopentone enantiomers in plasma. Thiopentone is metabolised principally through two pathways [11,12,13], side-chain oxidation to inactive barbiturates and desulphuration to pentobarbitone which is also active as an anaesthetic agent. It was therefore necessary to resolve thiopentone from pentobarbitone. The method reported here has met these requirements. It has also been used for the determination of thiopentone enantiomers in human plasma and would be suitable for pharmacokinetic studies of the pentobarbitone enantiomers.

2. Experimental

2.1. Materials and reagents

rac-Thiopentone sodium was obtained from Abbott Australia (N.S.W., Australia) and *rac*-pentobarbitone sodium was obtained from Boehringer Ingelheim (N.S.W., Australia). *R*(+)-citronellic acid was supplied by Aldrich (N.S.W., Australia). Phenylbutazone was from ICN Biochemicals (CA, USA). 2-Propanol was from Waters Associates (N.S.W., Australia). Diethyl ether (analytical reagent, AR), disodium hydrogen phosphate (AR) and sodium dihydrogen phosphate (AR) were from AJAX Chemicals (N.S.W., Australia). *n*-Hexane (Nanograde) was from BioLab Scientific (Vic., Australia). Orthophosphoric acid (AR) and sodium hydroxide were from BDH Chemicals (Vic., Australia). Water was purified by a Milli-Q system from Millipore (N.S.W., Australia).

2.2. Instrumentation

An AGP precolumn (10 mm × 3 mm I.D.) and AGP-CSP column (100 mm × 4 mm I.D.) were from ChromTech (Norsborg, Sweden). A Shimadzu Class-LC10 LC workstation (Kyoto, Japan) was used, which consists of a personal computer (IBM compatible, 486 DX2/66), a pump (LC-10AD), a communication bus module (CBM-10A), a UV–Vis detector (SPD-10A) and an auto-injector (SIL-10A).

2.3. Samples and standards

Plasma samples were obtained from adult Merino ewes. The samples for the pharmacokinetic study were obtained according to the method of Rutten et al. [14]. *rac*-Thiopentone (500 mg) was infused intravenously for 2 min and the arterial blood samples (1 ml) were collected into heparinized 1.5-ml Eppendorf tubes at a number of time points for 5 h. Standards were prepared by spiking a series of *rac*-thiopentone solutions (10 μl) with 990 μl drug free blood obtained from the same sheep before infusion. After centrifugation at 2000 *g* for 15 min, the plasma was harvested and the samples and standards were stored at −20°C until analysis. *rac*-Thiopentone stock solution (8 mg/ml) was prepared by adding 436.3 mg *rac*-thiopentone sodium to 50 ml Milli-Q water. Thiopentone standards were made by adding *rac*-thiopentone stock solution to drug-free sheep plasma to make a series of concentrations (0.1, 0.2, 0.5, 1, 5, 20, 40 and 80 μg/ml). Internal standard stock solution was prepared by adding 2 mg phenylbutazone to 4 ml 2 m*M* NaOH.

2.4. Extraction

Plasma samples (300 μl) were placed in 1.5-ml Eppendorf tubes. Internal standard solution (20 μl) and 20% ether in *n*-hexane (1.1 ml) were

then added to the tube followed by 3 *M* H_3PO_4 (20 μl) which decreased the pH of the aqueous phase to about 2.5. The mixture was vortex-mixed (1200 rpm) for 1 min, then centrifuged at 2000 *g* for 5 min and frozen in solid CO_2 for 5 min. The supernatant (organic phase) was decanted into 1.5-ml Eppendorf tubes. The solvent was spun and evaporated to dryness under a vacuum (16 mbar) at 40°C for 10 min in a micro-concentrator. The residue was reconstituted in 50 μl water containing 0.4 m*M* NaOH (ca. pH 10) and transferred into 0.25 ml insert vials of the HPLC auto-sampler for analysis.

2.5. Chromatography

Samples (2–40 μl) were chromatographed on an AGP-CSP column (100 mm × 4 mm I.D.) protected by an AGP (10 mm × 3 mm I.D.) precolumn with 4.5% propanol-2 in 0.1 *M* phosphate buffer (pH 6.2). The eluent flow-rate was 0.9 ml/min and the detection wavelength was at 287 nm for thiopentone and at 220 nm for pentobarbitone. This gave k' values of 1.92, 2.92, 5.71, 9.30 and 11.98 for *R*(+)-pentobarbitone, *S*(−)-pentobarbitone, *R*(+)-thiopentone, *S*(−)-thiopentone, and phenylbutazone, respectively. The detection wavelength was changed from 220 nm to 287 nm at 6 min; with this workstation module this could be done without affecting the baseline. Chromatography was performed at ambient temperature (ca. 23°C).

2.6. Enantiomeric synthesis

R(+)-Thiopentone and *R*(+)-pentobarbitone were synthesised from *R*(+)-citronellic acid using the method of Cook and Tallent [15] in which the possibility of racemisation was avoided during the procedure. These substances were characterised by standard methods and were used to identify the components of the racemates.

3. Results and discussion

First, the identities of *R*(+)-thiopentone and *R*(+)-pentobarbitone were confirmed by examination of ^1H and ^{13}C nuclear magnetic resonance (NMR) and mass spectra (MS).

The NMR and MS data for *R*(+)-thiopentone gave the following results. ^1H NMR (300 MHz, 26 mg per 0.5 ml, C^2HCl_3) δ 0.87 (t, 3H, $J = 7.4$ Hz), 0.88 (t, 3H, $J = 7.0$ Hz), 1.05 (d, 3H, $J = 6.9$ Hz), 1.18 (m, 2H), 1.45 (m, 2H), 2.13 (qd, 2H, $J = 7.4$ Hz, $J = 3.3$ Hz), 2.20 (m, 2H), 9.60 (bs, 2H). ^{13}C NMR (75 MHz, 26 mg per 0.5 ml, C^2HCl_3) δ 9.9, 14.0, 20.7, 28.6, 33.9, 43.0, 61.3, 170.5, 170.9, 176.4. Chemical ionisation mass spectrometry (CIMS, methane reagent gas) *m/z* $[M + 41]^+$ 283 (7), $[M + 29]^+$ 271 (19), $[M + 1]^+$ 243 (100), 213 (5), 173 (9).

The NMR and MS data for *R*(+)-pentobarbitone gave the following results. ^1H NMR (300 MHz, 20 mg per 0.5 ml, C^2HCl_3) δ 0.86 (t, 3H, $J = 7.4$ Hz), 0.89 (t, 3H, $J = 7.0$ Hz), 1.05 (d, 3H, $J = 6.9$ Hz), 1.18 (m, 2H), 1.46 (m, 2H), 2.11 (qd, 2H, $J = 7.4$ Hz, $J = 4.4$ Hz), 2.20 (m, 2H), 8.70 (bs, 2H). ^{13}C NMR (75 MHz, 20 mg per 0.5 ml, C^2HCl_3) δ 9.8, 14.0, 14.3, 20.8, 28.7, 34.0, 42.5, 61.0, 149.9, 172.7, 173.1. Chemical ionisation mass spectrometry (CIMS, methane reagent gas) *m/z* $[M + 41]^+$ 267 (7), $[M + 29]^+$ 255 (19), $[M + 1]^+$ 227 (100), 157 (13).

The optical rotations, measured on a photoelectric Perkin-Elmer Model 241 polarimeter, were found to be $[\alpha]_D$ +9.8° for *R*(+)-thiopentone $[\alpha]_D$ +12.7° for *R*(+)-pentobarbitone. These values were similar to those reported by Cook and Tallent ($[\alpha]_D$ +10.66° and $[\alpha]_D$ +13.12°, respectively) [15]. The melting points of *R*(+)-thiopentone and *R*(+)-pentobarbitone were 152–152.5°C and 122–123°C. These values also were similar to those reported by Cook and Tallent (151–151.5°C and 122–122.5°C, respectively) [15].

The chromatograms in Figs. 1B and 1C show that the synthesised *R*(+)-enantiomers were of very high purity and accounted for 98.9% of the peak areas of the final products: *S*-enantiomers accounted for the remaining 1.1%. For the racemates, the peak areas of the *R*(+)- and *S*(−)-enantiomers were approximately equal. The occurrence of *S*(−)-enantiomers was presumed to be due to optical impurity of the starting material, *R*(+)-citronellic acid, because a similar fraction of impurity of *S*-citronellic acid (2.1%) was

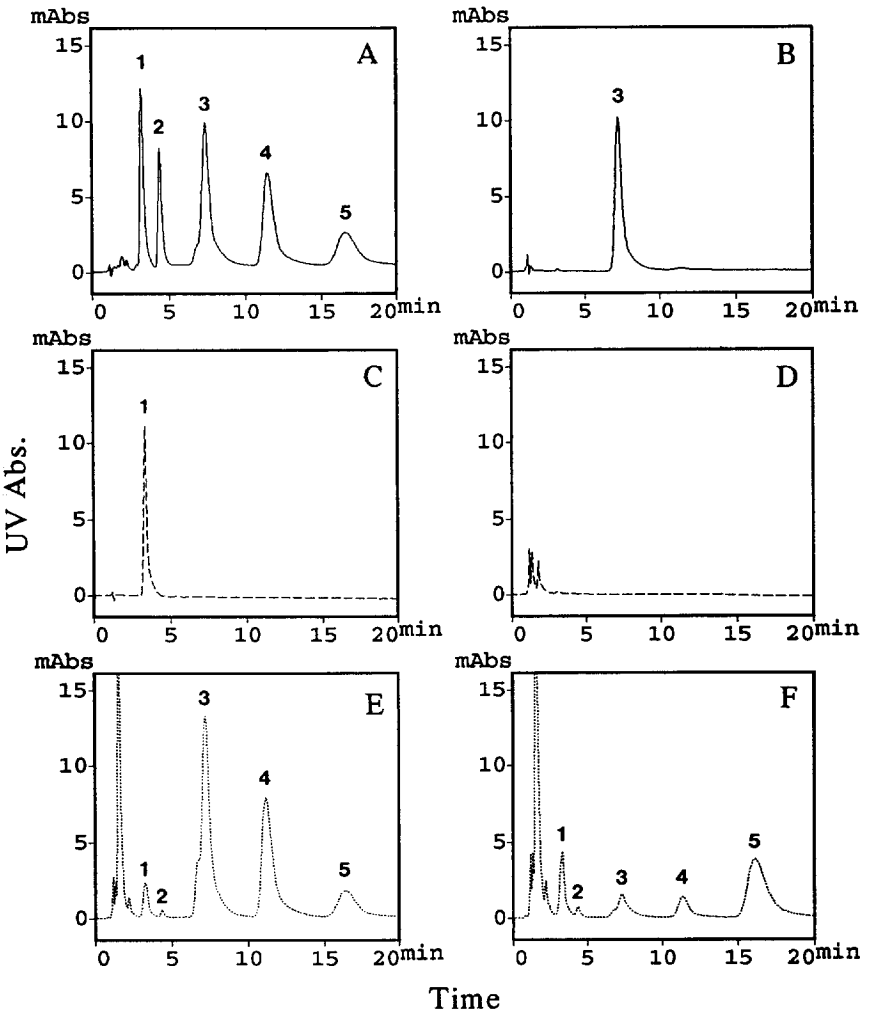

Fig. 1. Chromatograms of (A) *rac*-pentobarbitone 15 μg/ml, *rac*-thiopentone 10 μg/ml and phenylbutazone 7 μg/ml in water: peaks 1, 2, 3, 4 and 5 were *R*(+)-pentobarbitone, *S*(−)-pentobarbitone, *R*(+)-thiopentone, *S*(−)-thiopentone and phenylbutazone, respectively; (B) *R*(+)-thiopentone 6 μg/ml in water; (C) *R*(+)-pentobarbitone 7 μg/ml in water; (D) extract of blank plasma; (E) extract of a sample of 11 min; (F) extract of a sample of 90 min. The UV detection was 220 nm from 0 to 6 min and 287 nm afterward. The injection volumes were 25 μl for standard samples in water and 5 μl for plasma extract.

found in the starting material. Nevertheless, for the purpose to identifying the *R*(+) and *S*(−)-thiopentone by HPLC, the final products were entirely satisfactory.

A chromatogram is shown in Fig. 1A for the injection of *rac*-thiopentone, *rac*-pentobarbitone and phenylbutazone. The retention times of *R*(+)-pentobarbitone, *S*(−)-pentobarbitone, *R*(+)-thiopentone, *S*(−)-thiopentone and the internal standard phenylbutazone were 3.1, 4.1, 7.1, 10.8, and 15.7 min, respectively (Fig. 1A). Both *R*(+)-thiopentone and *R*(+)-pentobarbitone had shorter retention times than their *S*(−)-enantiomers. Chromatographic parameters of the assay are shown in Table 1: baseline separations were achieved for both the thiopentone and the pentobarbitone enantiomers.

A small shoulder was noted on the leading edge of the *R*(+)-thiopentone peak (Figs. 1A, 1E and 1F) and has also been noted for the

Table 1
Analytical data for thiopentone, pentobarbitone and phenylbutazone (internal standard)

Compound	Capacity factor (k')
$R(+)$-Pentobarbitone[a]	1.92
$S(-)$-Pentobarbitone[a]	2.92
$R(+)$-Thiopentone[b]	5.71
$S(-)$-Thiopentone[b]	9.30
Phenylbutazone	13.98

[a] Separation factor (α) = 1.52.
[b] Separation factor (α) = 1.62.

commercially available thiopentone sodium (i.e. racemate). This has been attributed to a thiopentone isomer, 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid, and was investigated previously by Stanski et al. in 1983 [16], who concluded that it has similar pharmacokinetic and anaesthetic properties to thiopentone. Since the impurity was found to exist in the pure *rac*-thiopentone solution (Fig. 1A) used for making the standard curve, its interference with the quantitation of $R(+)$-thiopentone was considered to be minimal. The synthetic route used in this study avoided this impurity in the thiopentone product (Fig. 1B).

The linearity of the standard range (0.01–40 μg/ml) was shown by a correlation coefficient >0.998 for both enantiomers. Inter-day coefficients of variation from six replicates of each thiopentone enantiomer measured at plasma concentrations of 0.02, 0.1 and 8 μg/ml were, respectively, 4.8, 4.4 and 3.5% for $R(+)$-thiopentone and, respectively, 5.0, 4.3 and 3.9% for $S(-)$-thiopentone. Intra-day coefficients of variation from six replicates of each enantiomer each measured on six days measured at the same concentrations were respectively 8.8, 8.0 and 8.8% for $R(+)$-thiopentone and respectively 8.8, 7.4 and 9.6% for $S(-)$-thiopentone. The limit of quantitation was 5 ng/ml for $R(+)$-thiopentone and 6 ng/ml for $S(-)$-thiopentone (signal to noise ratio >4:1). Putting these concentrations into context, plasma concentrations of thiopentone, undifferentiated into enantiomers, associated with sleep have been reported as 5–10 μg/ml [17] and those associated with clinical anaesthesia have been reported as >15 μg/ml [18]. After 500 mg *rac*-thiopentone was infused into the sheep, both thiopentone enantiomers were detectable with this method even after 24 h.

The internal standard phenylbutazone was suitable for the determination of thiopentone enantiomers. The injection of phenylbutazone in the HPLC system gave a peak at a retention time of 15.7 min and was totally resolved from the analytes. The extraction recovery of phenylbutazone (mean $\pm$ S.D., 87.9 $\pm$ 3.9%) was very close to the recovery of the thiopentone enantiomers ($R = 88.8 \pm 3.1\%$ and $S = 90.7 \pm 3.6\%$) in six spiked samples (8 μg/ml) using this method.

It was found that if the extracts in the last step of the extraction procedure were reconstituted in neutral or lower pH aqueous solution (but not organic solvent), the levels of thiopentone and phenylbutazone levels decreased. This was presumed to be due to the nonionized drugs binding to the plastic wall of the Eppendorf tubes and could be overcome by using dilute alkaline solution to reconstitute the sample. A similar phenomenon was also found for glass tubes.

Pentobarbitone is considered to be an active metabolite of thiopentone with a similar, but less potent, pharmacological profile [11–13]. Pentobarbitone was separated from the parent drug and was also resolved into its enantiomers (Fig. 1A) without interference from endogenous plasma substances (Fig. 1B). The extraction recovery was ca. 85% for pentobarbitone by this method. Compared to thiopentone, the concentrations of pentobarbitone in the plasma sample at 11 min were low (Fig. 1E), but significantly greater in the plasma sample at 90 min (Fig. 1F). This was consistent with the results of Stanski et al. [19] and Watson et al. [20] which indicated that 10% to 50% total blood barbiturate was pentobarbitone after patients received thiopentone infusion. A typical plasma concentration–time profile of $R(+)$- and $S(-)$-thiopentone, obtained using the above method, is shown in Fig. 2. This shows that the $R(+)$-thiopentone was eliminated faster than the $S(-)$-thiopentone by the sheep. This might be due to the faster $R(+)$-thiopentone desulphuration to $R(+)$-pentobarbitone re-

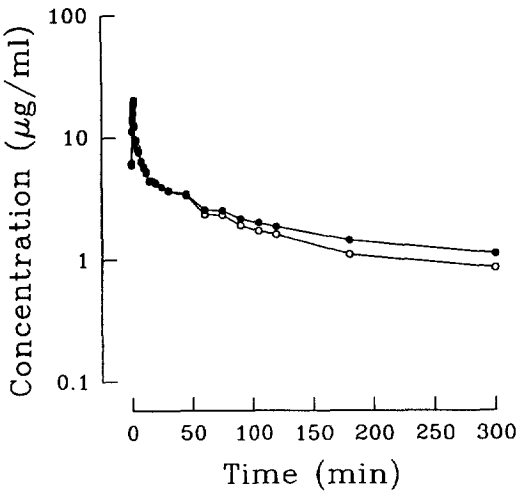

Fig. 2. Plasma concentrations of thiopentone enantiomers following a 2-min i.v. infusion of 500 mg *rac*-thiopentone in an adult Merino ewe. ○ = *R*(+)-Thiopentone; ● = *S*(−)-thiopentone.

sulting in the higher *R*(+)-pentobarbitone concentrations in the plasma (Fig. 1F). As an active metabolite, more attention should be paid to pentobarbitone for future pharmacodynamic studies of thiopentone.

Described here is a simple, selective, sensitive and precise method for the analysis of thiopentone enantiomers. One step extraction ensured the highest possible recovery ratio, simple operation and small manipulation error. A set of 100 samples could be treated in one batch by one operator and analysed overnight with the HPLC system. This method has proven to be suitable for routine analysis of thiopentone enantiomers in plasma as required for our pharmacokinetic studies in sheep and is also being used for studies in humans. It would be suitable for the routine analysis of pentobarbitone enantiomers in plasma.

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