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Short Communication

High-performance liquid chromatographic method for the simultaneous determination of *R*-(−)- and *S*-(+)-hexobarbital in rat plasma

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ABSTRACT

The enantiospecific determination of *R*- and *S*-hexobarbital in rat plasma is described. The method involves liquid–liquid extraction of racemic hexobarbital from plasma, separation of the underivatized enantiomers by high-performance liquid chromatography on an α_1-acid glycoprotein column and ultraviolet detection. The mobile phase consists of a phosphate buffer (pH 5.4) containing 0.4% 2-propanol as organic modifier. An α_1-acid glycoprotein guard column is used to increase the lifetime of the analytical column. Heptabarbital is the achiral internal standard. With detection limits of *ca.* 0.05 μg/ml for both *R*- and *S*-hexobarbital, the assay is suitable for pharmacokinetic studies of the enantiomers in rats.

INTRODUCTION

Hexobarbital, 1,5-dimethyl-5-(1′-cyclohexenyl)barbituric acid (Fig. 1), is used as a model substrate for metabolism studies, as it is completely metabolized by the liver [1], but there are marked pharmacokinetic differences between its enantiomers.

In recent years, enantioselective assays for hexobarbital have been developed, such as enantioselective radioimmunoassay [2], gas chromatography–mass spectrometry (GC–MS) [3], high-performance liquid chromatography (HPLC) with a chiral stationary phase [4] or chiral mobile phase additives [5] and gas chromatography (GC) with a chiral stationary phase [6].

Chiral α_1-acid glycoprotein (AAG) columns have been used for the enantioselective resolution of many substances [7]. The enantiomers of hexobarbital have been resolved on an AAG column [8], but this method was not further developed for application in biological samples.

The present study was undertaken to test the possibility of using HPLC with

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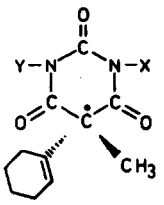
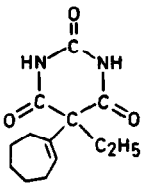


HEXOBARBITAL

	X	Y
R-(-)	CH_3	H
S-(+)	H	CH_3



HEPTABARBITAL

Fig. 1. Structures of the enantiomers of hexobarbital and of the internal standard heptabarbital.

separation on an AAG column for study of the stereoselective disposition of hexobarbital in rats.

EXPERIMENTAL

Reagents and materials

Racemic hexobarbital, 1,5-dimethyl-5-(1′-cyclohexenyl)barbituric acid, was supplied by Serva (Polylab, Antwerp, Belgium). *R*-(−)- and *S*-(+)-hexobarbital were kindly provided by Professor Dr. J. Knabe (Universität des Saarlandes, Saarbrücken, Germany). Heptabarbital was purchased from Brocacef (Maarssen, The Netherlands). The main metabolites of hexobarbital (α-1′,2′-epoxyhexobarbital, norhexobarbital and 3′-ketohexobarbital, the latter contaminated with 25% 6′-ketohexobarbital) were gifts from Professor Dr. N. P. E. Vermeulen (University of Leiden, Leiden, The Netherlands). Diethyl ether (analytical grade), 2-propanol (HPLC), $NaH_2PO_4 \cdot H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ were obtained from E. Merck (Darmstadt, Germany). Petroleum ether 40–60°C was obtained from Carlo Erba (Milan, Italy).

Equipment and chromatographic conditions

The HPLC system consisted of an LKB 2151 HPLC pump, an LKB 2151 variable-wavelength detector set at 210 nm, a Spectra-Physics 4290 integrator, and a Rheodyne Model 7125 injector (20 μl).

The column was an AAG column (100 mm × 4.0 mm I.D., 5 μm particle size, Chiral-AGP, Chromtech, Norsborg, Sweden), with a guard column (10 mm × 3.0 mm, Chiral-AGP, Chromtech), as advised by Chromtech.

Separation was performed at room temperature, using an isocratic mobile phase of phosphate buffer 0.01 *M* (pH 5.4) containing 0.4% 2-propanol, at a flow-rate of 0.9 ml/min.

After a day's use, the column was rinsed with water for 30 min, followed by a

30-min rinsing with 10% 2-propanol in water. With this procedure, problems with contamination of the column were not encountered.

Assay procedure

To 100 μl of plasma in a glass-stoppered glass centrifuge tube, 50 μl of internal standard solution (10 μg/ml heptabarbital) in methanol, 100 μl of water and 200 μl of phosphate buffer (pH 6.5) were added. The plasma was extracted with 3 ml of petroleum ether–diethyl ether–2-propanol (49:49:2, v/v). After vortex-mixing for 10 s and centrifugation for 10 min at 3015 *g*, the organic phase was transferred to a conical glass tube and evaporated to dryness under nitrogen at room temperature. The walls were rinsed with methanol, which was also evaporated. The residue was reconstituted in either 100 or 50 μl of the mobile phase.

To check for possible interference of the main metabolites of hexobarbital (norhexobarbital, 3′-ketohexobarbital contaminated with 25% 6′-ketohexobarbital and α-1′,2′-epoxyhexobarbital [9,10]), blank plasma samples were spiked with 1 and 5 μg/ml of these metabolites and processed as described above.

Standard curves

Standard curves were prepared by adding 0.05, 0.1, 0.2, 0.4, 0.8, 1.0 and 1.5 μg of racemic hexobarbital to 100 μl of drug-free rat plasma, using dilutions (100 and 10 μg/ml) of hexobarbital in methanol. A 10 μg/ml solution of heptabarbital in methanol was prepared, from which 50 μl (500 ng) was taken as the internal standard. The samples were then handled as described under *Assay procedure*. Curves were constructed for each of the enantiomers by plotting the peak-height ratios of enantiomer to internal standard *versus* the enantiomer concentrations. The calibration curves were calculated by least-squares linear regression analysis.

Accuracy and precision

For the calculation of the intra-day accuracy and precision, six blank rat plasma samples (100 μl) were spiked with racemic hexobarbital (0.1, 0.4 and 1.5 μg) and the samples were processed as described under *Assay procedure*. At the same time, calibration curves for both enantiomers were constructed.

For the calculation of the inter-day accuracy and precision, two pooled rat plasma samples (1 and 15 μg/ml racemic hexobarbital) were analysed on sixteen consecutive days, together with a calibration curve.

Recovery from plasma

In order to calculate the recovery of the extraction procedure, five spiked plasma samples (8 μg/ml racemic hexobarbital) were analysed, and the peak-height ratios of hexobarbital to internal standard were compared with those of non-extracted hexobarbital dissolved in the mobile phase (external standard addition).

To calculate the recovery of the I.S., five spiked plasma samples (5 μg/ml I.S.)

were analysed. The peak-height ratios of the internal standard to *S*-(+)-hexobarbital (4 μg/ml) were compared with those of non-extracted heptabarbital dissolved in the mobile phase, again containing *S*-(+)-hexobarbital as external standard. Results are expressed as mean ± standard error of the mean (S.E.M.)

Application of the assay

Hexobarbital concentrations in plasma were measured at different times after intravenous administration of 25 mg/kg racemic hexobarbital to a conscious rat with catheters inserted in both jugular veins [11]. Blood samples were centrifuged and plasma was stored at −20°C until assay.

RESULTS

Assay procedure

Representative chromatograms are shown in Fig. 2. For the enantiomers, the selectivity factor was 1.38, with a resolution factor of 1.41. The elution order, as determined by injection of the pure enantiomers, was *S*-(+)-hexobarbital (t_R = 4.5 min), *R*-(−)-hexobarbital (t_R = 5.8 min) and heptabarbital (t_R = 12.4 min).

After injection of a mixture of 3′- and 6′-ketohexobarbital (3:1), two peaks with retention times of 1.7 and 5.6 min were seen. The extraction recovery, determined in rat plasma samples spiked with the mixture of 3′- and 6′-ketohexobarbital, was *ca.* 30% for each peak. The other metabolites (norhexobarbital and α-1′,2′-epoxyhexobarbital) do not interfere.

Standard curves

Standard curves for *R*- and *S*-hexobarbital were linear over the concentration range studied (0.5–7.5 μg/ml). The slopes for the *R*- and *S*-isomers were 0.325 ± 0.0228 and 0.428 ± 0.0282%, respectively (n = 16), with a correlation coefficient of more than 0.998 for both enantiomers. Intercepts with the y-axis were not significantly different from zero. The minimum detectable level of *R*- and *S*-hexobarbital at a signal-to-noise ratio of 2 was *ca.* 0.05 μg/ml, using 100 μl of plasma.

Accuracy and precision

The intra-day coefficients of variation (C.V.) and the accuracy at concentrations of 1, 4 and 15 μg/ml racemic hexobarbital are given in Table I. The inter-day C.V. and the accuracy at concentrations of 1 and 15 μg/ml racemic hexobarbital are given in Table II.

Recovery from samples

With a mixture of diethyl ether, petroleum ether and 2-propanol as extraction solvent, the recovery was 88.81 ± 4.11% for *S*-(+)- and 87.39 ± 3.50% for *R*-(−)-hexobarbital (n = 5). The recovery of the internal standard, heptabarbital, was 92.58 ± 1.46% (n = 5).

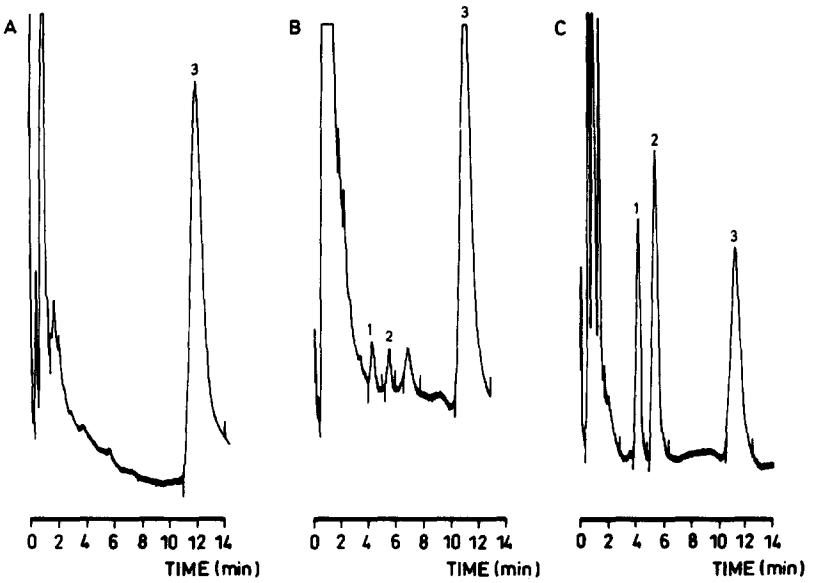


Fig. 2. Representative chromatograms of 0.1-ml extracts of rat plasma. (A) Blank plasma spiked with the internal standard heptabarbital (0.1 μg/ml); (B) blank plasma spiked with *RS*-hexobarbital (0.5 μg/ml) and the internal standard heptabarbital (0.1 μg/ml); (C) plasma obtained 20 min after the intravenous administration of 25 mg/kg *RS*-hexobarbital and spiked with the internal standard heptabarbital (0.1 μg/ml). Peaks: 1 = *S*-(+)-hexobarbital; 2 = *R*-(−)-hexobarbital; 3 = heptabarbital.

Application of the assay

The concentration–time profile of the hexobarbital enantiomers in the rat following intravenous administration of 25 mg/kg racemic hexobarbital is shown in Fig. 3. The terminal half-life of the *S*-enantiomer is 23 min and that of the *R*-enantiomer 26 min. The area under the curve of the *S*-enantiomer is smaller (144 *versus* 274 μg min/ml).

TABLE I

INTRA-DAY ACCURACY AND PRECISION (n = 6)

Concentration added (μg/ml)	Enantiomer	Mean concentration found (μg/ml)	Precision (C.V., %)	Accuracy (% error)
0.5	*R*-(−)	0.464	4.52	−7.2
	S-(+)	0.481	3.10	−3.8
2	*R*-(−)	2.129	3.82	+6.5
	S-(+)	1.982	3.08	−0.9
7.5	*R*-(−)	7.860	4.82	+4.8
	S-(+)	7.791	4.06	+3.9

TABLE II

INTER-DAY ACCURACY AND PRECISION (n = 16)

Concentration added (μg/ml)	Enantiomer	Mean concentration found (μg/ml)	Precision (C.V., %)	Accuracy (% error)
0.5	*R*-(−)	0.449	12.54	−10.2
	S-(+)	0.440	12.09	−12.0
7.5	*R*-(−)	7.211	7.52	−3.9
	S-(+)	7.185	6.45	−4.1

DISCUSSION

Our intention was to study the pharmacokinetics of the hexobarbital enantiomers in rats, so the possibility of using a chiral AAG column for the HPLC assay was investigated. There are some disadvantages in using chiral columns: they are rather expensive and they show a rather slow mass transfer with broader peaks as a result. However, the use of direct methods can avoid the need for time-consuming derivatizations, which are sometimes expensive. Moreover, supplementary steps in the analytical procedure can give rise to loss of drug or problems such as racemization, owing to severe reaction or extraction conditions [12,13].

Although the lifetime of certain chiral columns is limited, the AAG column is described as being stable and having a rather long lifetime [7]. In our hands, after injection of a limited number of samples, a void developed at the top of the

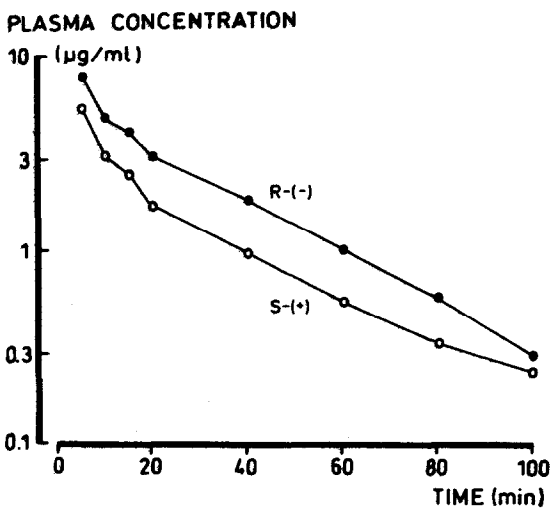


Fig. 3. Plasma concentration–time curves for *S*-(+)-and *R*-(−)-hexobarbital after intravenous administration of 25 mg/kg *RS*-hexobarbital to a male Wistar rat.

analytical column. When topped up, however, the column then remained unaltered. The stability of the column when used with a guard column, as specified by the manufacturer, was good, although the inlet and outlet filters had to be changed regularly, because of a rise in the back-pressure.

The degree of separation of the hexobarbital enantiomers is satisfactory, as indicated by the selectivity and resolution factors. Separation was performed at room temperature (24 ± 2°C): as the assay proved to be satisfactory, the influence of temperature was not investigated. Different pH values of the mobile phase were tested and pH 5.4 was chosen, because at this value plasma impurities in the rat samples, which were sometimes co-extracted, did not interfere. Decreasing the pH value decreases the resolution factor but, under the conditions used, the resolution was still sufficient. The resolution was also modified by changing the percentage of the organic modifier, 2-propanol: minor decreases or increases had a profound influence on the retention characteristics.

The metabolites of hexobarbital are more polar than the parent compound. As the extraction solvent is rather polar, all the metabolites were co-extracted and detected. Ketohexobarbital could possibly interfere with the assay, especially at the higher concentrations of the hexobarbital enantiomers, as its retention time lies between those of the two hexobarbital enantiomers. However, as the rat plasma samples contain so little ketohexobarbital [14] and as it can only be detected at a concentration of 5 μg/ml or greater, interference with the detection need not to be feared. The possibility of using this method to analyse the plasma of other species has to be studied, as interference of ketohexobarbital with the assay remains possible, depending on the plasma concentrations reached.

Other extraction solvents, such as chloroform, ethyl acetate, dichloromethane, methanol and acetonitrile, all gave rise to co-extracting plasma impurities and metabolites. An exception was hexane; however, with an extraction recovery of hexobarbital of *ca.* 60% and of the internal standard heptabarbital of 5–10%, this solvent proved to be inadequate for the analysis.

A series of other non-chiral barbiturates was tested for use as internal standard, but either no separation with the hexobarbital enantiomers could be obtained or the elution took too long. Heptabarbital, in contrast, proved to be satisfactory, and has also a structure similar to that of hexobarbital (Fig. 1).

The assay is simple, with linear standard curves over the concentration range studied. The detection limit is satisfactory, as the enantiomers reach rather high plasma concentrations in rats [1]. The accuracy and precision of the assay are also satisfactory. The preliminary pharmacokinetic findings of the experiments are in accordance with results obtained in other *in vivo* studies in rats [12,15].

It can be concluded that the assay described is suitable for small plasma samples, and that it provides the possibility of studying the pharmacokinetics of the hexobarbital enantiomers in rats.

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