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Note

Simultaneous quantitation of *d*- and *l*-hexobarbital in rat blood by high-performance liquid chromatography

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Hexobarbital has been widely used as a sedative/hypnotic, anesthetic and model substrate to investigate factors affecting the hepatic mixed function oxidase system [1,2]. Due to the racemic nature of hexobarbital, there has been considerable interest in the effects and disposition of its individual isomers. In the past, differences between hexobarbital enantiomers were characterized by the administration of its pure isomers [3–5]. In more recent years, stereoselective assays for hexobarbital have been developed and currently include an enantioselective radioimmunoassay [6], a gas chromatographic–mass spectrometric (GC–MS) assay [7] and a chiral capillary gas chromatography (GC) method [8]. The availability, expense and, in some instances, time-consuming extraction and/or synthesis required by these methods have diminished their broader application to routine work. These shortcomings have led to interest in alternative and less complicated methods for the separation and quantitation of hexobarbital enantiomers in biological samples.

Recent work has demonstrated the ability of the commercially available β-cyclodextrin bonded high-performance liquid chromatographic (HPLC) column to separate the enantiomers of hexobarbital [9–12]. The application of this chiral column to routine separation and quantitation of hexobarbital isomers in biological samples, however, has not been reported. The present study was undertaken to determine whether the Cyclobond I® column could be used to separate hexobarbital enantiomers extracted from small blood samples in order to characterize the stereoselective disposition of this drug in rat.

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EXPERIMENTAL

Materials

Racemic hexobarbital and phenobarbital were gifts from Dr. William C. Lubawy (College of Pharmacy, University of Kentucky, Lexington, KY, U.S.A.). Alphenal (5-allyl-5-phenylbarbituric acid) was purchased from Gane's Chemical Works (New York, NY, U.S.A.). The purified enantiomers of hexobarbital were kindly provided by Dr. Kazuko Miyano (Fukuoka University, Fukuoko, Japan) and Dr. J. Knabe (Universität des Saarlandes, Saarbrucken, F.R.G.). Monobasic potassium phosphate, dibasic potassium phosphate, methanol (HPLC grade) and methylene chloride (HPLC grade) were purchased from Fisher Scientific (Cincinnati, OH, U.S.A.). All chemicals and reagents were used without further purification.

Equipment and chromatographic conditions

The HPLC system used included a Beckman Model 160 UV dectector (214 nm), an Altex pump, a Rheodyne injector with a 20-μl sample loop, a guard column (5 cm $\times$ 4.1 mm, 30–38 μm C_{18} packing) and a Cyclobond I analytical column (25 cm $\times$ 4.6 mm, 10 μm β-cyclodextrin, Advanced Separation Technologies, Whippany, NJ, U.S.A.). Separation of solutes was accomplished at ambient temperature using an isocratic mobile phase of methanol–water (27:73, v/v) at a flow-rate of 1.3 ml/min.

Standards

Serial dilutions of a stock solution containing 1 mg/ml racemic hexobarbital in methanol were made in order to obtain concentrations of racemic hexobarbital ranging from 0.5 to 20 μg/ml. Solutions of alphenal and phenobarbital (6 μg/ml each) were prepared in methanol for use as internal standards.

Assay procedures

Standard curves were prepared by placing 100 μl of standard in a 10-ml PTFE-lined screw-capped borosilicate glass tube. After evaporation of methanol under a stream of nitrogen, 100 μl of rat blood were added to the residue followed by 70 μl of the alphenal internal standard. Each sample was subsequently acidified with 1 ml of phosphate buffer (0.367 *M*; pH 4.5) and extracted with 6 ml of methylene chloride. After vortexing and centrifugation, the aqueous layer was removed from this mixture by aspiration and the organic phase transferred in portions to a 3-ml Reacti-Vial® (Pierce, Rockford, IL, U.S.A.). The methylene chloride was evaporated under a stream of nitrogen and the residue reconstituted in 70 μl of mobile phase prior to injection.

Animal study and analysis

A male Fischer 344 rat (322 g) was employed to evaluate the application of this assay to routine biological samples. An indwelling cannula was inserted into the right jugular vein of the rat under diethyl ether anesthesia and exteriorized through the back of the neck. The animal was allowed one day for recovery fol-

lowing the surgery before entering it into the study. During this time, the rat was fasted but allowed free access to water.

A solution of hexobarbital (10 mg/ml) was prepared by dissolving the racemic drug in a solution of 0.1 *M* dibasic potassium phosphate buffer (pH 11.5). A 24-mg dose of this solution (2.4 ml) was then administered to the rat through an oral intubation tube. Blood samples (220 μl each) were subsequently collected from the rat at 0, 5, 10, 15, 20, 30, 40, 60, 90, 120, 180, 240 and 300 min after drug administration using a new microsampling technique [13]. Two 100-μl aliquots were removed from each sample, placed in 10-ml PTFE-lined screw-capped tubes and stored (−20°C) until analyzed in duplicate for *d*- and *l*-hexobarbital.

The blood concentration–time profile for each enantiomer was fitted to a one-compartment model using the PCNONLIN (Statistical Consultants, Lexington, KY, U.S.A.) computer package in order to obtain the half-life ($t_{1/2}$) and the total area under the concentration–time profile (AUC). The oral clearance (Cl_o) of each enantiomer was calculated using the equation: $Cl_o = D_o/\text{AUC}$, where D_o represents the oral dose of each enantiomer.

RESULTS AND DISCUSSION

Representative chromatograms of a blank blood sample (A) and a blood sample spiked with racemic hexobarbital and internal standard, alphenal (B) are shown in Fig. 1. Initially, retention times for *d*-hexobarbital, *l*-hexobarbital and alphenal were 21.0, 24.0 and 31.6 min, respectively. However, over a six-week period a progressive shortening in the retention times of all peaks were noted until the retention times of *d*- and *l*-hexobarbital and alphenal stabilized at 10.0, 11.1 and 17.2 min, respectively. This process of column aging created a special problem with respect to the selection of a proper internal standard. Earlier work with phenobarbital as an internal standard in this assay had to be abandoned due to the gradual loss in resolution of the *l*-hexobarbital and phenobarbital peaks. Although decreasing retention times also decreased the slopes of the *d*- and *l*-hexobarbital standard curves, this did not appear to adversely affect their linearity during a given run or to result in interference from endogenous blood substances.

The elution profile of hexobarbital enantiomers was determined by injecting the purified enantiomers of this drug (Fig. 2). The elution order of *d*- and then *l*-hexobarbital contrasts with a previous report indicating just the opposite elution profile [12]. The reason for this discrepancy is unknown. The same elution order was observed for hexobarbital enantiomers obtained from two different sources (Dr. Miyano, Japan and Dr. Knabe, F.R.G.) and the order of elution was not found to be affected by the process of column aging.

Standard curves for *d*- and *l*-hexobarbital were linear from 0.25 to 10 μg/ml with an intercept not significantly different from zero. The mean slopes for the *d*- and *l*-isomers were 0.247 and 0.227, respectively, with coefficients of variation for both slopes less than 7% once column aging was complete. The mean correlation coefficient (r) for *d*- and *l*-hexobarbital standard curves over a one-month time period was 0.999 for each isomer. The minimum detectable level of *d*- and *l*-

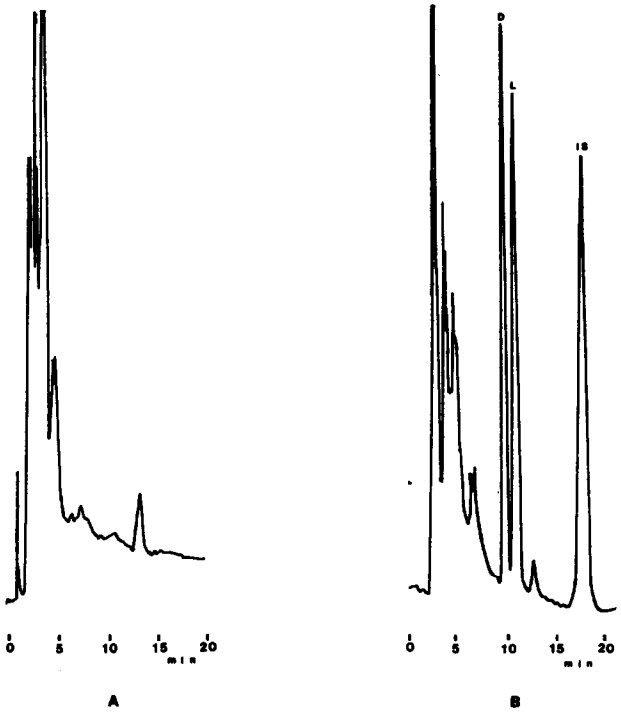

Fig. 1. Chromatograms of a blank blood sample (A) and a blood sample spiked with 1.0 μg of racemic hexobarbital and 0.42 μg of alphenal internal standard (B). Retention times for *d*-hexobarbital (D), *l*-hexobarbital (L) and alphenal (IS) were 10.0, 11.1 and 17.2 min, respectively.

hexobarbital, set at a signal-to-noise ratio of 2, was 0.05 μg/ml for this assay. The range in linearity of this assay compares favorably with the results reported using GC–MS [7] and the chiral GC method for quantitating the levels of hexobarbital enantiomers [8]. The lower limit of quantitation for this assay was also similar to that obtained with GC–MS (0.05 μg/ml) [7] and the chiral GC (0.5 μg/ml) assays previously reported [8].

The reproducibility of this assay was assessed by evaluating the intra-day and inter-day variation in hexobarbital concentrations from spiked blood samples. The intra-day coefficient of variation in *d*- and *l*-hexobarbital at concentrations of 0.5, 5 and 10 μg/ml ranged from 4.2 to 8.4% for the *d*-isomer and 4.2 to 7.5% for the *l*-enantiomer (Table I). This variation was similar to the inter-day variation determined at the same concentrations over a three-week period (Table I). Furthermore, the variation observed in this assay compared favorably with the 4% intra-day to 20% inter-day variability obtained with GC–MS and the 6.2% intra-day to 9.4% inter-day variation found with the chiral GC method for quantitation of hexobarbital isomer concentrations [7,8].

The concentration–time profiles of *d*- and *l*-hexobarbital following administration of a 24-mg oral dose of racemic hexobarbital to a rat are shown in Fig. 3. The marked differences in the disposition of these enantiomers are well illustrated in this rat. The terminal half-life for the *d*-isomer was 6.7 min and for the *l*-enan-

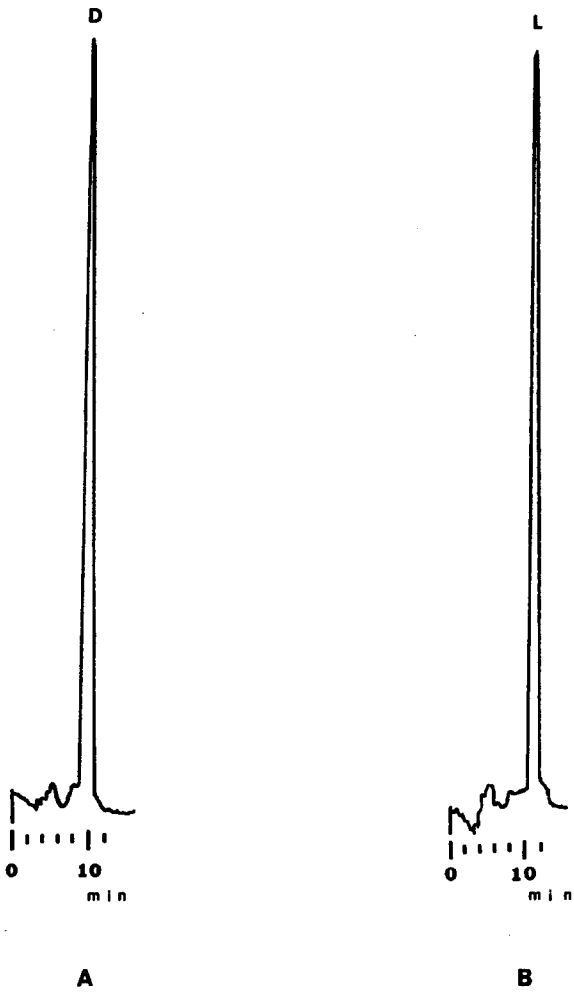

Fig. 2. Chromatograms following injection of *d*-hexobarbital (A) and *l*-hexobarbital (B).

tiomer 24.0 min. The difference in the oral clearances of these enantiomers was even more pronounced. The *d*-isomer had an estimated oral clearance of 2359 ml/min while the estimated oral clearance of the *l*-isomer was only 161 ml/min. This finding is similar to the results obtained in other in vivo rat studies using hexobarbital [3–5,7] and supports in vitro evidence indicating more rapid hepatic microsomal metabolism of the *d*-enantiomer [3,14–17].

The major advantage of this assay lies in its simplicity and lower cost relative to other stereoselective hexobarbital assays. The GC–MS method [7] requires expensive analytical equipment and the costly and time-consuming preparation of pseudoracemic hexobarbital mixtures. The chiral GC method [8] requires a rather lengthy extraction and the use of an expensive chiral column which has a very limited life span due to its sensitivity to oxygen and lability at higher temperatures. An enantioselective radioimmunoassay kit for hexobarbital is not commercially available and its preparation requires familiarity with the prepa-

TABLE I

INTRA- AND INTER-DAY REPRODUCIBILITY IN *d*- AND *l*-HEXOBARBITAL CONCENTRATIONS

Stereoisomer	Spiked concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)
Intra-day (n=7)			
d	0.50	0.49 ± 0.04	8.4
d	5.00	4.94 ± 0.21	4.2
d	10.00	10.20 ± 0.73	7.2
l	0.50	0.44 ± 0.02	5.4
l	5.00	4.94 ± 0.21	4.2
l	10.00	10.14 ± 0.76	7.5
Inter-day (n=9)			
d	0.50	0.51 ± 0.03	6.5
d	5.00	5.02 ± 0.39	7.8
d	10.00	9.92 ± 0.35	3.6
l	0.50	0.51 ± 0.04	7.6
l	5.00	5.09 ± 0.33	6.4
l	10.00	9.87 ± 0.38	3.8

ration of antibodies to haptens and additional testing to rule out cross-reactivity of the final product. By comparison, this assay is relatively simple and cheap in that the chiral HPLC columns are comparable in cost to other achiral columns and the extraction involves only a single step. The application of this assay to small blood samples from the rat provides an opportunity to address the role which endogenous and exogenous factors may have on the stereoselective disposition of this drug.

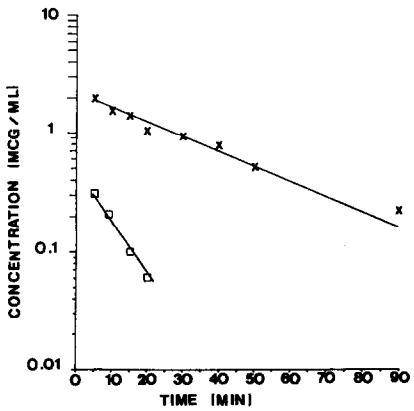

Fig. 3. Plot of log concentration versus time for *d*- (□) and *l*- (×) hexobarbital following oral administration of racemic hexobarbital (24 mg dose) to a male Fischer rat. The solid lines represent the computer best-fit equations to the postabsorptive concentration–time data points.

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REFERENCES

1 M.T. Bush and W.L. Weller, Drug Metab. Rev., 1 (1972) 249.
2 R.R. Holcomb, N. Gerber and M.T. Bush, J. Pharmacol. Exp. Ther., 188 (1974) 15.
3 R.L. Furner, J.S. McCarthy, R.E. Stitzel and M.W. Anders, J. Pharmacol. Exp. Ther., 169 (1969) 153.
4 D.D. Breimer and J.M. van Rossum, Eur. J. Pharmacol., 26 (1974) 321.
5 M. van der Graaff, N.P.E. Vermeulen, R.P. Joeres and D.D. Breimer, Drug Metab. Dispos., 11 (1983) 489.
6 C.E. Cook, in D.D. Breimer and P. Speiser (Editors), Topics in Pharmaceutical Sciences, Elsevier, Amsterdam, 1983, p. 87.
7 M. van der Graaff, P.H. Hofman, D.D. Breimer, N.P.E. Vermeulen, J. Knabe and L. Schamber, Biomed. Mass Spectrom., 12 (1985) 464.
8 M. van der Graaff, N.P.E. Vermeulen, P.H. Hofman and D.D. Breimer, J. Chromatogr., 375 (1986) 411.
9 D.W. Armstrong, J. Liq. Chromatogr., 7 (Suppl. 2) (1984) 353.
10 Z.-Y. Yang, S. Barkan, C. Brunner, J.D. Weber, T.D. Doyle and I.W. Wainer, J. Chromatogr., 324 (1985) 444.
11 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, Science, 232 (1986) 1132.
12 W.L. Hinze and T.E. Riehl, Anal. Chem., 57 (1985) 234.
13 S.L. Chang, K. Emmick and P.J. Wedlund, J. Pharm. Sci, 75 (1986) 456.
14 E. Degwitz, V. Ullirch, H. Staudinger and W. Rummel, Hoppe-Seyler's Z. Physiol. Chem., 350 (1969) 547.
15 J.S. McCarthy and R.E. Stitzel, J. Pharmacol. Exp. Ther., 176 (1971) 772.
16 D.R. Feller and W.C. Lubawy, Pharmacology, 9 (1973) 129.
17 K. Miyano, Y. Fujii and S. Toki, Drug Metab. Dispos., 8 (1980) 104.