ELSEVIER

Journal of Chromatography B, 703 (1997) 289–294

JOURNAL OF CHROMATOGRAPHY B

Short communication

Capillary electrophoresis stereoselective determination of *R*-(+)- and *S*-(−)-pentobarbital from serum using hydroxypropyl-γ-cyclodextrin, solid-phase extraction and ultraviolet detection

Karthik Srinivasan, Michael G. Bartlett*

Department of Medicinal Chemistry, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA

Received 2 May 1997; received in revised form 21 July 1997; accepted 25 July 1997

Abstract

Pentobarbital is a sedative hypnotic which is used in the treatment of people suffering from anxiety related conditions and to manage elevated intracranial pressures and cerebral ischemia due to neurosurgical procedures. This paper develops and validates a rapid and sensitive method for the determination of *S*-(−)- and *R*-(+)-enantiomers in serum using capillary electrophoresis (CE) and UV detection. Stereoselective resolution was accomplished using 40 m*M* hydroxypropyl-γ-cyclodextrin contained in 50 m*M* phosphate buffer pH 9.0. The method involves a solid-phase extraction of both the enantiomers and the internal standard, aprobarbital, from serum using C_{18} Bond-Elut cartridges. The CE system consists of a 52 cm×75 μm I.D. fused-silica capillary maintained at a run voltage of 15 kV with sample detection performed at 254 nm. The detection and quantitation limits for *S*-(−)- and *R*-(+)-pentobarbital are 1 μg/ml from serum. Linear calibration curves from 1 to 60 μg of both *S*-(−)- and *R*-(+)-enantiomers show a coefficient of determination of more than 0.999. The precision and accuracy of the method calculated as R.S.D. and error are 0.20–2.20% and 0.00–4.40%, respectively for *R*-(+)-pentobarbital and 0.30–2.19% and 0.30–6.40%, respectively for *S*-(−)-pentobarbital.

Keywords: Pentobarbital; Hydroxypropyl-γ-cyclodextrin

1. Introduction

Enantiomers often differ in their effect on living matter, as tissues are composed of chiral building blocks like amino acids, sugars and nucleic acids. Consequently, determination of individual enantiomers is essential for elucidating the distribution, metabolism and excretion profiles of the enantiomers [1]. To date, chiral high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been the most widely used techniques. The advantages of capillary electrophoresis (CE) like low sample volumes, high efficiency and ease of operation provide powerful alternatives to existing chromatographic methodologies [2–8]. Resolution of enantiomers using CE has been performed using various chemically diverse agents like macrocyclic antibiotics (vancomycin, teicoplanin) [9], bile salts (taurocholic acid, glycocholic acid etc.) [10], native and derivatized cyclodextrins [11], bovine serum albumin [12] and ergot alkaloids [13]. Cyclodextrin mediated capillary zone electrophoresis (CZE) is a versatile and probably the most well understood separation principle to date. Hydroxypropyl-γ-cyclo-

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00399-X

dextrin is one of the most soluble cyclodextrins with a solubility of about 45 g/100 ml. A solution of this cyclodextrin may be stored for several weeks at room temperature. Its cavity size is 8.3 Å and as such accommodates slightly larger substrates. Stereoselective and nonstereoselective quantitation of barbiturates using HPLC and CE have been done previously [14–16] but to date there has been no report of CE stereoselective determination of pentobarbital.

Pentobarbital is a sedative hypnotic used to relieve anxiety prior to surgical procedures. It is also used to manage elevated intracranial pressures and cerebral ischemia from head injuries, asphyxiation and neurosurgical procedures. Pentobarbital shows some adverse reactions at a dose of 0. 15 to 1.5 mg/kg and is reported to cause bradicardia, hypotension and syncope. It is reported that calcium current is selectively blocked by *S*-(−)-pentobarbital and not by *R*-(+)-pentobarbital which might be the cause of the cardiovascular adverse reactions observed on administration of the high doses of the drug [17]. *S*-(−)-Pentobarbital also enhances GABA response which may account for the higher sedative effect of *S*-(−)- over the *R*-(+)-enantiomer [17].

2. Experimental

2.1. Reagents and chemicals

Racemic pentobarbital, *S*-(−)- and *R*-(+)-enantiomers were obtained from National Institute for Drug Abuse. Phosphoric acid (85%), sodium dihydrogenphosphate monohydrate and ammonia solution were obtained from J.T. Baker (Phillipsburg, NJ, USA). Hydroxypropyl-γ-cyclodextrin was obtained from Research Biochemicals International (Natick, MA, USA). Aprobarbital, β-cyclodextrin and dimethyl-β-cyclodextrin were obtained from Sigma (St. Louis, MO, USA). Drug free human serum was obtained from Biological Specialty (Colmar, PA, USA). C_{18} solid-phase extraction (SPE) columns of 1 ml capacity (100 mg/ml) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). All solutions were filtered through a 0.2 μm nylon filter (Acrodisc 13, Gelman Sciences, Ann Arbor, MI, USA).

2.2. Preparation of stock and standard solutions

Individual stock solutions were prepared in 20% methanol–water to give a concentration of 1.0 mg/ml of *S*-(−)- and *R*-(+)-pentobarbital and the internal standard, aprobarbital. Appropriate volumes of the individual *S*-(−)- and *R*-(+)-pentobarbital and internal standard were pipetted into a 2 ml volumetric flask and evaporated. Then 1 ml serum was added and mixed well. A stock solution of sodium dihydrogen phosphate was prepared in double distilled, deionized water and the pH was adjusted to 9.0 using 100 m*M* sodium hydroxide and concentrated phosphoric acid.

2.3. Electrophoretic system

All CE experiments were performed using a P/ACE System 5000 (Beckman, Fullerton, CA, USA) equipped with a UV detection system. An uncoated fused-silica capillary 62 cm (effective length 52 cm)×75 μm I.D (Polymicro Technologies, Phoenix, AZ, USA) was used for analysis. The capillary was thermostatted at 25°C and the voltage applied was 15 kV. The typical running current was approximately 100 μA. A 0.5 cm detection window was created by stripping the polyamide coating of the capillary. The detection was towards the cathodic end. The run buffer consisted of aqueous solution of 50 m*M* phosphate buffer pH 9.0 (adjusted with 100 m*M* sodium hydroxide) containing 40 m*M* hydroxypropyl-γ-cyclodextrin. The analytes were monitored at a wavelength of 254 nm.

2.4. Electrophoretic conditions

New capillaries were conditioned by rinsing with 1 *M* sodium hydroxide for 10 min followed by 10 min each with water, 0.1 *M* hydrochloric acid and run buffer solutions. The sample introduction was performed using vacuum injection (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 10 s. Before each analysis, the capillary was rinsed for 2 min first with 0.1 *M*

sodium hydroxide and 2 min with the run buffer solution.

2.5. Assay procedure

Sample clean up was done using Bond-Elut C_{18} SPE cartridges. The cartridge was pretreated using 3 ml of methanol and then with 3 ml of pH 9.0 phosphate buffer. The sample which contains *R*-(+)- and *S*-(−)-pentobarbital and the internal standard aprobarbital in 1 ml of serum was added to the cartridge and allowed to flow down under low vacuum. The cartridge was not allowed to dry between the pretreatment and sample application steps. The column was then washed with 3 ml of the buffer and allowed to dry for 5 min. The analytes were then eluted with 3 ml of methylene chloride. The eluent was filtered with a nylon filter prior to evaporation using a nitrogen stream. The samples were reconstituted in 1 ml of 30% methanol in water mixture and vacuum injected into the capillary for 10 s. Absolute recoveries were calculated by comparing the drug peak height in the spiked serum analyte sample to the unextracted stock solutions which had been injected directly into the electrophoretic system. SPE with Bond Elut C_{18} cartridges gave absolute recoveries of 98–99% for both analytes and the internal standard. The drug concentration used to study the absolute recovery was 30 μg/ml. Calibration curves were constructed using 1–60 μg/ml. Linear regression analysis of concentration versus drug/internal standard peak height ratios gave slope and intercept data for each analyte, which were used to calculate the concentration of unknown analytes in serum samples.

3. Results and discussion

The chemical structures of *R*-(+)- and *S*-(−)-pentobarbital and the internal standard aprobarbital are shown in Fig. 1. Baseline resolution (R_s=4.66) of the pentobarbital enantiomers was achieved using 40 m*M* hydroxypropyl-γ-cyclodextrin as a chiral complexing agent. The separation efficiency was studied at pH values 7.5, 8.0 and 9.0 and was found to be best at pH 9.0. Baseline resolution was

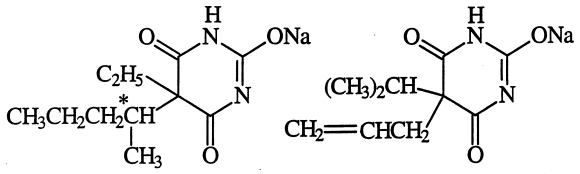

Fig. 1. Chemical structures of pentobarbital and aprobarbital.

achieved at hydroxypropyl-γ-cyclodextrin concentrations of 25, 30, 35 and 40 m*M* but depletion on repeated runs leading to a decrease in resolution was prominent at concentrations less than 40 m*M*.

Migration times were also not significantly affected by increasing the concentration of the buffer. Baseline resolution could not be achieved with native β-cyclodextrin or trimethyl-β-cyclodextrin. The remaining derivatised cyclodextrins showed poor separation efficiencies. The analyte peak shape symmetries were found to be best at 50 m*M* phosphate buffer and the migration time was around 10 min. Fig. 2A shows the electropherogram of blank serum and Fig. 2B shows the electropherogram of spiked *R*-(+)- and *S*-(−)-pentobarbital with the internal standard, aprobarbital.

Cyclodextrins separate enantiomers using the phenomenon of host–guest complexation where a transient diastereomeric complex is formed between the cyclodextrin and analyte. The affinity of the cyclodextrin for the analyte is due to hydrophobic interactions between the cyclodextrin cavity and analyte and the hydrogen bonding of the functional groups on the analyte with the hydroxyl groups in the cyclodextrin ring [18]. Counter-current flow of analyte and cyclodextrin was effected by negatively charging the analyte and moving it against the flow of cyclodextrin. A pH of 9.0 was used to charge both analyte and cyclodextrin, thereby inducing electrostatic interaction between both and significantly improving the resolution [19]. Migration time reproducibility was high with less than 0.5% R.S.D. (n=9) for all three peaks.

To decrease the detection limit and to reduce band broadening to achieve sharper peaks, the sample was prepared in a lower conductivity solvent (methanol–water) than the electrolyte solution. When a voltage of 15 kV is applied across the capillary, a greater

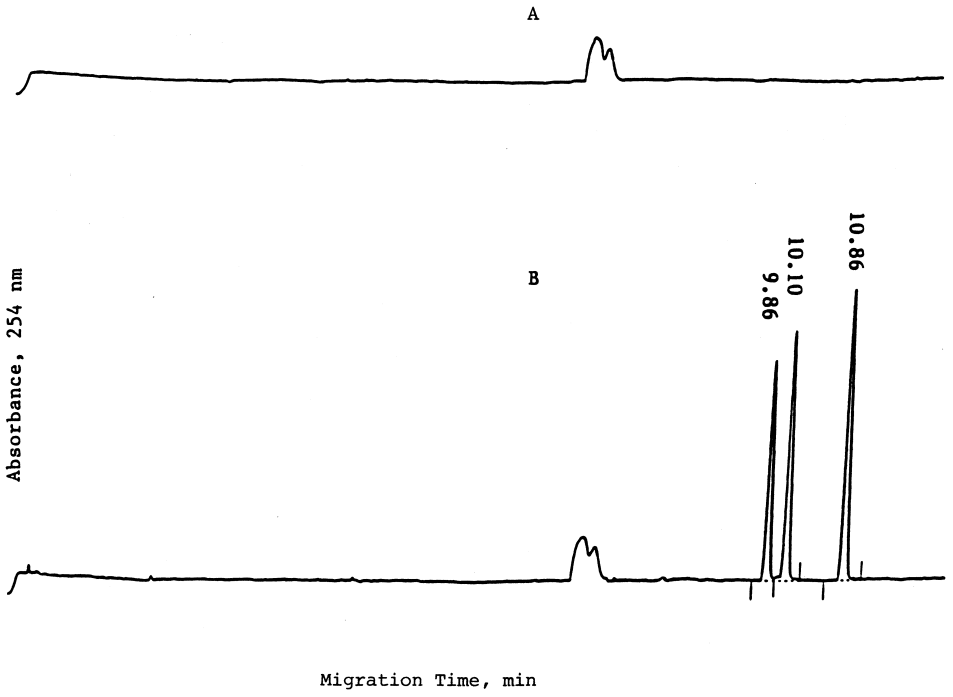

Fig. 2. Typical chromatogram of (A) blank serum and (B) serum spiked with *R*-(+)-pentobarbital (9.86 min), *S*-(−)-pentobarbital (10.10 min) and internal standard aprobarbital (10.86 min) on a 57 cm×75 μm I.D. fused-silica capillary. The run buffer contained 40 m*M* hydroxypropyl-γ-cyclodextrin in 50 m*M* phosphate buffer (pH 9.0) with detection at 254 nm. The capillary was thermostatted at 25°C and run voltage was 15 kV.

field develops across the sample plug. This causes the ions to move faster. When the ions reach the buffer they slow down due to the reduced field to which they are subjected, thereby resulting in analyte stacking within a narrow zone of the capillary [20,21].

Pentobarbital is sometimes administered in combination with the anticonvulsants diazepam, clonazepam and phenytoin. Fig. 3A shows the separation of the pentobarbital enantiomers, aprobarbital, diazepam and clonazepam and Fig. 3B shows the separation of the pentobarbital enantiomers, aprobarbital and phenytoin. Thus, the coadministered drugs do not interfere or affect the method developed.

The calibration curve showed a good linearity in the range of 1 to 60 μg/ml for both *R*-(+)- and *S*-(−)-pentobarbital. The range of concentrations encompass the typical therapeutic range for pentobarbital (20–40 μg/ml). The coefficient of determination was 0.999 ($n=3$). Representative linear regression equations obtained for *R*-(+)- and *S*-(−)-pentobarbital were $y=50.87x-7.08$ and $y=46.19x-6.83$, where y and x were concentration and drug to internal standard peak height ratios, respectively. The intra-day precision and accuracy (n=3) as expressed by R.S.D. and error were 0.20–2.20%, 0.00–4.40%, respectively for *R*-(+)-pentobarbital and 0.30–1.50% and 3.40–4.40%, respectively for *S*-(−)-pentobarbital. The inter-day precision and accuracy over three days ($n=9$) expressed by R.S.D. and error were 0.77–1.80% and 0.30–2.60%, respectively for *R*-(+)-pentobarbital and 1.80–2.19% and 0.30–6.40%, respectively for *S*-(−)-pentobarbital. Table 1 gives inter- and intra-day precision and accuracy values of this method. The methods limit of quantitation was found to be 1.0 μg/ml at a signal-to-noise (S/N) ratio of 3/1.

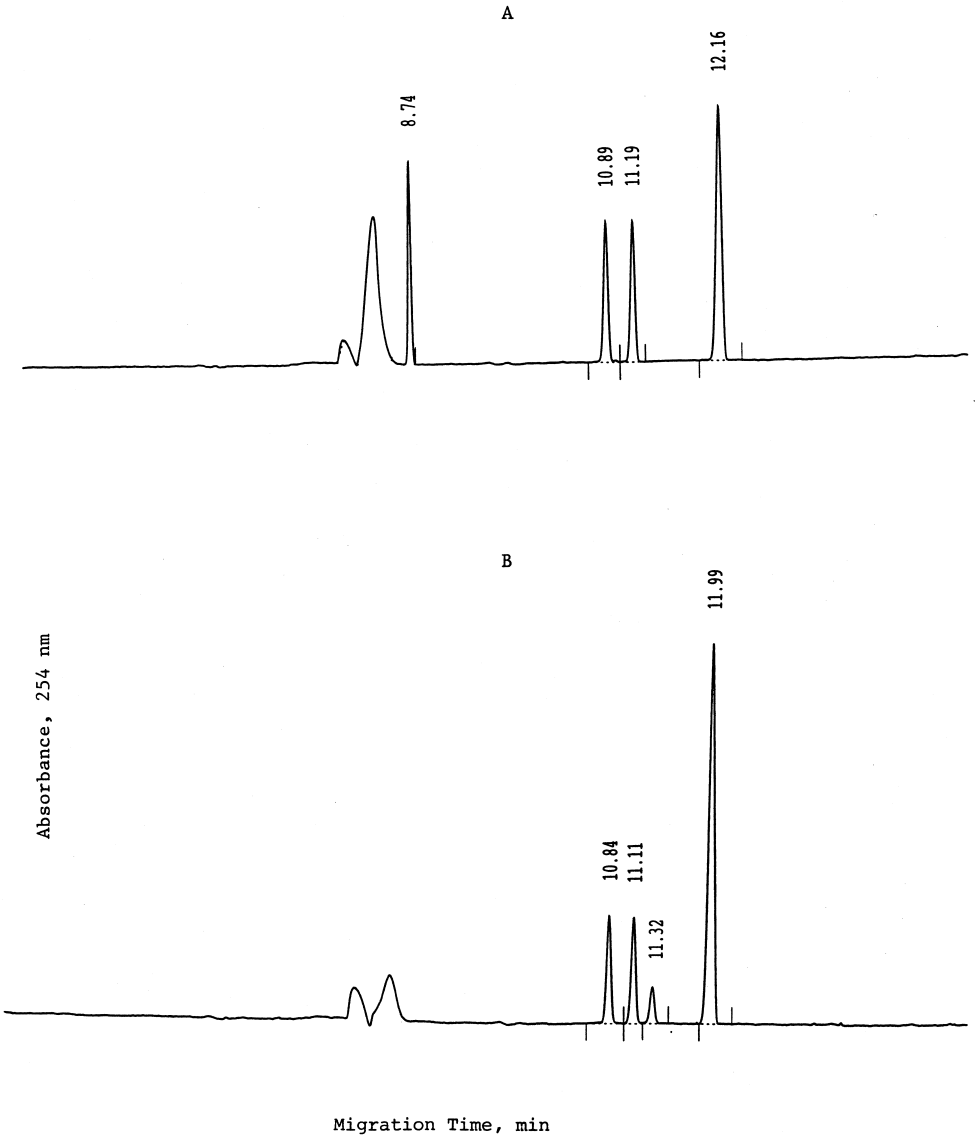

Fig. 3. Chromatogram of (A) diazepam and clonazepam (8.74 min), *R*-(+)-pentobarbital (10.89 min), *S*-(−)-pentobarbital (11.19 min) and aprobarbital (12.16 min) and (B) *R*-(+)-pentobarbital (10.84 min), *S*-(−)-pentobarbital (11.11 min), phenytoin (11.32 min) and aprobarbital (11.99 min) on a 57 cm×75 μm I.D. fused-silica capillary. The run buffer contained 40 m*M* hydroxypropyl-γ-cyclodextrin in 50 m*M* phosphate buffer (pH 9.0) with detection at 254 nm. The capillary was thermostatted at 25°C and run voltage was 15 kV.

In summary, the high-performance CE assay described herein is sensitive and suitable for simultaneous determination of *R*-(+)- and *S*-(−)-enantiomers of pentobarbital from serum. The SPE method provides excellent sample clean up with no endogenous interferences and almost complete recovery. This method also shows good linearity, precision and accuracy within the linear range of 1–60 μg/ml. The method is rapid and sensitive and would be a good alternative to chiral HPLC or GC methods.

Table 1
Accuracy and precision of pentobarbital enantiomers added to serum

	Concentration added (μg/ml)	Concentration found (μg/ml)	R.S.D. (%)	Error (%)
Intra-day ($n=3$)				
R-(+)-pentobarbital	10	10.00±0.22	2.20[a]	0.00
	30	31.37±0.06	0.20	4.40
S-(−)-pentobarbital	10	10.34±0.16	1.50	3.40
	30	31.30±0.09	0.30	4.40
Inter-day ($n=9$)				
R-(+)-pentobarbital	10	9.97±0.18	1.80[b]	0.30
	30	30.79±0.24	0.77	2.60
S-(−)-pentobarbital	10	9.97±0.18	1.80	0.30
	30	31.92±0.70	2.19	6.40

[a]Mean±S.D., based on $n=3$ for intra-day assay.
[b]Mean±S.D., based on $n=9$ for inter-day assay.

References

[1] D. Stevenson and I.D. Wilson, Chiral Separations, Plenum Press, New York, 1988, p. 1.
[2] J. Snopek, I. Jelinek, E. Smolkova-Keulemansova, J. Chromatogr. 438 (1988) 211.
[3] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg, B.L. Karger, J. Chromatogr. 448 (1988) 41.
[4] S. Terabe, Trends Anal. Chem. 8 (1989) 129.
[5] A.G. Ewing, R.A. Wallingford, T.M. Olefirowicz, Anal. Chem. 61 (1989) 292A.
[6] S. Terabe, H. Shibata, Y. Miyashita, J. Chromatogr. 480 (1989) 403.
[7] S. Fanali, P. Bocek, Electrophoresis 11 (1990) 757.
[8] C. Desiderio, Z. Aturki, S. Fanali, Electrophoresis 15 (1994) 864.
[9] D.W. Armstrong, K.L. Rundlettand, J.R. Chen, Chirality 61 (1994) 496.
[10] S. Terabe, K. Otsuka, H. Nishi, J. Chromatogr. A 666 (1994) 295.
[11] A. Guttman, S. Brunet, C. Jurado, N. Cooke, Chirality 7 (1995) 409.
[12] D. Ebele, R.P. Hummel, R. Kuhn, J. Chromatogr. A 759 (1997) 185.
[13] B.A. Ingelse, M. Flieger, H.A. Classens, F.M. Everacts, J. Chromatogr. A 755 (1996) 251.
[14] H.Y.A. Enein, V. Serignese, L.I.A. Basha, J. Bojarski, Pharmazie 51 (1996) 159.
[15] E. Francotte, S. Cherkaoui, M. Faupel, Chirality 5 (1993) 516.
[16] S. Eto, H. Noda, A. Noda, J. Chromatogr. 579 (1992) 253.
[17] J.M.M. Ffrench, J.L. Barker, M.A. Rogawski, J. Neurosci. 13 (1993) 3211.
[18] M. Siluveru, J.T. Stewart, J. Chromatogr. B 691 (1997) 217.
[19] B. Chankvetadze, G. Endresz, G. Blaschke, Electrophoresis 15 (1994) 804.
[20] D.S. Burgi, R.L. Chien, Anal. Chem. 63 (1991) 2042.
[21] R.L. Chien, D.S. Burgi, J. Chromatogr. 559 (1991) 141.