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Characterization of the stereoselective metabolism of thiopental and its metabolite pentobarbital via analysis of their enantiomers in human plasma by capillary electrophoresis

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Abstract

Using capillary zone electrophoresis (CZE) with a 75 m*M* phosphate buffer at pH 8.5 containing 5 m*M* hydroxypropyl-γ-cyclodextrin (OHP-γ-CD) as chiral selector, the separation of the enantiomers of thiopental and its oxybarbiturate metabolite, pentobarbital, is reported. Enantiomer assignment was performed via preparation of enantiomerically enriched fractions using chiral recycling isotachophoresis (rITP) processing of racemic barbiturates and analysis of rITP fractions by chiral CZE and circular dichroism spectroscopy. Thiopental and pentobarbital enantiomers in plasma were extracted at low pH using dichloromethane and extracts were reconstituted in acetonitrile or 10-fold diluted, achiral running buffer. The stereoselectivity of the thiopental and pentobarbital metabolism was assessed via analysis of 12 plasma samples that stemmed from patients undergoing prolonged or having completed long-term racemic thiopental infusion. The data obtained revealed a modest stereoselectivity with *R*-(+)-thiopental/*S*-(−)-thiopental and *R*-(+)-pentobarbital/*S*-(−)-pentobarbital plasma ratios being <1 ($P<0.05$ compared to data obtained with racemic controls) and >1 ($P<0.001$), respectively. The total *S*-(−)-thiopental plasma concentration was found to be on average about 24% higher compared to the concentration of *R*-(+)-thiopental, whereas the total *R*-(+)-pentobarbital plasma level was observed to be on average 29% higher compared to the *S*-(−)-pentobarbital concentration.

Keywords: Enantiomer separation; Pharmaceutical analysis; Thiopental; Pentobarbital; Barbiturates

1. Introduction

Thiopental and pentobarbital are intravenous anesthetic agents which are also used to reduce intracranial pressure in case of neurological and neurosurgical emergencies like closed head injuries. Pentobarbital is also a metabolite of thiopental which is formed via substitution of sulfur with oxygen (for structures refer to Fig. 1). The mean plasma half lifes of thiopental and pentobarbital are 9 and 27 h, respectively. About 1% of both drugs are excreted unchanged in the urine [1]. Both thiopental and pentobarbital are chiral compounds (Fig. 1) and are administered as racemates. For thiopental two studies showed that pharmacokinetic data are slightly different for the two enantiomers [2,3]. The total clearance and the apparent volume of distribution at steady-

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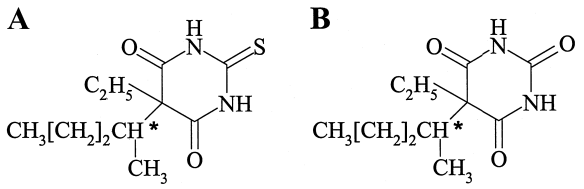

Fig. 1. Chemical structures of (A) thiopental and (B) pentobarbital. Asterisks mark the chiral C atoms.

state for *R*-(+)-thiopental are somewhat greater than for *S*-(−)-thiopental. This is explained with the difference in the unbound protein fraction. The *R*-form of thiopental was determined to be less protein-bound than the *S*-form. Thus, the total serum concentration of the *R*-enantiomer was found to be slightly lower compared to that of the *S*-enantiomer. The pharmacological EEG effect, however, could not be assigned to one or the other enantiomer [3]. For pentobarbital, the *S*-form is reported to cause a longer duration of sedation in man than the *R*-enantiomer. Cook et al. determined that there is a stereoselective elimination and protein binding of pentobarbital in man [4]. *S*-(−)-Pentobarbital is reported to be stronger protein bound and to have a lesser clearance. Furthermore, after intravenous administration of thiopental, Nguyen et al. [2] found no significant difference in the total plasma concentrations of the two pentobarbital enantiomers. However, due to different protein binding, the free fraction of the *R*-enantiomer is present at a significant higher concentration than *S*-(−)-pentobarbital.

As of today, thiopental and pentobarbital enantiomers were mainly determined by high-performance liquid chromatography (HPLC) using expensive, enantioselective columns [2,3,5,6]. Pentobarbital enantiomers were also determined by enantioselective radioimmunoassays [4]. Recently, capillary electrophoresis (CE) techniques have been shown to represent attractive approaches for drug monitoring and characterization, including the analysis of thiopental and pentobarbital in body fluids [7–10]. CE in the presence of a chiral selector is well suited to achieve separation of enantiomers [11–14]. Using cyclodextrin-based CE methods has lead to the characterization of the stereoselective metabolism of various drugs, including cicletanine [15], mephenytoin [16], oxprenolol [17], zoplicone [18], dimethindene [19], methadone [20], 3,4-methylenedioxymethamphetamine (MDMA) [21] and debrisoquine [22]. Enantiomeric separation for thiopental and pentobarbital was reported by Nishi et al. using a micellar buffer (pH 9.0) that was fortified with 30 to 40 m*M* γ-cyclodextrin and 20 to 40 m*M* sodium d-campher-10-sulphonate [14]. Using micellar buffers with various cyclodextrins as chiral selectors, enantiomeric resolution of other barbiturates was studied by Francotte et al. [23]. Furthermore, Srnivasan and Bartlett reported a chiral assay for the determination of serum levels of pentobarbital enantiomers. This method involves solid-phase extraction followed by CE at pH 9.0 using a micelle-free buffer containing (2-hydroxypropyl)-γ-cyclodextrin (OHP-γ-CD) as chiral selector [24]. In our laboratory, the micellar configuration of Nishi et al. was previously employed to monitor thiopental and pentobarbital enantiomers in serum [25,26]. The data obtained with a couple of patient samples suggested that there is no measurable stereoselectivity involved in the hepatic thiopental to pentobarbital conversion. This is somewhat in contrast to the modest stereoselectivity reported by others [2,3]. No other CE report dealing with the stereoselective metabolism of thiopental and pentobarbital was found. Recently, using CE with non-micellar buffers, the assessment of the stereoselective metabolism of the two barbiturates was revisited in our laboratory.

In this paper, simultaneous analysis of thiopental and pentobarbital enantiomers in plasma and serum by cyclodextrin-based capillary zone electrophoresis (CZE) is discussed and shown to be a simple and economical approach for the simultaneous assessment of the stereoselective metabolism of thiopental and pentobarbital. Furthermore, as no pure enantiomeric standards were available, assignment of enantiomers is shown to be possible with chiral CZE and circular dichroism spectroscopy analysis of samples that are partly enriched in one of the two enantiomers. Such samples were prepared by chiral recycling isotachophoresis (rITP) of a racemic mixture, an approach that has recently been investigated and described in detail using methadone as a model compound [27].

2. Experimental

2.1. Chemicals, origin of patient specimens and preparation of calibrator and control samples

All chemicals used were of analytical or research grade. Pentobarbital was of European Pharmacopoeia quality and was received from the hospital pharmacy (Inselspital, Berne, Switzerland). Pentobarbital stock solutions were made with methanol (1 mg/ml). Thiopental was used as sodium salt from a Pentothal ampoule (Abbott, Baar, Switzerland) which contains 2.5 g thiopental and 150 mg sodium carbonate. Stock solutions were made with bidistilled water. 3-isobutyl-1-methylxanthin (EGA-Chemie, Steinheim, Germany) was used as internal standard (I.S.). OHP-γ-CD was from Fluka, Buchs, Switzerland. Plasma samples stemmed from patients undergoing prolonged or having completed long-term (up to several days) Pentothal infusion and were collected over a 5-year period in our departmental drug assay laboratory where they were received for drug monitoring. The samples were stored at −20°C until analysis by CZE. Our own plasma or a bovine serum albumin (BSA) solution (6%, w/v, BSA in Krebs–Ringer buffer) were used as blank matrices. Two control samples containing racemic mixtures of the two drugs were prepared via addition of aliquots of stock solutions to the BSA solution (thiopental/pentobarbital enantiomer concentrations of 3.5/2 and 12.5/5 μg/ml, respectively). Five calibrator samples (thiopental enantiomers: 0.50–25 μg/ml; pentobarbital enantiomers: 0.25–12.5 μg/ml) were prepared the same way.

2.2. Sample pretreatment

For extraction of thiopental and pentobarbital, 300 μl control plasma or patient plasma, 5 μl of IST solution (400 μg/ml in acetonitrile), 150 μl of 1 *M* HCl and 1 ml dichloromethane were combined and vortexed for 1 min. Then, the sample was centrifuged at 9000 *g* for 1 min. The upper phase was discarded and the dichloromethane phase was evaporated to dryness under a gentle stream of nitrogen at room temperature and the residue was redissolved in 50 μl acetonitrile or 10-fold diluted running buffer without OHP-γ-CD (see below). Attempts to prepare the plasma via acetonitrile precipitation of the proteins [9] were discontinued, as injection of the protein-free supernatant did not lead to reproducible results.

2.3. Electrophoretic instrumentation and running conditions

Initial work was performed on an instrumental setup comprising a Prince autosampler and power supply (Lauerlabs, Emmen, Netherlands), an uncoated 58.6 cm (39.6 cm to the point of detection)× 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) and a fast forward scanning UVIS 206 detector (Linear Instruments, Reno, NV, USA) were used. The capillary was kept at a temperature of 27–30°C. The sample injection time and applied pressure were 0.1 min and 20 mbar, respectively. Various buffers (see Section 3.1) were used and the applied voltage was between 11 and 22 kV (currents: 80 to 100 μA). For all other measurements a BioFocus 3000 capillary electrophoresis system (Bio-Rad Labs., Hercules, CA, USA) was employed. It was equipped with an untreated 60 cm (55.4 cm to the detector)×50 μm I.D. fused-silica capillary (Polymicro Technologies) which was mounted in a user assembled cartridge (Bio-Rad). Injection of sample was effected by applying positive pressure (5–7 psi·s). A 75 m*M* NaH_2PO_4 buffer (titrated to pH 8.5 with 1 *M* NaOH) containing 5 m*M* OHP-γ-CD was used. The chiral selector was added freshly every day. A constant voltage of 15 kV (current about 45 μA) was applied, the temperature of the cartridge was maintained at 20°C and the carousel temperature was at 25 or 30°C. Solute detection was effected either by scanning from 195 to 360 nm (5 nm interval) or by simultaneous monitoring at three wavelengths (200/245/300 nm). Before every experiment the capillary was rinsed with 0.1 *M* NaOH, water and running buffer (2 min each). BioFocus Integration software (version 5.2, Bio-Rad) was employed for data conversion and evaluation.

2.4. Data analysis

Quantitation was based upon multilevel, internal calibration using relative peak areas (i.e. peak areas divided by detection times). Statistical data analysis was performed with SigmaStat Statistical Analysis System version 1.01 (Jandel Scientific, Corte Madera, CA, USA). Differences between groups were evaluated using the *t*-test or the Mann–Whitney rank sum test. $P<0.05$ was considered statistically significant.

2.5. Instrumentation and running conditions for recycling isotachophoresis

rITP was performed on a MinipHor (Protein Technologies, distributed through Rainin, Woburn, MA, USA) as described previously [27]. It was operated under a constant voltage of 800 V (initial current: about 80 mA, end current: about 25 mA). The cathode was at the terminator side (side of recycling channel 1), electrolyte chambers were separated from the separation cell by dialysis membranes and the temperature was monitored to be about 12°C (cooling bath 5°C). The advancing barbiturate boundary was detected in channel 17 by a 2138 Uvicord S (Pharmacia-LKB, Uppsala, Sweden) with a 206 and 280 nm filter for pentobarbital and thiopental, respectively. The counterflow inlet and outlet were placed into channels 20 and 1, respectively. The counterflow was generated using a low pulse peristaltic pump (Minipuls 3, Gilson Medical Electronics, Middleton, WI, USA). It was regulated manually with registration of the output signal from the boundary detector on a strip chart recorder. For rITP operation, the separation cell was first filled with the 10 m*M* leading electrolyte containing OHP-γ-CD (10 m*M* formic acid/ammediol (pH 8.7) with 5 and 30 m*M* OHP-γ-CD for thiopental and pentobarbital, respectively) and the multichannel peristaltic pump was started. The sample (10 mg thiopental or pentobarbital in 4 ml of 10 m*M* leader) was then slowly injected in channel 2 followed by application of power. At the occurrence of the absorbance change in the monitoring loop (channel 17), the counterflow was started so as to maintain a constant absorbance level thereby immobilizing the isotachophoretic zone. In the leading electrolyte chamber a 100 m*M* formic acid/ammediol buffer (pH 8.7) was employed. The terminating electrolyte was 100 m*M* β-alanine/ammediol at pH 9.5.

2.6. Analysis of rITP fractions

For pH measurement a pH meter, model 720, and a ROSS pH electrode, model 8103 (both from Orion Research, Cambridge, MA, USA) were used. The conductivity was measured with a conductivity meter model 101 (Orion Research) equipped with a model PW 9510/65 cell (Philips, Eindhoven, Netherlands). The absorbance (determined in 10- or 100-fold diluted fractions) was measured at 206 nm (pentobarbital) or 280 nm (thiopental) using a UV–Vis spectrophotometer Lambda 15 (Perkin-Elmer, Ueberlingen, Germany). The CZE assays used for analysis of the enantiomeric composition of the fractions were (i) that employed for the assessment of the stereoselective metabolism of the two compounds (see above) and (ii) that described by Srinivasan and Bartlett [24] which comprises a 50 m*M* phosphate buffer (pH 9.0) containing 40 m*M* OHP-γ-CD. rITP fractions were analyzed on the BioFocus 3000 (Bio-Rad) under the conditions described in Section 2.3, having detection wavelengths of 280 and 245 nm for thiopental and pentobarbital, respectively. Most of the fractions were diluted (up to 10 times) prior to analysis. Data were expressed in relative peak areas, i.e. the peak area divided by its detection time. rITP fractions were also analyzed by circular dichroism spectroscopy. Circular dichroism spectra (ellipticity in millidegree (mdeg) vs. wavelength) were recorded on a Model J-710 spectropolarimeter (Jasco, Tokyo, Japan) and at room temperature using a cell of 1 mm optical path length. The wavelength was scanned at 20 nm/min between 350–200 nm, the band width was 1 nm and the resolution 0.5 nm.

3. Results and discussion

3.1. Separation of pentobarbital and thiopental enantiomers

In analogy to the achiral work of Shihabi [9], separation of thiopental and pentobarbital was in-

vestigated in alkaline buffers around pH 8.5. Under these conditions, barbiturates are negatively charged and migrate against the electroosmotic flow. The latter displacement, however, is stronger such that the net transport is towards the cathode. Pentobarbital (pK_a 8.0 [1]) has a higher pK_a value than thiopental (pK_a 7.6 [1]). Thus, pentobarbital reaches the detector before thiopental (Fig. 2A). First, the use of 300 m*M* boric acid buffers at pH 8.5, 8.75 or 9.0 was investigated. With a OHP-γ-CD concentration of

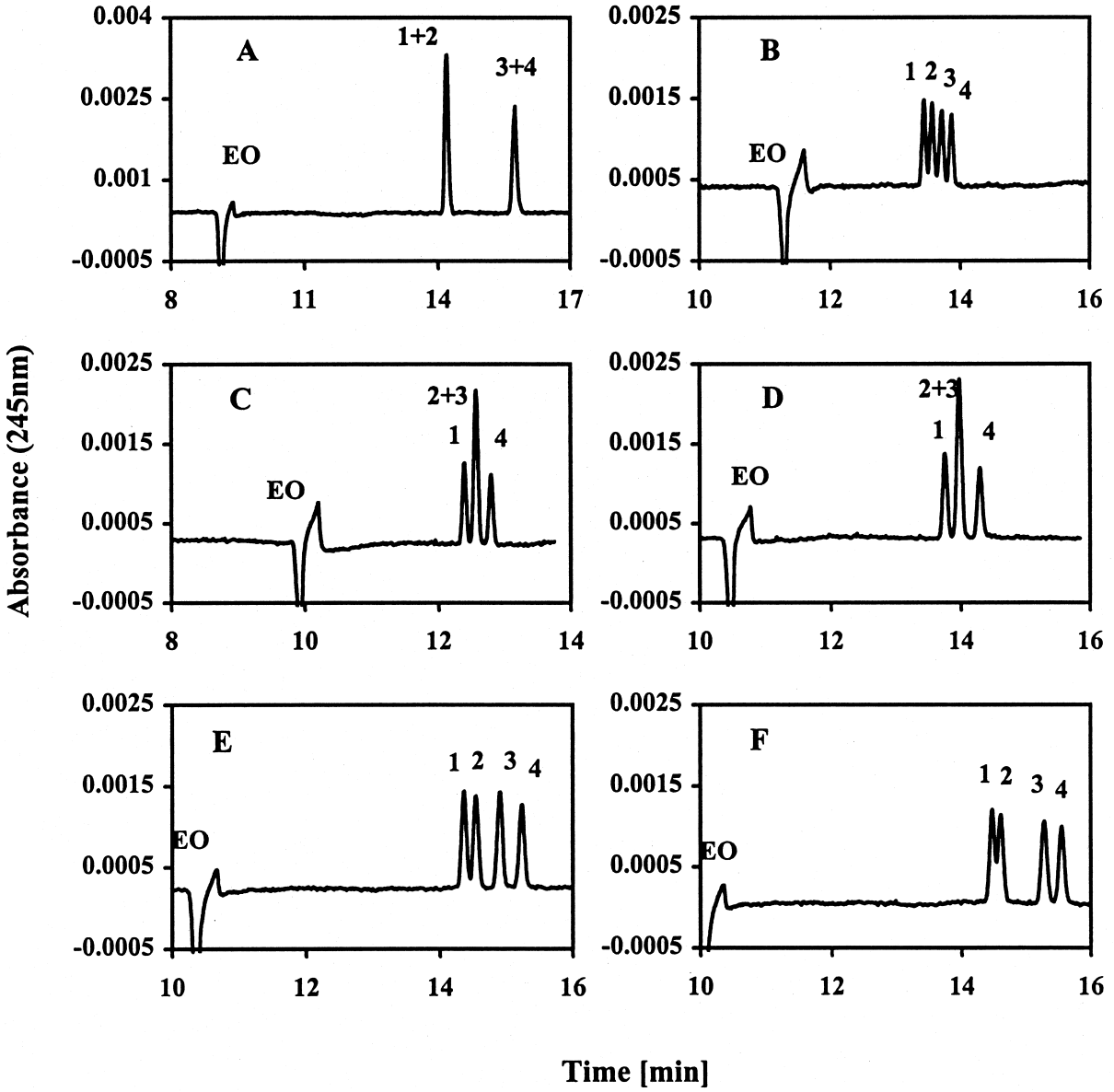

Fig. 2. CZE electropherograms obtained with the Prince having a sample composed of thiopental and pentobarbital (20 μg/ml each) dissolved in water (20%), methanol (20%) and acetonitrile (60%) and a 75 m*M* phosphate buffer (pH 8.5) with (A) 0, (B) 40, (C) 20, (D) 10, (E) 5, and (F) 2.5 m*M* OHP-γ-CD. Voltages applied were (A–C) 12 kV and (D–F) 11 kV (currents: 86–99 μA). Key: 1,2: pentobarbital, 3,4: thiopental, EO: electroosmotic flow.

40 m*M*, incomplete separation of the enantiomers was observed (data not shown). Improved results were obtained with a 50 m*M* phosphate buffer containing 40 m*M* OHP-γ-CD. First the pH was altered. At pH 8.0, thiopental and pentobarbital were well separated, but no enantiomeric separation was attained. With a pH of 8.5, incomplete separation of the enantiomers was observed. Finally at pH 9.0, the enantiomeric separation became complete, but thiopental and pentobarbital comigrated. Next the ionic strength of the buffer was altered. With 75 m*M* phosphate all four enantiomers could be separated (Fig. 2B). Further increase of the phosphate concentration to 100 m*M* did not improve separation of the four enantiomers. Next, the impact of the OHP-γ-CD concentration was investigated. Examples of these electropherograms are presented in Fig. 2. Not surprisingly, without OHP-γ-CD no chiral discrimination was observed (Fig. 2A). Best separation of all four substances was obtained with 5 m*M* OHP-γ-CD. All further experiments were thus made with the 75 m*M* phosphate buffer (pH 8.5) containing 5 m*M* OHP-γ-CD.

3.2. rITP of barbiturates and enantiomer assignment via analysis of rITP fractions

First, anionic capillary isotachophoresis (cITP) was performed with a leader composed of 10 m*M* formic acid/ammediol at pH 8.7 and a terminator comprising 10 m*M* β-alanine/ammediol at pH 9.5. Using both the BioFocus 3000 and the Tachophor [27], thiopental and pentobarbital were found to form isotachophoretic zones. Furthermore, cITP experiments with 5–40 m*M* OHP-γ-CD indicated partial separation of thiopental and pentobarbital enantiomers (data not shown). Then, anionic rITP with 10 mg thiopental in absence of a chiral selector was found to form an isotachophoretic zone between channels 10 to 17 (data not shown). Having 5 m*M* OHP-γ-CD in the leader, the data presented in Fig. 3A were obtained. UV absorbance, pH, and conductivity distributions are shown in the top panel, whereas the enantiomeric composition assessed by CZE (expressed in relative peak area values) is depicted in the bottom panel. In that rITP run the total run time was 245 min (counterflow was commenced after 11 min) and the consumption of counterflow was 156 ml. Similar data were obtained with a total run time of 72 min (24 ml of counterflow). Chiral CZE analysis of the fractions revealed that there is an enrichment of the first detected thiopental enantiomer at the rear end of the rITP zone (fractions 11–15) but hardly no enrichment of the second detected thiopental enantiomer at the rITP front. An electropherogram of fraction 14 is depicted in Fig. 4A. In that sample, the enrichment of the first detected enantiomer was determined to be 60.1% (enantiomeric excess, ee, of 20.2%, for definition of ee refer to [27]). The rITP data presented in panel B of Fig. 3 are those obtained with 10 mg pentobarbital and a total run time of 110 min (98 min under counterflow conditions; 60 ml counterflow was applied). In that case, clear enrichment of both enantiomers (first and second detected enantiomer at rear isotachophoretic boundary and at the isotachophoretic front, respectively) was observed. Fractions 8, 12 and 16 are characterized by ee-values of 38.5, 16.5 and 20.2%, respectively. The electropherogram of fraction 12 is presented in Fig. 4B.

Having a pentobarbital rITP fraction with enantiomer enrichment, assignment of the enantiomers was simply possible by analyzing the sample in the buffer of Srinivasan and Bartlett [24], a configuration in which the detection order of pentobarbital enantiomers is known (Fig. 4C). In that system, *R*-(+)-pentobarbital is detected before *S*-(−)-pentobarbital. Comparison of Figs. 4B and C reveals that there is equal detection sequence in the two buffers. Thus, peaks 1 and 2 can be unambiguously assigned to *R*-(+)-pentobarbital and *S*-(−)-pentobarbital, respectively. *S*-(−)-pentobarbital has the higher electrophoretic mobility (therefore enrichment at the isotachophoretic front, Fig. 3B) than *R*-(+)-pentobarbital (enrichment at rear side, Fig. 3B). Furthermore, analysis of fractions 10–12 by circular dichroism spectroscopy revealed a small negative response. The same was found to be true for thiopental fractions 12–14 (Fig. 4A), this suggesting that the detection sequence of the thiopental enantiomers be the same. Peaks 3 and 4 can thus be assigned to *R*-(+)-thiopental and *S*-(−)-thiopental, respectively. As the two substances only differ in one atom which is not near the chiral center (Fig. 1), comparable detection order of the enantiomers of the two compounds is not unexpected.

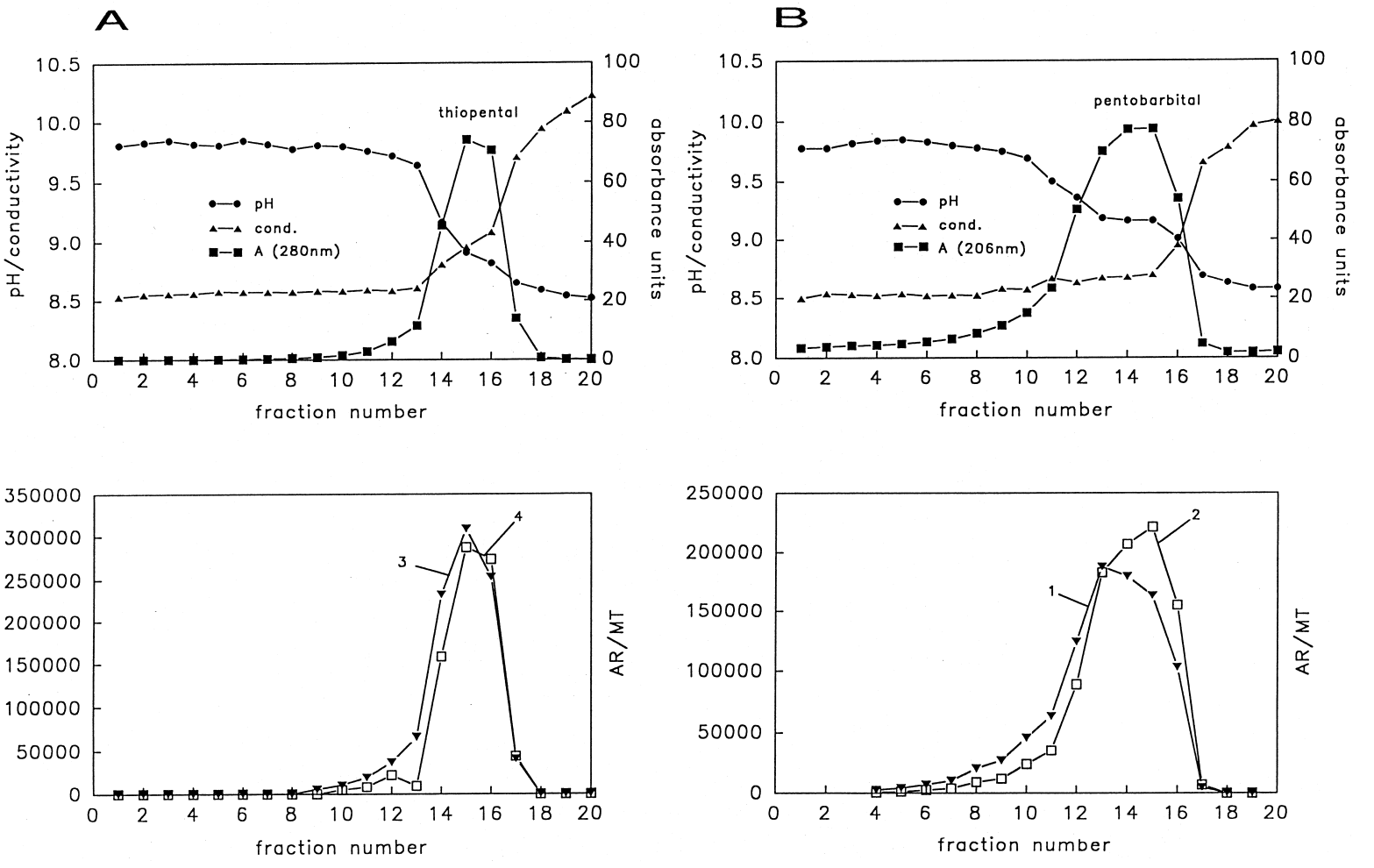

Fig. 3. Chiral rITP data obtained with the MinipHor apparatus for (A) thiopental and (B) pentobarbital. The upper panels show UV absorbance ((A) 280 nm, (B) 206 nm), pH and conductivity data of the collected fractions. Conductivity data are in mS/cm and are altered by $1.6x+8.3$ (x=measured conductivity) for presentation purposes. The lower panels show the enantiomeric composition of the fractions as assessed by chiral CZE (for electropherograms see Fig. 4). Key as for Fig. 2.

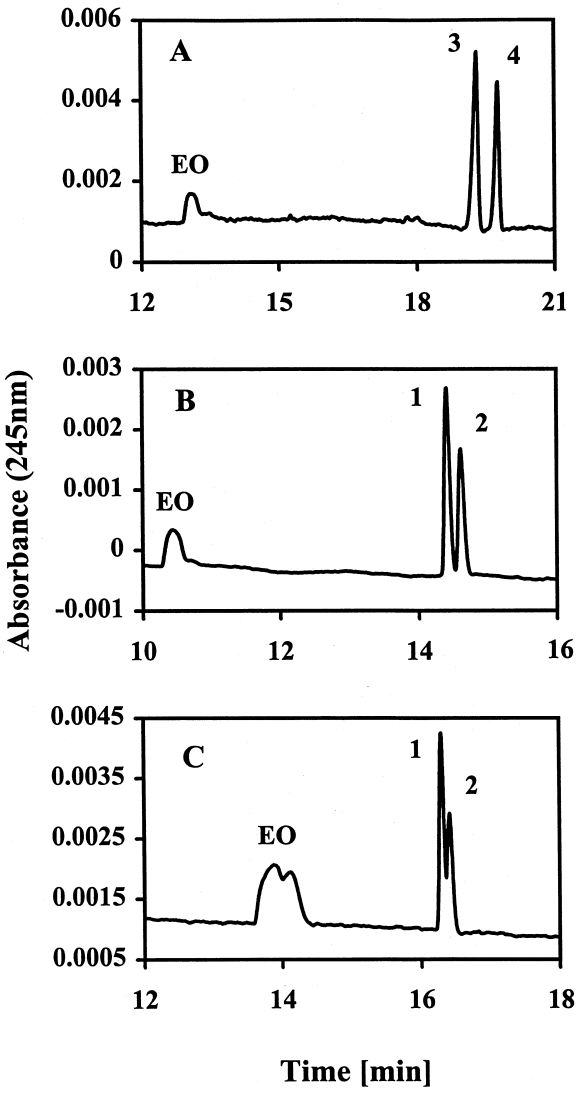

Fig. 4. Chiral CZE data of 10-fold diluted rITP fractions analyzed with the BioFocus 3000 (7 psi·s injection; 1 p.s.i.=6894.76 Pa) using (A,B) the 75 m*M* phosphate buffer at pH 8.5 with 5 m*M* OHP-γ-CD and a current of 44 μA, and (C) the 50 m*M* phosphate buffer at pH 9.0 with 40 m*M* OHP-γ-CD (current: 18 μA). Samples: (A) fraction 14 of Fig. 3A, (B,C), fraction 12 of Fig. 3B. Key as for Fig. 2.

3.3. Determination of pentobarbital and thiopental enantiomers in plasma

Thiopental and pentobarbital enantiomers were extracted with dichloromethane as described in Section 2.2. Electropherograms obtained with a blank, a control sample and a patient sample are depicted in Fig. 5. Furthermore, three-dimensional data obtained with spiked human plasma and a patient plasma are presented in Figs. 6A and B, respectively. These data

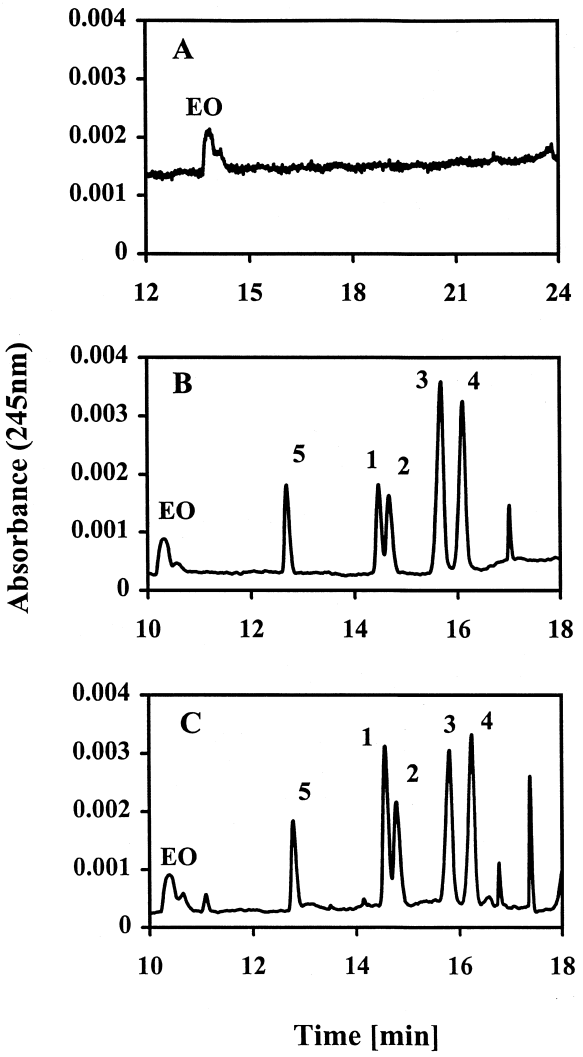

Fig. 5. Chiral CZE data of extracts prepared in 10-fold diluted running buffer (no OHP-γ-CD) obtained of (A) a blank plasma, (B) a control sample containing 6.67 μg/ml I.S., 25 μg/ml racemic thiopental and 10 μg/ml racemic pentobarbital, and (C) a patient sample containing 10.3/11.6 μg/ml *R*-(+)-thiopental/*S*-(−)-thiopental and 8.1/6.0 μg/ml *R*-(+)-pentobarbital/*S*-(−)-pentobarbital. The samples were analyzed on the BioFocus 3000 (7 p.s.i.·s injection). Key: 1: *R*-(+)-pentobarbital, 2: *S*-(−)-pentobarbital, 3: *R*-(+)-thiopental, 4: *S*-(−)-thiopental, 5: internal standard.

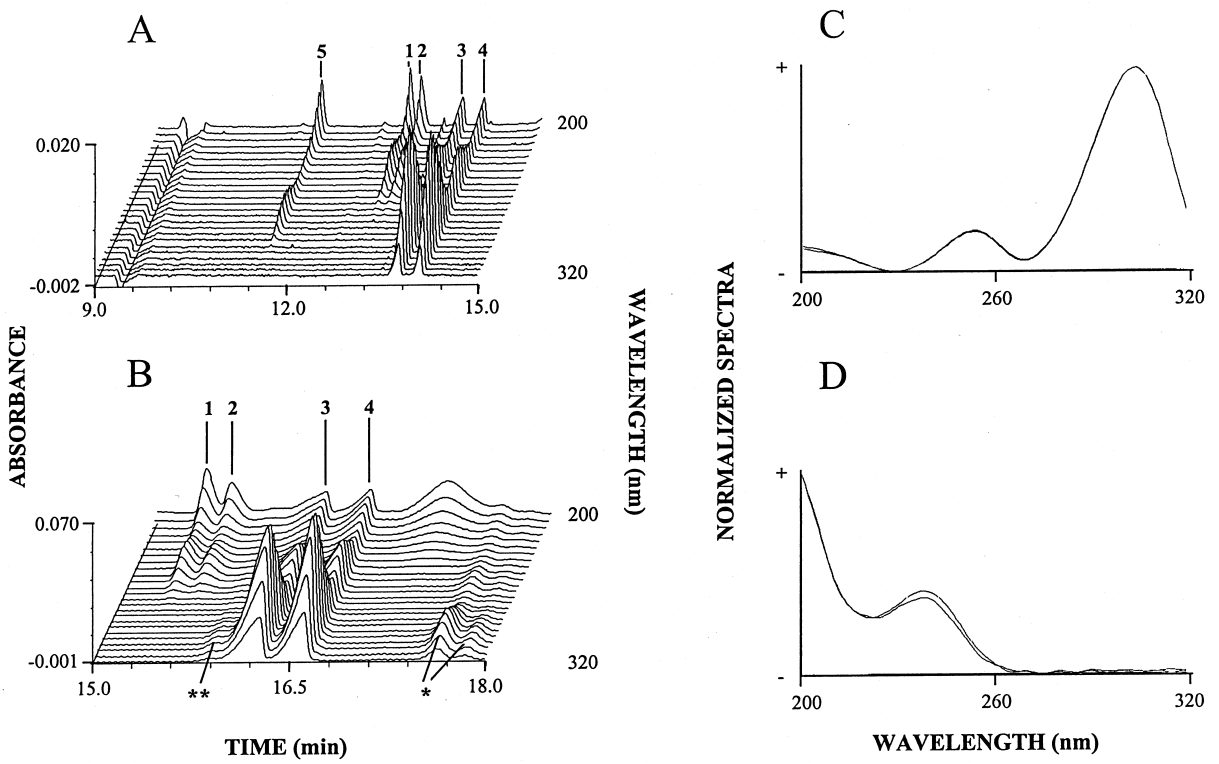

Fig. 6. Three-dimensional (absorbance vs. time vs. wavelength) electropherograms of extracts obtained for (A) human plasma blank fortified with 6.67 μg/ml I.S., 40 μg/ml racemic thiopental, 18 μg/ml racemic pentobarbital and (B) the same patient sample as for Fig. 5C. Extracts were prepared in (A) acetonitrile–water (1:1) and (B) 10-fold diluted running buffer (no OHP-γ-CD). Experiments were performed on (A) the BioFocus (cf. Section 2.3) and (B) a manual instrument featuring a 98.2 cm (58.6 cm to the detector)×75 μm I.D. capillary, vacuum sample injection (2 s) and an applied voltage of 16 kV (current: 85 μA). Panels C and D depict normalized spectra (no background subtraction) of thiopental and pentobarbital enantiomers, respectively, that were extracted from the data presented in panel B.

reveal interesting aspects that merit some discussion. The data presented in panel A of Fig. 5 were registered in the scanning mode which provides increased noise compared to data gathering in the triple-wavelength mode (panels B and C). The three-dimensional electropherograms nicely depict the appearance of thiopental and pentobarbital enantiomers and illustrate spectral differences between the two barbiturates (panels C and D of Fig. 6). Extracts of patient specimens revealed the presence of two additional peaks (marked with an asterisk in Fig. 6B) which have similar spectra as thiopental but were detected more than 1 min after thiopental. If the two peaks should correspond to the enantiomers of a thiopental metabolite, significant stereoselectivity is present. Furthermore, the peak marked with two asterisks (Fig. 6B) is also characterized by a thiopental-like spectrum. No such peaks were monitored in the calibrators and controls, samples that were prepared with human plasma blank or BSA solution and were fortified with Pentothal, the pharmaceutical preparation employed for thiopental administration to patients. Thus, our assay appears to pick up one or several additional thiopental metabolite(s). The peaks marked with an asterisk are observed to be more mobile than thiopental and might thus have a higher negative charge (lower pK_a value). It could represent the carboxy-propyl metabolite (carboxylic acid analogue of thiopental) described as thiopental metabolite in the literature [28,29]. The CZE behavior of another known metabolite, hydroxy-butyl thiopental [28], is more difficult to predict. The isomer known to be present in Pentothal [7,30], on the other hand, can be excluded as it would appear in the controls as

well. Due to lack of standards, assignment of the unknown peaks could not be confirmed.

Data evaluation for thiopental and pentobarbital can be performed at 245 nm (Fig. 6). Data at lower wavelengths are unsuitable as the absorbance of thiopental is too low and interferences might falsify the result. Higher wavelengths, particularly around 300 nm, provide highest sensitivity for thiopental, but no response for pentobarbital. Thus, data were typically registered at three wavelengths (200, 245 and 300 nm) and data evaluation for thiopental, pentobarbital and the I.S. were executed with electropherograms at 300, 245 and 245 nm, respectively. Next, using the calibrators, data evaluation based upon peak heights and peak areas was compared. In electropherograms registered with extracts containing racemic thiopental and pentobarbital were typically characterized by R/S peak height ratios >1 (e.g. 1.11 and 1.14, respectively, Fig. 5B). At other pentobarbital concentrations, R/S peak height ratios >1 were monitored as well. For thiopental enantiomer concentrations >30 μg/ml, however, peak height ratios <1 were noted (data not shown). Such a concentration dependent change cannot be tolerated for assessment of the stereoselective metabolism of thiopental. Fortunately, R/S ratios based on relative peak areas were found not to significantly change in a concentration dependent manner. R/S values for thiopental and pentobarbital were found to be around 1.08 (1.06 to 1.11) and around 1.00 (1.00 to 1.04), respectively. Thus, data evaluation could only be performed on the basis of relative peak areas.

Finally, reconstitution in acetonitrile and 10-fold diluted running buffer provided comparable data. However, due to evaporation of acetonitrile, quantitative work was performed with extracts prepared in 10-fold diluted running buffer (without chiral selector) only. Using relative peak area ratios, all calibration graphs were determined to be linear with F values >180 ($P<0.001$) and >860 ($P<0.001$) for thiopental and pentobarbital enantiomers, respectively. The y-intercepts were observed to be smaller than the smallest calibrator values and were thus negligible. Imprecisions for thiopental enantiomers (12.5 μg/ml) and pentobarbital enantiomers (5 μg/ml) were determined to be <12 and <4%, respectively ($n=3$, independent samples that were not analyzed consecutively but at the same day).

3.4. Assessment of the stereoselective metabolism of thiopental and pentobarbital

For the assessment of the stereoselective metabolism of thiopental and pentobarbital, data of 12 control (six of each level) and 12 patient samples were evaluated. Box plots of R/S plasma ratios are presented in Fig. 7. Relative peak areas, i.e. peak areas divided by detection time, and the responses measured for 300 nm (thiopental) and 245 nm (pentobarbital) served as the basis for data evaluation. Total thiopental and pentobarbital concentrations within the patient samples were determined to be in the range of 0–38 and 2–14 μg/ml, respectively. As patient samples are often collected after completion of the thiopental infusion and as thiopental is metabolized to pentobarbital, pentobarbital was found in all samples whereas thiopental could not be detected in five of the 12 patient samples. Nevertheless, statistical analysis using the Mann–Whitney rank sum test revealed a statistically significant difference between the two thiopental groups of data ($P=0.013$) (Fig. 7A). Mean (median) R/S plasma values for the racemic controls and patient samples were found to be 1.131 (1.100) and 0.861 (0.790), respectively. Thus, the total S-(−)-thiopental plasma concentration in these patient samples is on average 23.9% larger than the total R-(+)-thiopental plasma level. This is in agreement with the findings measured by HPLC in other laboratories [2,3,6] and thereby also supports the thiopental enantiomer assignment discussed above. Comparable stereoselectivity was also noted for the thiopental metabolism in sheep [31].

The fact that the R/S plasma ratios in racemic controls was determined to be >1 [which is in contrast to the ratio observed for pentobarbital (Fig. 7B)] calls for some further comments. The normalized spectra of the two thiopental peaks were found not to differ at all (Fig. 6C). Evaluation of samples containing >5 μg/ml thiopental enantiomers revealed mean values of 1.065 and 0.770 for the R/S ratio of six controls and four patient specimens, respectively (decrease of 27.7%). Thus, inaccuracies associated with CZE monitoring of concentrations close to the detection limit could at best only partly be the reason for the R/S ratio deviation from unity. Furthermore, Pentothal is known to contain an

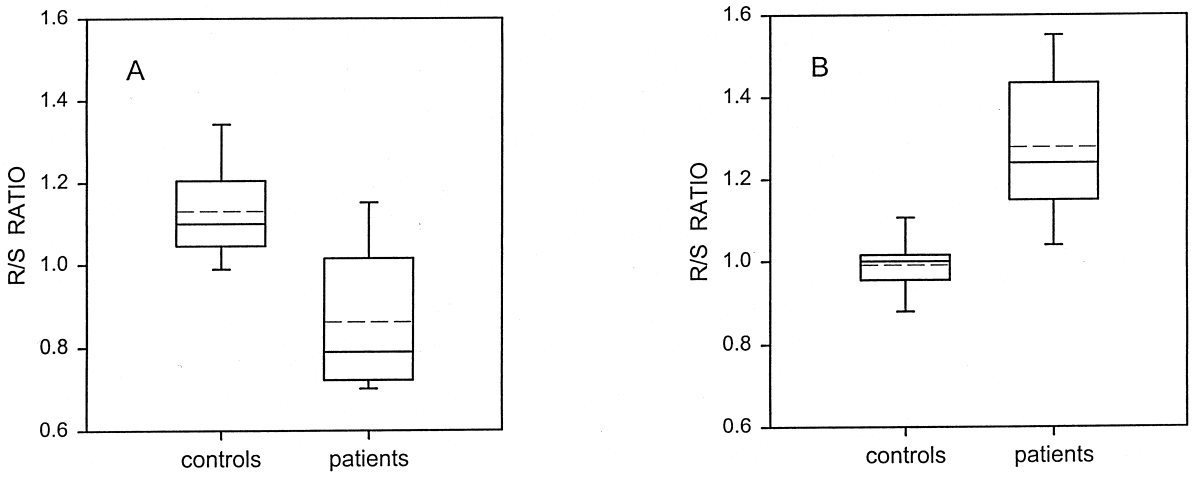

Fig. 7. Box plots of (A) R/S plasma ratios of thiopental enantiomers and (B) R/S plasma ratios of pentobarbital enantiomers for control samples (left) and patient samples (right). The boxes are drawn for the 25th and 75th percentiles with median (solid line) and mean (broken line). The capped bars mark the 10th and 90th percentiles. With the exception of seven patient plasma samples containing thiopental, data evaluation was based upon 12 samples.

achiral isomer of thiopental with an occurrence of 6–10% [30] that might comigrate with *R*-(+)-thiopental. This assumption is not unreasonable, as no additional peak with a thiopental-like spectrum is monitored in the electropherograms of the controls (see Fig. 6A). Analysis of plain barbiturate standards that were not extracted revealed mean ($n=4$) R/S values of 1.20 and 1.01 for thiopental and pentobarbital, respectively. This strongly suggests that the isomer is indeed comigrating with *R*-(+)-thiopental and that the isomer content in the employed Pentothal is about 9%. Using a different racemic thiopental standard that was obtained from Alltech (State College, PA, USA) provided similar results. Unfortunately, due to the unavailability of a pure standard, the CZE behavior of the isomer could not be investigated on its own. The isomer and thiopental are reported to have similar pharmacokinetic properties [30]. Thus, its comigration with *R*-(+)-thiopental is not expected to hinder our assessement of the stereoselectivity of the thiopental metabolism. Furthermore, using an achiral micellar buffer, the isomer and thiopental could be nicely separated [7,8]. In a chiral environment, however, complete separation of the isomer, the thiopental enantiomers and possible thiopental metabolites was found to be difficult [25,26].

For pentobarbital, statistical analysis revealed a significant difference between the two groups ($P<0.001$). Mean (median) R/S ratios of 12 racemic controls and 12 patient samples were determined to be 0.991 (1.00) and 1.278 (1.24), respectively. The total *R*-(+)-pentobarbital plasma concentration in these patient samples is on average 29.0% higher than the total *S*-(−)-pentobarbital plasma level (Fig. 7B). Evaluation of samples containing >2.5 μg/ml per enantiomer revealed similar data. Mean R/S plasma values for six controls and nine patient samples were determined to be 1.000 and 1.221, respectively (increase of 22.1%). This result differs from that reported by Nguyen et al.. Having a thiopental infusion of 2 to 3 h only, no significant difference between the two pentobarbital enantiomers was found [2]. Our samples stemmed from patients that were typically subjected to thiopental infusions that lasted one or several days. Furthermore, after oral administration of 100 mg racemic pentobarbital, Cook et al. observed somewhat higher concentrations of *S*-(−)-pentobarbital [4].

4. Conclusions

CZE with OHB-γ-CD as chiral selector is shown to provide a simple, attractive and inexpensive method for the enantiomeric separation of thiopental

and its oxybarbiturate metabolite pentobarbital. No expensive stereospecific separation column and no sample derivatization are required to separate the enantiomers. Furthermore, chiral rITP processing of racemic barbiturates is shown to provide enantiomerically enriched samples that can be used for identification of enantiomers in absence of pure chiral standards. rITP fractions were analyzed by CZE and circular dichroism spectroscopy. Comparison of the electropherograms obtained for pentobarbital with those reported in Ref. [24] revealed proper assignment for that compound. Then, the absolute configuration of the thiopental enantiomers could be determined via comparison of the circular dichroism spectra obtained with enantiomerically enriched rITP fractions. With a capillary of 55 cm effective length and run times of less than 20 min, it was possible to show that the metabolism of thiopental and its metabolite pentobarbital are modestly enantioselective. For the 12 plasma specimens analyzed, significantly higher total plasma concentrations of *S*-(−)-thiopental and *R*-(+)-pentobarbital compared to *R*-(+)-thiopental and *S*-(−)-pentobarbital, respectively, were noted. This stereoselectivity has to be regarded in view of the patient plasma samples analyzed, specimens that were collected during or after completion of long-term (up to several days) Pentothal infusion and were collected over a 5-year period. Human plasma that is stored at −20°C for a prolonged time period was observed to increase the amount of thiopental desulfurated to pentobarbital, an instability that has recently been described under different conditions by Russo et al. [32]. Stereoselectivity of this process, however, is not expected and should thus not have an impact on *R*/*S* ratios of thiopental and pentobarbital.

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