ELSEVIER

Journal of Chromatography B, 665 (1995) 133–137

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Enantioselective determination of thiamylal in human serum by high-performance liquid chromatography

Masanori Sueyasu[a], Taeko Ikeda[a], Kenji Otsubo[a,*], Takuro Taniyama[b], Toshinobu Aoyama[a,1], Ryozo Oishi[a]

[a]*Department of Hospital Pharmacy, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan*
[b]*Intensive Care Unit, Faculty of Medicine, Kyushu University 3-1-1, Maidashi, Higashi-ku, Fukuoka 812, Japan*

First received 16 September 1994; revised manuscript received 11 November 1994; accepted 11 November 1994

Abstract

Thiamylal, a widely used anesthetic drug, has two enantiomers. We developed a novel and simple method for measuring thiamylal enantiomers in human serum using reversed-phase high-performance liquid chromatography. *R*(+)- and *S*(−)-Thiamylal were separated using a chiral mobile phase containing β-cyclodextrin, and detected at the range of 50 ng/ml–25 μg/ml in serum. The relative standard deviations of *R*(+)- and *S*(−)-thiamylal were 3.4–8.7% and 2.8–8.7% for the intra-day assay, and 2.8–12.0% and 2.8–13.0% for the inter-day assay. This method may be applied to enantioselective pharmacokinetic studies of thiamylal.

1. Introduction

Thiamylal (TH), 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid, is an ultrashort-acting anesthetic agent (Fig. 1). TH has a chiral carbon at the 1′-position and the racemate has been widely used as a parenteral product. Pharmacokinetic differences between both enantiomers have been found in some chiral barbiturates [1–4]. However, there have been few studies on TH, because no means had been developed with which to discriminate the enantiomers in body fluids.

An enantioselective radioimmunoassay for the determination of pentobarbital enantiomers in human plasma has been described [1]. TH enantiomers may be determined with this assay, but complex procedures for antibody production and the use of radioactive compounds are necessary. An HPLC method using a chiral mobile phase containing heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin has been developed to determine barbiturate enantiomers with a chiral center in the aliphatic side chain [5]. However, this method is too time-consuming and expensive to apply to biological samples. On the other hand, for some barbiturates with a chiral center in the pyrimidine ring, HPLC using a reversed-phase

* Corresponding author.
[1] Present address: Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-chou, Minami-ku, Fukuoka 815, Japan.

0378-4347/95/$09.50
SSDI 0378-4347(94)00527-3

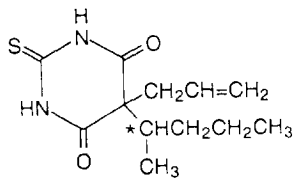

Fig. 1. Structure of the thiamylal enantiomers. The asterisk represents the chiral carbon atom.

column and a mobile phase containing β-cyclodextrin (β-CyD) has been described [6,7]. Here, we describe an improved enantioselective HPLC method for the enantiomers of TH.

2. Experimental

2.1. Reagents and solutions

The sodium salt of TH was supplied by Yoshitomi Pharmaceutical Industries (Osaka, Japan). β-Cyclodextrin (β-CyD) was purchased from Tokyo-Kasei Kogyo (Tokyo, Japan). Acetonitrile, ethanol and methanol (Cica-Merck, Tokyo) were of HPLC grade and all other chemicals were of reagent grade. Lyophilized drug-free serum was purchased from Bio-Rad Lbs. (Anaheim, CA, USA) for the preparation of the standard sera. Bond-Elut C_{18} extraction cartridges (50 mg/1 ml) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). Distilled water was passed through a Milli-Q water purification system (Millipore Japan, Tokyo).

The sodium salt of TH was dissolved in water to prepare the stock solution (1 mg/ml). Standard solutions (2.5–25 μg/ml) were prepared by diluting the stock solution with water. An internal standard (I.S.) was prepared by dissolving *n*-propyl-*p*-hydroxybenzoate in a small amount of methanol and diluting with water to a concentration of 25 μg/ml. The TH stock and I.S. solutions were stable for at least a month when stored at −20°C.

2.2. Apparatus and HPLC conditions

The HPLC system consisted of a pump (Model 510, Waters Division of Millipore Japan, Tokyo), a variable-wavelength UV detector set at 288 nm (Model 490, Waters Division), an integrator (C-R1B, Shimadzu, Kyoto, Japan) and a Reodyne 7125 sample injector with a 50-μl sample loop (Cotati, CA, USA). A reversed-phase column contained TSK-gel ODS 80TM (75 × 4.6 mm I.D., 5 μm particle size; Tosoh, Tokyo). The mobile phase was prepared as follows: β-CyD (17 mmoles) was suspended in 150 ml of ethanol and 10 m*M* KH_2PO_4 was added gradually to a final volume of 1 l. The solution was filtered through a 0.45-μm membrane and ultrasonically degassed. The flow-rate was 0.9 ml/min and the column temperature was 25°C.

2.3. Serum sample preparation

Blood samples obtained from patients undergoing TH therapy as part of the current treatment protocol utilized in the Operating Room and Intensive Care Unit of the Kyushu University Hospital (Fukuoka, Japan) were centrifuged at 1500 *g* for 10 min. The sera were stored at −20°C until use. TH in the serum samples was stable for at least two weeks under these conditions.

Standard sera (0.25–2.5 μg/ml for each enantiomer) were prepared by mixing the TH standard (2.5–25 μg/ml) and the concentrated drug-free serum (1:4, v/v), which was obtained by dissolving the lyophilized product in 4/5 aliquot of water (8 ml).

2.4. Extraction procedure

Serum was solid-phase extracted as follows. The Bond-Elut C_{18} cartridge was washed with 2 × 1 ml of methanol and 2 × 1 ml of 10 m*M* KH_2PO_4 before use. Serum (100 μl) was spiked with the I.S. solution (50 μl), diluted with 500 μl of 10 m*M* KH_2PO_4, and the mixture was passed through the cartridge using a Vac-Elut SPS 24 system (Analytichem International, Harbor City, CA, USA). The cartridge was washed with 2 × 1 ml of water. TH and I.S. were eluted with 300 μl of methanol and the eluate was evaporated. The residue was dissolved in 100 μl of 50% (v/v) ethanol aq. solution, and 50-μl aliquots were injected onto the HPLC system.

2.5. Circular dichroism spectra of enantiomers

The eluate fractions corresponding to the $R(+)$- and $S(-)$-enantiomers separated by the HPLC system were collected. The enantiomers were extracted with diethyl ether under acid conditions. The ether layer was evaporated to dryness using a Speed Vac concentrator (Savant Instruments, Hicksville, NY, USA). The residue was dissolved in methanol, and the circular dichroism (CD) spectrum was measured by a Model J-600 spectropolarimeter (Japan Spectroscopic Co., Tokyo).

3. Results and discussion

Hibi et al. [8] have described a reversed-phase HPLC method for measuring TH racemates in serum. They used acetonitrile–phosphatebuffer (10 m*M*, pH 3.0) (1:1, v/v) as the mobile-phase. Since β-CyD forms an inclusion complex with each enantiomer [9–11], TH can be enantioselectively determined by reversed-phase HPLC using a mobile phase containing β-CyD. We applied such a HPLC system to determine the TH enantiomers. First, we used acetonitrile and phosphate buffer containing β-CyD as the mobile phase. However, β-CyD was insoluble in acetonitrile resulting in turbidity of the mixture. We examined ethanol and methanol as organic solvents in the mobile phase, and finally selected ethanol because of the higher solubility of β-CyD and the shorter retention times of TH and the internal standard compared with methanol.

The separation of the TH enantiomers was not influenced by a change in pH over the range 3.0–4.6. The hydrophobic cavity of β-CyD can selectively include the non-ionic species. The formation of a TH inclusion complex may not be influenced under acidic conditions, since the pK_a of TH is 7.5 and TH is almost non-ionic. The pH of the 10 m*M* KH_2PO_4 solution was found to be 4.6, which was acceptable for the method and simple to prepare. The amount of ethanol and the optimal concentration of β-CyD in the mobile phase were determined for sufficient resolution of the TH enantiomers, and the HPLC conditions described in the Experimental were selected.

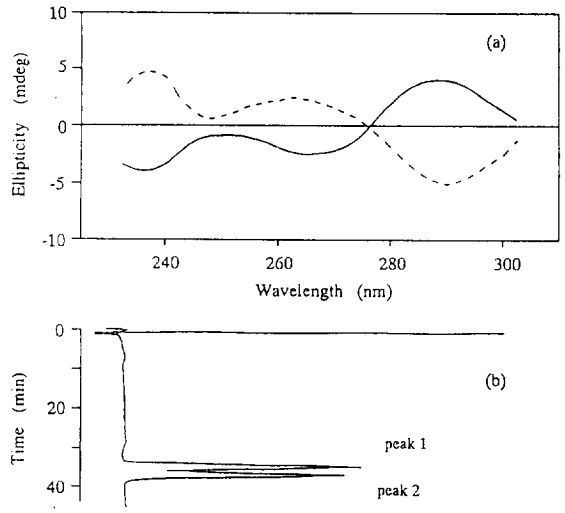

Fig. 2. (a) CD spectra of the peak fractions. The solid line represents the spectrum derived from peak 1 and the dotted line represents that from peak 2. (b) Typical chromatogram of racemic TH dissolved in water.

Fig. 2a shows the CD spectra derived from the peak fractions on the chromatogram (Fig. 2b) obtained for the authentic TH racemate. The CD spectrum derived from the first peak agreed with that of the $S(-)$-enantiomer reported by Carroll and Philip [12]. So we assigned the $S(-)$-TH to the isomer corresponding to the first peak and the $R(+)$-isomer to the second peak.

Fig. 3 shows a typical chromatogram obtained from serum of a patient who received a dose of TH. $S(-)$-, $R(+)$-TH and I.S. had retention times of 37, 39 and 25 min, respectively. Drug-free serum showed no peaks at the retention times corresponding to these compounds. A combination of drugs such as muscle relaxants

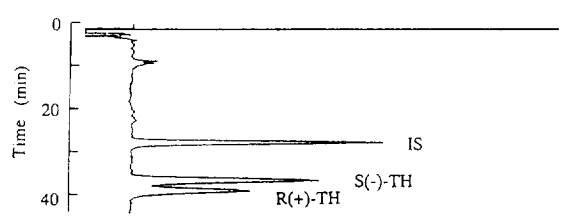

Fig. 3. Typical chromatogram of a serum sample obtained from a patient 3.5 h after an intravenous infusion of racemic TH (200 mg/h for 4.3 h).

and benzodiazepines, did not interfere with the peaks of interest.

The peak-height ratios of the TH enantiomers were linear over the concentration range 0.25–2.5 μg/ml [*S*(−)-TH, $y = 0.469x - 0.008$, $r = 0.999$; *R*(+)-TH, $y = 0.436x - 0.007$, $r = 0.999$, duplicate determinations]. The minimum detectable concentrations of the TH enantiomers were 50 ng/ml in serum (signal-to-noise ratio = 3). The calibration range almost completely covered the range of TH enantiomer concentrations found in patient sera. When the serum concentrations of the enantiomers were higher than 2.5 μg/ml, the sample was diluted with water. Both *R*(+)- and *S*(−)-TH were sufficiently detectable in the range 50 ng/ml–25 μg/ml in serum.

The intra- and the inter-day precision of the present method were investigated by using standard sera (0.25, 1.25, 2.5 μg/ml) (Table 1). The precision was satisfactory for monitoring the serum concentrations in patients under TH therapy.

The recovery of both TH enantiomers from the extraction procedure was ca. 97%, and the relative standard deviations were below 5% (TH racemate 2.5 μg/ml, five determinations).

Table 1
Intra- and inter-day precision of the TH enantiomer assay

	Spiked concentration (μg/ml)	Measured concentration[a] (μg/ml)	R.S.D. (%)
Intra-day			
S(−)-TH	0.25	0.23 ± 0.02	8.7
	1.50	1.45 ± 0.05	3.4
	2.50	2.43 ± 0.12	4.9
R(+)-TH	0.25	0.23 ± 0.02	8.7
	1.50	1.43 ± 0.04	2.8
	2.50	2.42 ± 0.13	5.4
Inter-day			
S(−)-TH	0.25	0.25 ± 0.03	12.0
	1.50	1.45 ± 0.04	2.8
	2.50	2.52 ± 0.07	2.8
R(+)-TH	0.25	0.23 ± 0.03	13.0
	1.50	1.44 ± 0.05	3.5
	2.50	2.50 ± 0.07	2.8

[a] Mean ± S.D. ($n = 5$).

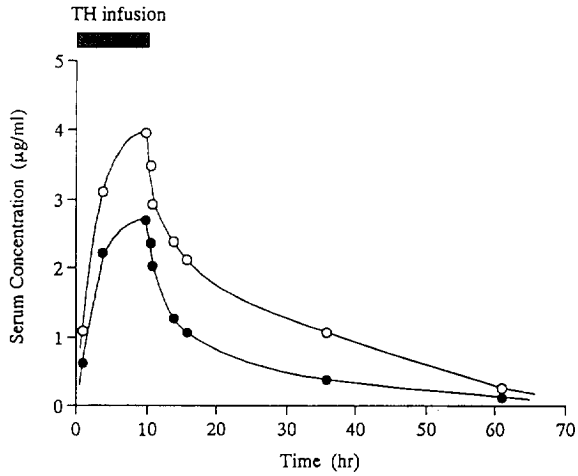

Fig. 4. Time–concentration profiles of TH enantiomers after an intravenous infusion of racemic TH in a patient. (○) *S*(−)-TH, (●) *R*(+)-TH. TH was intravenously infused as the racemate at a constant rate of 125 mg/h for 10 h.

The concentrations of the individual enantiomers in human serum samples were summed and compared with the total concentration of TH determined by achiral HPLC [8]. The linear correlation between the total concentrations determined by two methods was excellent.

The regression equation was Total = 0.9833 Sum + 0.0397 ($r = 0.996$, $n = 110$). This confirmed the validity of the present chiral HPLC method.

Fig. 4 shows the serum concentration–time profile of the TH enantiomers after the start of continuous intravenous infusion of racemic TH (125 mg/h for 10 h) in a patient. The serum concentrations of *S*(−)-TH were always higher than those of *R*(+)-TH, suggesting that the pharmacokinetics of TH in human are stereoselective.

4. Conclusion

We developed a simple and accurate HPLC method for measuring the levels of TH enantiomers in serum using reversed-phase HPLC and a mobile phase containing β-CyD. The method

should be useful for pharmacokinetic and pharmacodynamic studies of TH enantiomers.

References

[1] C.E. Cook, T.B. Seltzman, C.R. Tallent, B. Lorenzo and D.E. Drayer, *J. Pharmacol. Exp. Ther.*, 241 (1987) 779.
[2] M.H. Chandler, S.R. Scott and R.A. Blounin, *Clin. Pharmacol. Ther.*, 43 (1988) 436.
[3] W.D. Hooper and M.S. Qing, *Clin. Pharmacol. Ther.*, 48 (1990) 633.
[4] H. Watanabe, S. Ohdo, M. Ishikawa and N. Ogawa, *J. Pharmacol. Exp. Ther.*, 263 (1992) 1036.
[5] J. Zukowski, D. Sybilska, J. Bojarski and J. Szejtli, *J. Chromatogr.*, 436 (1988) 381.
[6] D. Sybilska, J. Zukowski and J. Bojarski, *J. Liq. Chromatogr.*, 9 (1986) 591.
[7] S. Eto, H. Noda and A. Noda, *J. Chromatogr.*, 579 (1992) 253.
[8] K. Hibi, A. Wada, M. Takeuchi and M. Hatta, *Bunseki Kagaku*, 35 (1986) 245.
[9] K. Uekama, F. Hirayama, K. Ikeda and K. Inaba, *J. Pharm. Sci.*, 66 (1977) 706.
[10] J. Debowski, J. Jurczak and D. Sybilska, *J. Chromatogr.*, 282 (1988) 83.
[11] A.D. Cooper and T.M. Jefferies, *J. Pharm. Biomed. Anal.*, 8 (1990) 847.
[12] F.I. Carroll and A. Philip, *J. Med. Chem.*, 19 (1976) 521.