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Short communication

# Improved method of determining thiamylal enantiomers in human serum by high-performance liquid chromatography

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**Abstract**

Thiamylal, a widely used anesthetic drug, has two enantiomers. We developed a simple and rapid method for measuring the thiamylal enantiomers in human serum. The method involves a liquid–liquid extraction procedure followed by chiral resolution using a 5 μm silica-bonded $\alpha_1$-acid glycoprotein column (Chiral-AGP). The thiamylal enantiomers and internal standard were eluted within 15 min and were well-resolved. At concentrations of 1, 5 and 20 μg $ml^{-1}$, the relative standard deviations of *R*(+)- and *S*(−)-thiamylal were 1.35–2.88% and 1.37–3.01%, respectively, for the intra-day assay, and 2.93–4.46% and 2.46–4.84%, respectively, for the inter-day assay. This method facilitates the routine monitoring and pharmacokinetic studies of thiamylal enantiomers. 

*Keywords:* Enantiomer separation; Thiamylal

## 1. Introduction

Thiamylal, 5-allyl-5-(1-methylbutyl)-2-thiobarbituric acid (Fig. 1A), is an ultrashort-acting barbiturate originally used for the induction and/or maintenance of anaesthesia [1]. It has also been used for the treatment of status epilepticus [2] and for brain protection after cerebral surgery [3]. Thiamylal has two enantiomers derived from one chiral carbon at the 1′-position, *R*(+)- and *S*(−)-thiamylal, and the racemic mixture has been used as a parenteral product.

The pharmacodynamic activity of a racemic drug resides predominantly in one of its enantiomers. In the case of thiamylal, the *S*(−)-enantiomer was reported to be more potent than optical antipode [4], a finding similar to that in the other 5-(1-methylbutyl)-barbituric acid derivatives thiopental [4,5], pentobarbital [4] and secobarbital [4,6].

Differential properties between enantiomers may also occur in pharmacokinetic behaviors, such as absorption, distribution, metabolism and excretion. In order to clarify the pharmacokinetic differences among enantiomers, enantioselective analytical methods for use with biological specimens must be developed. We previously established a high-performance liquid chromatography (HPLC) method for measuring the levels of thiamylal enantiomers in human serum, using an ODS column and β-cyclo-

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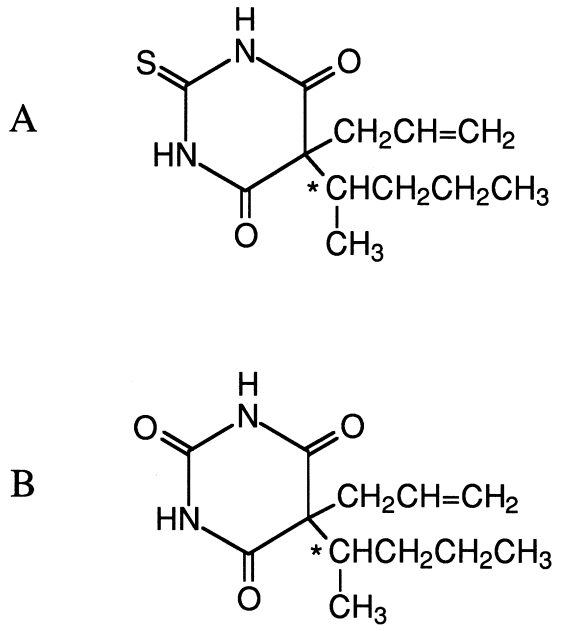


Fig. 1. Structures of thiamylal (A) and secobarbital (B).

dextrin as a chiral mobile phase additive [7]. Despite its simplicity and accuracy, this method was much too time-consuming, and the consequent long retention time, about 45 min, lowered the applicability of the method to routine monitoring and pharmacokinetic studies of thiamylal enantiomers.

Chiral-AGP, which is composed of an α1-acid glycoprotein binding covalently to silica gel, has recently been used for the enantioselective determination of several drugs such as thiopental [8,9], ketamine [10] and mepivacaine [11]. In the present study, we improved our HPLC method for the measurement of thiamylal enantiomers by using Chiral-AGP.

## 2. Experimental

### 2.1. Materials and reagents

Racemic thiamylal sodium was obtained from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Diethylether, *n*-hexane and *n*-propyl-*p*-hydroxybenzoate (internal standard) were obtained from Wako Pure Chemical Industries (Osaka). Methanol, 2-propanol, phosphoric acid and $KH_2PO_4$ were purchased from Kanto Chemical Co. (Tokyo, Japan). All chemicals were of HPLC or analytical grade. Lyophilized drug-free serum was purchased from Bio-Rad Labs. (Anaheim, CA, USA) to prepare the standards. Distilled water was passed through a Milli-Q Water Purification System (Millipore Japan, Tokyo).

### 2.2. Standards

Thiamylal sodium was dissolved with water (1 mg $ml^{-1}$), and this stock solution was stored at −20°C. Thiamylal standard solutions (0.5–25 μg $ml^{-1}$ for each enantiomer) were prepared by diluting the stock solution with water and adding it to 9 vol. of drug-free serum. An internal standard stock solution was prepared by dissolving *n*-propyl-*p*-hydroxybenzoate with methanol (0.25 mg $ml^{-1}$) and stored at 4°C in the dark. This solution was diluted with methanol when used. The stock solutions were stable for at least three months under these conditions.

### 2.3. Apparatus

The chromatographic system consisted of a pump (LC-10AD, Shimadzu, Kyoto, Japan), a variable-wavelength UV–Vis detector (SPD10-A, Shimadzu), an integrator (C-R6A, Shimadzu), and a Reodyne 7725 sample injector equipped with a 20 μl sample loop (Cotati, CA, USA). The separation was performed on a Chiral-AGP column (100×4.0 mm, ChromTech AB, Hägersten, Sweden) equipped with a prefilter (SUMIPAX PG-OH, Sumika Chemical Analysis Service, Osaka, Japan) and a guard column (Chiral-AGP, 10×3.0 mm, ChromTech AB).

### 2.4. Chromatographic conditions

The mobile phase consisted of 20 m*M* $KH_2PO_4$ containing 3% (v/v) 2-propanol (pH 4.7) and was degassed in an ultrasonic bath before being used. The flow-rate was 0.9 ml $min^{-1}$. Chromatography was performed at ambient temperature (ca. 25°C).

The hold-up time of the column ($t_0$) was determined with an injection of 50% (v/v) methanol.

After each day's use, the HPLC system was washed with 4.5% (v/v) 2-propanol aq. solution.

### 2.5. Serum sample preparation

Blood samples were obtained from patients undergoing thiamylal therapy as part of the current treatment protocol utilized in the Operating Room and Intensive Care Unit, Kyushu University Hospital (Fukuoka, Japan) and were centrifuged at 1 500 *g* for 10 min. The sera were stored at −20°C until use. Thiamylal in the serum sample was stable for at least one month under these conditions.

### 2.6. Extraction procedure

Liquid–liquid extraction was performed. The serum sample or standard solution (~300 μl), internal standard solution (100 μl), 3 *M* phosphoric acid (20 μl) and 1 ml of 20% (v/v) diethylether in *n*-hexane were placed into a 1.5-ml plastic microtube. The mixture was vortex-mixed for 1 min, centrifuged at 13 000 *g* for 5 min and left in a freezer (−80°C) for 10 min. After freezing the aqueous layer (lower layer), the supernatant (organic layer) was decanted into another microtube and evaporated to dryness under a gentle stream of nitrogen gas at ambient temperature. The residue was reconstituted in ~200 μl of 50% (v/v) methanol aq. solution, and then 20 μl aliquots were injected into the HPLC system.

## 3. Results and discussion

The manufacturer of Chiral-AGP recommends 10 m*M* phosphate buffer (pH 7.0) containing 5% 2-propanol as the starting mobile phase to determine weak acidic compounds. However, a satisfactory resolution of the thiamylal enantiomers was not obtained with this mobile phase. The concentration of organic modifier, the ionic strength and the pH of the mobile phase have a profound effect on the retention and the resolution of enantiomers on this stationary phase [12]. Therefore, the ratio of 2-propanol and the concentration of $KH_2PO_4$ was examined, and we finally obtained sufficient resolution of thiamylal enantiomers using the optimal HPLC conditions described in the Experimental section. When the sample residue was dissolved in mobile phase, the relative standard deviations of intra-day precision were more than 20%. This might due to the low solubility of thiamylal in mobile phase. So, we reconstituted the sample residue in 50% methanol. This solvent had no influence on the retention times and the peak shapes of thiamylal enantiomers.

Chromatograms of blank serum, serum spiked with racemic thiamylal and the serum obtained from a patient who underwent thiamylal administration are shown in Fig. 2A–C, respectively. We previously found that the concentration of *S*(−)-thiamylal was higher than that of *R*(+)-thiamylal in all patients at any period following racemic thiamylal administration [13]. As shown in the patient's chromatogram (Fig. 2C), the second peak of thiamylal enantiomer is higher than the first peak. Thus, we assigned *R*(+)-thiamylal to the enantiomer corresponding to the first peak and *S*(−)-thiamylal to the second peak. The order of elution for the thiamylal enantiomers was the same as those of thiopental and pentobarbital [8,9]. This was considered reasonable since these barbiturates have identical steric features. The retention times were 10.0 and 11.7 min for *R*(+)-thiamylal and *S*(−)-thiamylal, respectively, and 5.3

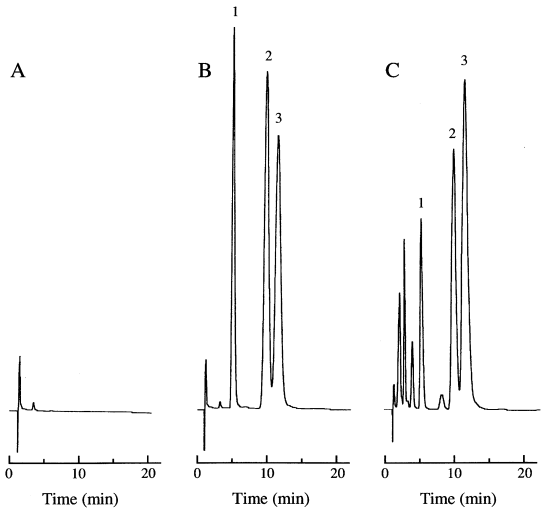


Fig. 2. Chromatograms of (A) extract of blank serum; (B) extract of rac-thiamylal 10 μg ml$^{-1}$ spiked to serum: peaks 1, 2 and 3 were internal standard, *R*(+)-thiamylal and *S*(−)-thiamylal, respectively; (C) extract of serum from a patient, who underwent thiamylal treatment; *R*(+)-thiamylal 8.0 μg ml$^{-1}$, *S*(−)-thiamylal 12.6 μg ml$^{-1}$.

Table 1
Intra-day accuracy and precision of thiamylal enantiomer determination

| Racemic thiamylal spiked ($\mu g\ ml^{-1}$) | *R*(+)-thiamylal | | | *S*(−)-thiamylal | | |
|---|---|---|---|---|---|---|
| | Measured concentration[a] ($\mu g\ ml^{-1}$) | RSD[b] (%) | Mean recovery (%) | Measured concentration[a] ($\mu g\ ml^{-1}$) | RSD[b] (%) | Mean recovery (%) |
| 2.0 | 0.937±0.027 | 2.88 | 93.4 | 0.897±0.027 | 3.01 | 92.8 |
| 10.0 | 4.97±0.11 | 2.21 | 91.2 | 5.03±0.11 | 2.19 | 91.5 |
| 40.0 | 19.3±0.26 | 1.35 | 91.0 | 19.7±0.27 | 1.37 | 92.4 |

[a] Data are mean±SD of five experiments.
[b] RSD: relative standard deviation.

min for *n*-propyl-*p*-hydroxybenzoate (internal standard).

In this system, secobarbital (Fig. 1B), one of the thiamylal metabolites [14,15], was eluted at 4.4 min without enantioseparation, and did not interfere with the thiamylal enantiomer assay, because secobarbital has a maximum UV absorbance at 217 nm and no absorbance at 288 nm.

The enantiomers of thiamylal were well-resolved from each other, the resolution factor being 1.4. The capacity factors ($k'$) were 6.4 and 7.6 for *R*(+)-thiamylal and *S*(−)-thiamylal, respectively, and the enantioselectivity factor ($\alpha$) was 1.19.

The retention times and the enantioselectivity varied with an increasing number of analyzed samples. This phenomenon has also been reported by other workers [16,17].

The peak height ratios of the thiamylal enantiomers were linear in the concentration range of 0.5–30 $\mu g\ ml^{-1}$ with a correlation coefficient greater than 0.999 for both enantiomers. Aarons et al. [18] defined the limit of quantitation as the level having a relative standard deviation of 20%. According to this criterion, the lower limit of quantitation of thiamylal enantiomers was 5 ng. The limit of detection was 1.5 ng (with a signal-to-noise ratio of 3).

The intra-day accuracy and precision were investigated at the concentrations of 2, 10, and 40 $\mu g\ ml^{-1}$ racemic thiamylal in drug-free serum (Table 1). The relative standard deviations were less than 3.0%. The measured values of *R*(+)- and *S*(−)-thiamylal were 93.7–99.3% and 89.7–100.6% of the target value, respectively. The mean recoveries of each enantiomer obtained from five experiments were more than 90%. The inter-assay precision at the same concentrations investigated throughout five days are shown in Table 2. The relative standard deviations were less than 5.0%. The accuracy, precision and reproducibility were satisfactory for monitoring the serum/plasma concentrations in patients undergoing thiamylal therapy.

The serum concentrations of individual enantiomers and total thiamylal in seven patients who underwent thiamylal therapy were measured using

Table 2
Inter-assay reproducibility of thiamylal enantiomers

| Racemic thiamylal spiked ($\mu g\ ml^{-1}$) | *R*(+)-thiamylal | | *S*(−)-thiamylal | |
|---|---|---|---|---|
| | Measured concentration[a] ($\mu g\ ml^{-1}$) | RSD[b] (%) | Measured concentration[a] ($\mu g\ ml^{-1}$) | RSD[b] (%) |
| 2.0 | 1.01±0.045 | 4.46 | 0.971±0.047 | 4.84 |
| 10.0 | 5.12±0.15 | 2.93 | 5.14±0.16 | 3.11 |
| 40.0 | 20.4±0.63 | 3.09 | 20.7±0.51 | 2.46 |

[a] Data are mean±SD of five experiments for five days.
[b] RSD: relative standard deviation.

the present method and conventional achiral HPLC [19]. The sum of the *R*(+)- and *S*(−)-thiamylal concentrations (SUM) was compared with the total thiamylal concentration (TOTAL). The linear correlation between the total concentrations determined by the two methods was excellent, and the regression equation was SUM=1.05×TOTAL−1.50 ($r$=0.993, $n$=32), strongly supporting the validity of the present chiral HPLC method.

## 4. Conclusion

We developed a simple and accurate HPLC method for measuring the levels of thiamylal enantiomers in serum using an α1-acid glycoprotein column (Chiral-AGP) with 20 m*M* potassium dihydrogen phosphate containing 3% (v/v) 2-propanol as a mobile phase. The two thiamylal enantiomers were well-resolved and eluted within 15 min. This method will be useful for pharmacokinetic and pharmacodynamic studies of thiamylal enantiomers.

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