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Determination of thiopental in human serum and plasma by high-performance capillary electrophoresis–micellar electrokinetic chromatography

PETER MEIER and WOLFGANG THORMANN*
Department of Clinical Pharmacology, University of Bern, Murtenstrasse 35, CH-3010 Bern (Switzerland)

ABSTRACT

The quantitation of thiopental in human serum and plasma was investigated using high-performance capillary electrophoresis (HPCE) in a micellar configuration and the results were compared with reversed-phase high-performance liquid chromatography (HPLC). Thiopental and an internal standard (carbamazepine for HPCE and thiamylal for HPLC) were extracted from serum or plasma using pentane and a phosphate buffer (pH 6.4). HPCE analysis took place in a phosphate–borate buffer with 50 m*M* sodium dodecyl sulphate using an automated instrument and HPLC was performed with a C_8 column and a mobile phase of phosphate buffer–acetonitrile (65:35, v/v). HPCE and HPLC data from 66 patient samples compared well based on linear regression analysis. However, estimates obtained with the inclusion of the internal standard were lower than those based on the sample peak only. This example allows the elucidation of the advantages of using HPCE as an assay methodology for the therapeutic monitoring of thiopental and other drugs.

INTRODUCTION

The barbiturate thiopental is used for anaesthetic and pre-anaesthetic medication and for the treatment of head trauma with severe brain injury. It has a low therapeutic index and is therefore prone to cause poisoning. Monitoring its concentration in body fluids is important for the optimization of pharmacotherapy. The therapeutic range of thiopental is not yet clearly established, but plasma concentrations in the range 1–40 μg/ml have been found to be efficient in many treatments [1,2]. Several immunoassays [3–5] and chromatographic methods [6–9] have been developed for the determination of thiopental in serum. The immunological techniques are attractive because of their ease of performance, speed of analysis and sensitivity. However, these assays are not specific and sensitive enough to monitor thiopental in the presence of other barbiturates, including its metabolite pentobarbital. Chromatographic procedures, and high-performance liquid chromatography (HPLC) in particular, have been successfully applied to the determination of thiopental, although they are rather time-consuming.

High-performance capillary electrophoresis (HPCE) and micellar electrokinetic

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capillary chromatography (MECC; an interface between electrophoresis and chromatography) have been shown to be attractive approaches to the determination of drugs in body fluids [10–15]. It has previously been shown that barbiturates in human urine and serum can be determined by an HPCE–MECC technique where the separation is based on a combination of differential partitioning and differences in electrophoretic mobilities [16]. This paper reports the determination of thiopental in human plasma using HPCE–MECC (abbreviated as HPCE throughout this paper) and data are compared with those obtained by reversed-phase HPLC.

EXPERIMENTAL

Drugs and chemicals

Thiopental (Pentothal, containing 6% sodium carbonate) was from Abbott Labs. (Cham, Switzerland) and thiamylal, carbamazepine and sodium dodecyl sulphate (SDS) were from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (both HPLC grade) were from Rathburn Chemicals (Walkerburn, UK); *n*-pentane (99% pure), potassium dihydrogenphosphate, sodium dihydrogenphosphate, sodium borate and phosphoric acid (85%) were from Merck (Darmstadt, Germany). Bovine plasma, used as the calibration material, was prepared by centrifugation (1500 *g* for 10 min) of bovine blood which was obtained from the local slaughter house.

Patient samples

Patient samples were received from the Department of Anesthesiology and Intensive Care, Inselspital (Bern, Switzerland) from patients undergoing short-term thiopental infusion (100 mg/min for 6–9 min). Blood samples were drawn during the infusion period and at increasing time intervals over a period of 24 h thereafter.

Preparation of standard solutions

Methanolic standard solutions of thiopental (100 μg/ml), thiamylal (20 μg/ml) and carbamazepine (2 mg/ml) were prepared and stored at 4°C. Blank and patient sera were spiked by the addition of known aliquots of these standard solutions to body fluids prior to sample extraction.

Extraction procedure

Liquid–liquid extraction of thiopental and the internal standards was achieved with pentane at pH 6.4, as described by Elbling *et al.* [17]. Briefly, 0.5 ml of patient serum spiked with 100 μl of a solution containing the internal standard (or 0.5 ml of bovine plasma spiked with thiopental and internal standard), 1 ml of pH 6.4 phosphate buffer (about 0.07 *M*) and 5 ml of *n*-pentane were added to a 11-ml screw-capped Sovirel test-tube. After vigorous shaking for 10 min and centrifugation at 1500 *g* for 10 min the upper (organic) phase was transferred into a centrifuge glass tube with a short conical bottom and then evaporated to dryness under a gentle stream of nitrogen at 40°C. For HPCE (HPLC) the residue was dissolved in 200 μl of running buffer (200 μl of mobile phase) and vortexted for about 60 s.

HPCE

A Model 270A capillary electrophoresis system (Applied Biosystems, San Jose,

CA, USA) was used. This apparatus features automated capillary rinsing, sampling and execution of the electrophoretic run. For these experiments it was equipped with a 50 μm I.D. fused-silica capillary of 50 cm effective separation length. A Model D-2000 chromato-integrator (Merck-Hitachi, Darmstadt, Germany) was used for recording the pherograms and for quantitation by peak-area measurements. The integrator sampling period was set to one data point per 200 ms. As an alternative a PC integration pack (version 2.50, Kontron Instruments, Zürich, Switzerland), together with a Mandax AT 286 computer system, were used for data acquisition, raw data storage and integration of the signals. The pack features automatic range switching and a dynamic sampling rate allowing sampling every 10 ms for quickly changing signals. Before each run the capillary was rinsed with 0.1 *M* sodium hydroxide (1 min) and buffer (2 min). The running buffer, if not stated otherwise, was composed of 50 m*M* SDS, 9 m*M* sodium borate and 15 m*M* sodium dihydrogenphosphate (pH about 7.8). Injection of the sample was by vacuum suction (typically 2 s). In most experiments a constant voltage of 30 kV (current 55–60 μA) was applied, the temperature was set at 40°C and detection occurred at 290 nm. Under these conditions, thiopental and carbamazepine (internal standard) eluted after about 4 and 7.5 min, respectively, and the total analysis time was about 10.5 min.

HPLC

HPLC analyses were performed using a Model M45 solvent delivery system, a WISP 712 autosampler (both from Waters Assoc., Milford, MA, USA), a reversed-phase C_8 column (Nucleosil 5 C_8, 250/8/4, Macherey Nagel, Oensingen, Switzerland) and a Spectroflow Model 757 UV detector (Kratos Analytical, Ramsey, NJ, USA). Chromatograms were recorded and integrated by a Model 3390A integrator (Hewlett-Packard, Basel, Switzerland). The integrator sampling rate was set to five data points per min (peak width 0.20 min). The mobile phase consisted of a mixture of an aqueous phosphate buffer (0.2 m*M* phosphoric acid and 0.175 m*M* potassium dihydrogenphosphate) and acetonitrile (65:35, v/v). Thiamylal served as the internal standard, the injection volume was 5 μl, the flow-rate was 1.2 ml/min, the temperature was ambient and detection was at 290 nm. Under these conditions, thiopental and thiamylal eluted after about 10 and 12 min, respectively, and the total analysis time was 15 min. After each sample series the column was first rinsed with mobile phase (30 min) and then for about 60 min with a mixture of acetonitrile–water (35:65, v/v).

RESULTS AND DISCUSSION

The electropherograms in Fig. 1 represent the analysis of a model mixture of seven barbiturates (20 μg/ml each) detected at 215, 254 and 290 nm. This result confirms the HPCE data with fast-scanning polychrome detection reported elsewhere [16]. Thiopental is monitored at 290 nm selectively and with the highest sensitivity. Peak 5a represents an isomer of thiopental found in the commercial thiopental product (Fig. 2), a barbiturate which can also be detected by HPLC [9]. Each component is characterized by its retention and migration behaviour, with barbital being the fastest and pentobarbital the slowest of the components investigated. For detection at 290 nm, none of these barbiturates can be used as an internal standard for the determination of thiopental. Therefore, thiamylal (Fig. 2), which was often used for the HPLC

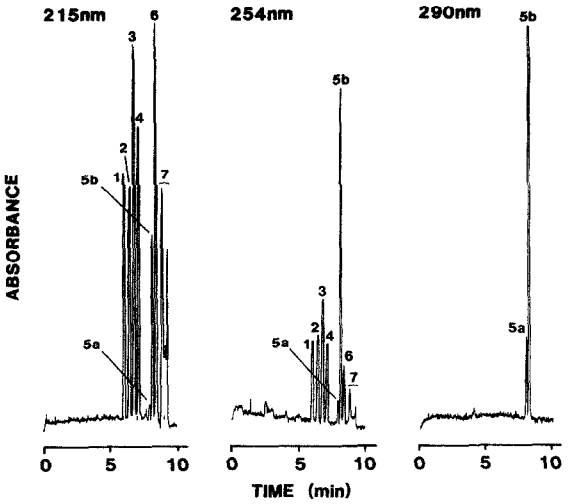

Fig. 1. HPCE data of a model mixture of seven barbiturates (20 μg/ml each) monitored at three different wavelengths. The injection time was 5 s and the applied voltage was a constant 20 kV (current 35 μA). Peaks: 1 = barbital; 2 = allobarbital; 3 = phenobarbital; 4 = butalbital; 5a = isomer of thiopental; 5b = thiopental; 6 = amobarbital; 7 = pentobarbital.

determination of thiopental [9,17], was considered. Retention of that compound, however, was found to be only slightly larger than thiopental. Modifications of the buffer composition, such as pH, SDS concentration (between 20 and 100 m*M*) and addition of methanol (up to 10%, v/v) did not sufficiently alter the separation of the two compounds to obtain full resolution in a capillary of 50 cm length. Resolution was attained with much longer columns, but the analysis times became too long for practical purposes. Therefore, carbamazepine (a compound also used in HPLC [7]) was used as the internal standard for all HPCE determinations of thiopental reported here.

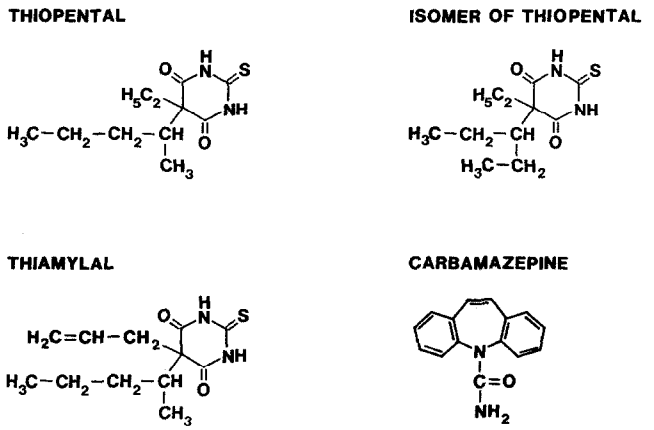

Fig. 2. Structures of thiopental, its isomer, thiamylal and carbamazepine.

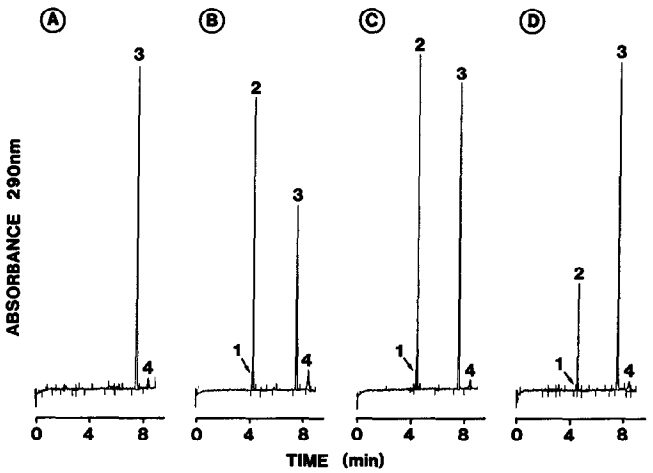

Fig. 3. Electropherograms of two calibration samples [(A) bovine plasma spiked with carbamazepine (0.4 mg/ml); (B) bovine plasma spiked with internal standard and 40 μg/ml thiopental] and two patient samples [(C) 23.6 μg/ml; (D) 6.4 μg/ml thiopental]. All experiments were performed with a 2-s injection and a constant 30-kV voltage (55–60 μA). Peaks: 1 = isomer of thiopental; 2 = thiopental; 3 = carbamazepine; 4 = unknown compound.

Fig. 3 shows typical electropherograms obtained after the extraction of two calibration (panels A and B) and two patient (panels C and D) samples. Thiopental (2), its isomer (1), carbamazepine (3) and an unknown compound (4) were detected (290 nm) within about 8 min of current flow beginning. Typical chromatograms of the HPLC determination of thiopental with thiamylal as the internal standard are shown in Fig. 4. Here excellent separation of the two barbiturates and the isomer of thiopental was obtained with an analysis time of about 12 min. All the calibration chromatograms contained an unknown peak (1) in front of the isomer and all data panels showed an impurity of unknown origin (4) which eluted between thiopental (3) and thiamylal (5). Comparison of the data in Figs. 3 and 4 reveal that the HPCE peaks are significantly sharper than those monitored in HPLC.

The reproducibility of the HPCE determination of thiopental is given in Table I. Here typical means and the relative standard deviations of ten consecutive injections evaluated with the D-2000 chromato-integrator and the PC integration pack are given. Different capillaries (and samples) were used for the two sets of data. With both methods the carbamazepine peak was integrated with a higher reproducibility than that of thiopental. The peak of the isomer in front of that of thiopental may be attributed to this difference. Retention times, although gradually increasing within the sets of ten injections, showed the best reproducibility of all parameters tested. These data are comparable to those reported with model compounds [18].

The quantitation of thiopental in human serum was performed (i) by the internal standard method and (ii) by direct calibration with the thiopental peak, *i.e.* without the inclusion of an internal standard. Calibration graphs were constructed with spiked bovine plasma in the concentration range 2–60 μg/ml (six data points). Peak areas were used as the basis for data evaluation, providing graphs with good

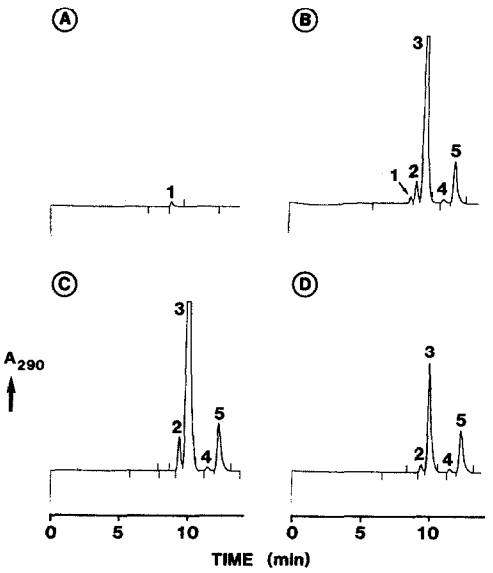

Fig. 4. Chromatograms of (A) bovine plasma blank, (B) bovine plasma spiked with thiamylal (internal standard, 4 μg/ml) and 40 μg/ml thiopental, and (C, D) of two patient samples [(C) 31.4 μg/ml thiopental; (D) 7.1 μg/ml thiopental] spiked with thiamylal (4 μg/ml). Peaks: 1 = unknown compound extracted from bovine plasma; 2 = isomer of thiopental; 3 = thiopental; 4 = unknown impurity; 5 = thiamylal.

linearities and small y-intercepts. Table II lists the statistical data of seven HPCE and HPLC calibrations which were based on peak-area measurements using the D-2000 and the HP 3390A instruments, respectively. Evaluations based on the inclusion of the internal standards are referred to as "ratio", whereas those with calibration using peak areas of thiopental only are referred to as "area". An increased concentration range to 80 μg/ml thiopental (or higher) could best be fitted with a quadratic regression analysis (data not shown). Similar results were obtained when (i) peak heights

TABLE I

REPRODUCIBILITY DATA FOR TEN CONSECUTIVE INJECTIONS

Integration method	D-2000 chromato-integrator[a]		PC integration pack[b]	
	Mean	Relative standard deviation (%)	Mean	Relative standard deviation (%)
Retention time of thiopental (min)	4.617	0.95	5.22	1.11
Area of thiopental (μV s)	39965	3.73	81420	6.93
Area of carbamazepine (μV s)	69180	2.04	39000	3.54
Area ratio thiopental/carbamazepine	0.577	2.08	2.087	5.22
Thiopental concentration (μg/ml)	25.83	1.93	–	–

[a] Patient sample spiked with carbamazepine (0.4 mg/ml).

[b] Calibration sample spiked with thiopental (40 μg/ml) and carbamazepine.

TABLE II

STATISTICAL EVALUATION OF SEVEN CALIBRATION GRAPHS

Calibration graph	Slope	*y*-Intercept	Correlation coefficient
HPCE ratio[a]	40.177 ± 4.087	−0.022 ± 2.878	0.977 ± 0.003
HPCE area[b]	0.00136 ± 0.000938	−2.990 ± 3.778	0.994 ± 0.007
HPLC ratio[a]	4.035 ± 0.690	0.159 ± 0.904	0.994 ± 0.013
HPLC area[b]	6.186 ± 0.565	−0.659 ± 1.133	0.998 ± 0.001

[a] Data evaluation with inclusion of internal standard.
[b] Data evaluation without inclusion of internal standard.

were used as the basis for data evaluation and (ii) the PC integration pack was used instead of the integrators.

Sixty-six serum samples of four patients obtained during and within about 24 h after short-term (see under Experimental) thiopental infusion were analysed using the two methods. A comparison of all the data evaluated on the basis of peak-area ratios,

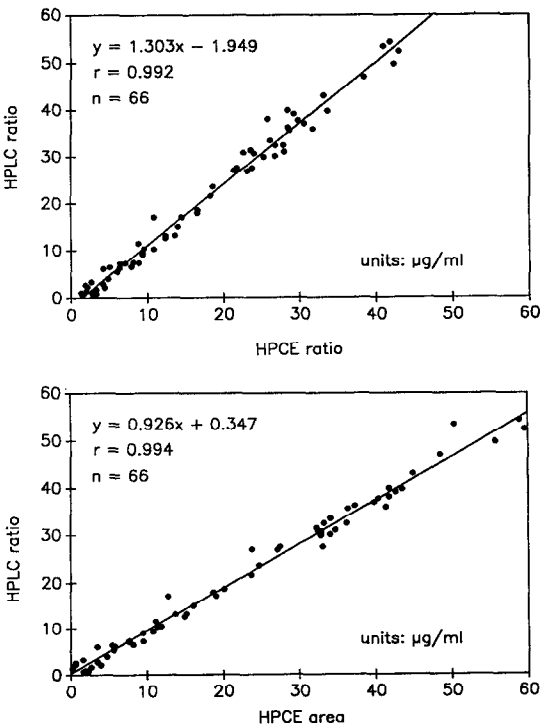

Fig. 5. Comparison of HPCE and HPLC data of 66 patient samples. The acronym "area" represents data evaluated based on thiopental areas and without inclusion of internal standard, whereas "ratio" stands for evaluation based on area ratios (inclusion of internal standard). All electropherograms were integrated with the D-2000 instrument.

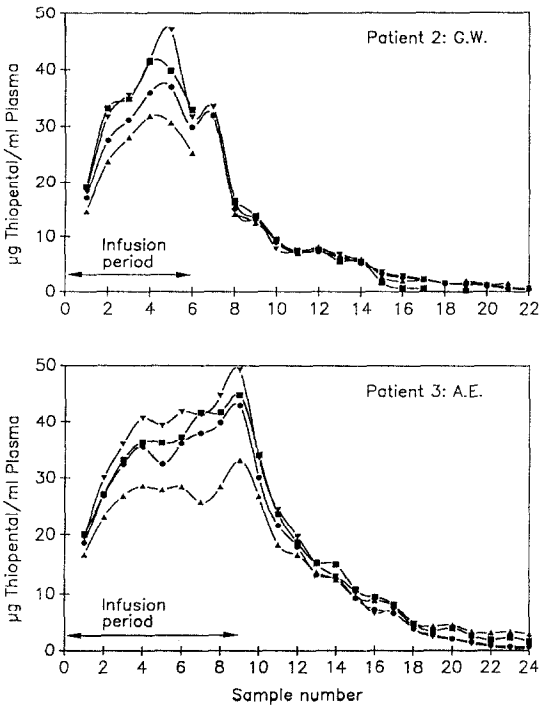

Fig. 6. HPCE and HPLC data of two patients plotted as a function of sample number which were obtained during thiopental infusion and (at increasing time intervals) over about 24 h thereafter. Other conditions are identical to those in Fig. 5. Symbols: (●) HPLC ratio; (▲) HPCE ratio; (■) HPCE area; (▼) HPLC area.

i.e. with calibrations established using the ratio of the area of thiopental divided by the area of the internal standard, are presented in the upper panel of Fig. 5. The data show a good linear correlation which, however, is not identical to the line of equality. It is interesting that the correlation of HPLC data based on area ratios with HPCE data obtained without the use of the internal standard correlated better (lower panel of Fig. 5). Linear regression analysis of the HPCE data based on area ratios *versus* those based on the thiopental peak area only (slope 1.40; y-intercept -2.39; correlation coefficient 0.994; $n = 66$) showed much stronger deviations from the line of equality than the same analysis of the HPLC results (slope 1.15; y-intercept -0.68; correlation coefficient 0.999; $n = 24$). The discrepancy of the estimates obtained by the different procedures is also seen by plotting the thiopental concentrations of single patients as a function of sample number (Fig. 6). No explanation has yet been found to account for these differences, which are most pronounced in the HPCE data obtained with the inclusion of the internal standard.

CONCLUSIONS

The results reported in this paper suggest that HPCE can be easily used for the determination of thiopental in human serum or plasma. After extraction, this barbit-

urate can selectively and sensitively be monitored at 290 nm. Peak integration can be executed with a stand-alone integrator or a PC integration pack, both developed for the evaluation of chromatograms. For the concentration range 2–60 μg/ml and using different quantitation procedures, such as peak areas with and without internal standard, calibration graphs using bovine plasma spiked with thiopental always showed good linear correlations. HPLC and HPCE data from 66 patient samples compared well based on linear regression analysis. However, the graphs always deviated from the line of equality. The reasons for this behaviour are the subject of on-going investigations. For therapeutic drug monitoring, such differences are relatively insignificant. Using HPCE instead of HPLC for the therapeutic monitoring of thiopental has several important advantages. These are a high degree of automation, small sample size, no requirement for large amounts of organic solvents and rapidity of analysis. Furthermore, HPCE allows a fast change from one buffer configuration to another separation medium.

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