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Short Communication

Serum antipyrine concentrations determined by micellar electrokinetic capillary chromatography

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ABSTRACT

We developed an assay for antipyrine in serum using micellar electrokinetic capillary chromatography. A capillary electrophoresis system with a 70-cm fused-silica capillary and pH 8.2 borate–sodium dodecyl sulfate buffer was used. Standard curves ranging from 0.5 μg/ml to 25 μg/ml were analyzed. The resulting electropherograms showed no interfering endogenous peaks and the assay was linear in the range of concentrations analyzed. The intra-day and inter-day relative standard deviations ranged from 0.7 to 5.2% and 1.7 to 8.1%, respectively. Analytical recovery of antipyrine ranged from 78.2 to 105.0%. The theoretical plate number for antipyrine was calculated to be 561 000 ± 79 000 plates/m.

INTRODUCTION

Antipyrine has been extensively used as a marker compound for hepatic oxidative metabolism and for the estimation of total body water in humans and in animals [1,2]. Antipyrine disposition has been widely used as a method to investigate the influence of disease [3], hydration [4], xenobiotics [5], and diet on drug metabolism [6]. Antipyrine is almost exclusively metabolized in the liver by oxidative microsomal enzymes and the measurement of antipyrine clearance provides a measure of cytochrome P-450 metabolizing activity. Furthermore, antipyrine is not significantly bound to plasma or tissue proteins and is rapidly distributed throughout total body water.

Several high-performance liquid chromatographic (HPLC) methods have been described

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for antipyrine [7–14]. Although chromatographic methods offer an acceptable lower limit of detection, chromatographic assays often require large sample volumes. While chromatographic methods may be ideally suited for analysis of large volume samples, capillary electrophoresis may provide an additional method for the analysis of micro-volume samples. The purpose of the present study was to develop a reproducible microanalytical method of measuring antipyrine concentrations in serum using micellar electrokinetic capillary chromatography (MECC).

EXPERIMENTAL

Materials

All chemicals were analytical-grade reagents and used as received without further purification. Antipyrine, acetaminophen, sodium dodecyl sulfate, and sodium tetraborate were purchased from Sigma (St. Louis, MO, USA). Boric acid was purchased from Fisher Scientific (Pittsburgh, PA, USA). Hydrochloric acid, phosphoric acid and sodium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade acetonitrile was used for all deproteination procedures (J.T. Baker). Deionized water was obtained from an 18 megohm · cm service deionization system (Continental Water System Corp., Roswell, GA, USA).

Animals

Ten male Sprague–Dawley rats (300–330 g, Harlan Sprague Dawley, Indianapolis, IN, USA) were used during this study. All procedures were approved by the Committee for Animal Use for Research and Education at the Medical College of Georgia prior to the initiation of the study. All procedures were in accordance with the guidelines established by the National Institutes of Health for the humane treatment of animals.

Apparatus

Sample analysis was performed using a commercially available capillary electrophoresis apparatus (CES-I, Dionex, Sunnyvale, CA, USA). Separations were performed in an untreated fused-silica capillary (Dionex, 70 cm × 0.75 μm I.D.). The exterior of the capillary was coated with polyimide. The total length between injection and detection was 65 cm. Prior to analysis, the capillary was rinsed with 0.5 ml of phosphoric acid (pH 2.3, 0.5 *M*) followed by 0.5 ml of deionized water. The capillary was then rinsed with 0.5 ml of sodium hydroxide (pH 13.4, 0.5 *M*), followed by 0.5 ml of deionized water and subsequently filled with a borate buffer solution described below. This treatment prepared the column for sample analysis. All experiments were performed using a constant voltage of 20 kV with the anode at the site of the source buffer, and the cathode at the site of the destination buffer. Sample introduction was accomplished by gravity injection. In brief, one end of the capillary was placed in a vial holding the sample solution, and then the vial was raised 100 mm above the height of the opposite end of the capillary (which was placed in determination buffer) for 10 s. The sample vial was then lowered, and the capillary removed from the sample solution and placed into the source buffer vial. For all analyses, an electrolyte buffer system consisting of 10 m*M* sodium tetraborate, 50 m*M* boric acid, and 50 m*M* sodium dodecyl sulfate was used. The buffer solution was filtered through a 0.45-μm membrane filter (Millipore, Corp., Milford, MA, USA) and degassed in vacuum prior to use. The pH of the buffer was adjusted to 8.2 with hydrochloric acid (10 *M*) or sodium hydroxide (10 *M*). All assays were performed at ambient temperature with a detection wavelength of 242 nm.

Rat samples

Rats were administered a single intravenous injection of antipyrine (20 mg/kg in normal saline over 15 s) and blood samples (100 μl) were obtained through an indwelling jugular catheter prior to and at 20, 40, 60, 90, 120, 180, 240, 300, and 360 min following the administration of the dose. Blood samples were allowed to clot at ambient temperature, serum was separated by centrifugation (3000 *g* for 5 min), and samples were stored at −70°C prior to analysis (within 2 weeks).

Sample preparation

Antipyrine standards were prepared in drug-free rat serum with a concentration range of 0.5 μg/ml to 25 μg/ml. All samples were stored at −70°C prior to analysis (within 2 weeks). Serum samples were allowed to equilibrate at room temperature for 60 min before the extraction procedure was started. Samples were vortex-mixed, and a 25-μl aliquot of each sample was transferred to a polypropylene microcentrifuge tube. To each aliquot, 100 μl of cold acetonitrile containing acetaminophen (2.5 μg/ml) as internal standard were added. The mixture was vortex-mixed for 15 s, and then centrifuged at 3000 *g* for 5 min. Following centrifugation, the supernatant was removed and placed into another polypropylene microcentrifuge tube. The samples were dried under a stream of filtered air at ambient temperature for 2 h. Samples were then reconstituted with 25 μl of deionized water and analyzed. Each sample was analyzed a single time. Sample data was collected and analyzed with the use of a chromatographic computer software system (Baseline 810, Millipore, Ventura, CA, USA).

Data analysis

The number of theoretical plates per meter, N, was calculated from the equation:

$$N = 2\pi\left(\frac{t_r \cdot h}{A}\right)^2\left(\frac{1}{L}\right)$$

where t_r, h, A, and L represent the migration time (s), antipyrine peak height (μV), antipyrine peak area (μV s), and total capillary length (m), respectively [15].

Sample injection volume was determined by the following equation:

$$V = \frac{2.84 \cdot 10^{-8} \cdot H \cdot T \cdot D^4}{L}$$

where V, H, T, D, and L represent the injection volume (nl), height the sample was raised (mm), sampling time (s), capillary I.D. (μm), and the total capillary length (cm), respectively [16].

Variability in migration time was determined by the following equation:

$$\text{R.S.D. (\%)} = \frac{\sigma_{tr}}{\chi_{tr}}$$

where χ_{tr} and σ_{tr} represent the mean and standard deviation of the migration time, respectively.

The ratio of antipyrine peak height to internal standard peak height was used for the construction of standard curves and for determination of the antipyrine concentration in the analytical samples. Linearity of standard curves was determined by least-squares regression analysis. All data is represented as mean ± S.D. [R.S.D.(%)], unless otherwise stated.

RESULTS

The application of 20 kV to the buffer system resulted in a field potential of 285 V/cm with a current of 65 μA. The range of the standard curves analyzed was 0.5 to 25 μg/ml, with a limit of detection (three times baseline noise) of 0.25 μg/ml. The resulting electropherograms showed no interferences from endogenous components (Fig. 1). The injection volume was approximately 13 nl. Variability in injection volume was

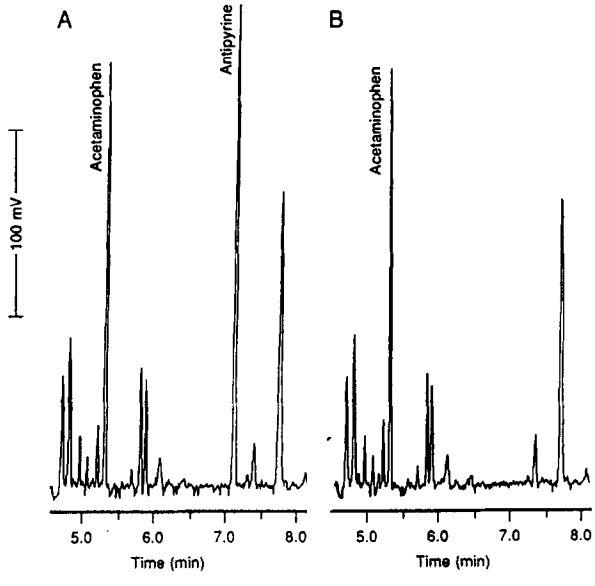

Fig. 1. Representative electropherograms of rat serum containing acetaminophen (internal standard) and antipyrine 10 μg/ml (A), and acetaminophen only (B).

TABLE I

INTRA-DAY PRECISION OF SERUM ANTIPYRINE ANALYSIS ($n = 5$)

Added concentration (μg/ml)	Peak-height ratio	Measured concentration (μg/ml)	Analytical recovery (%)
0.5	0.055 ± 0.002 (2.8)	0.46 ± 0.03 (6.3)	86.2 ± 2.4 (2.8)
1	0.106 ± 0.005 (5.2)	0.93 ± 0.03 (3.7)	78.2 ± 4.1 (5.2)
2.5	0.270 ± 0.007 (2.5)	2.40 ± 0.06 (2.7)	84.2 ± 2.1 (2.5)
5	0.565 ± 0.015 (2.6)	5.05 ± 0.12 (2.4)	87.6 ± 2.3 (2.6)
10	1.143 ± 0.014 (1.2)	10.22 ± 0.10 (1.0)	101.0 ± 1.2 (1.2)
25	2.946 ± 0.087 (0.7)	24.91 ± 0.03 (0.1)	92.9 ± 0.6 (0.7)

found to be <5%. Acetaminophen migrated through the capillary first, and was detected at 5.3 min. Antipyrine was detected at 7.2 min. The migration time R.S.D.(%) for antipyrine was 0.38% for 40 consecutive samples. The number of theoretical plates for the antipyrine analysis was 561 000 ± 79 000 plates/m.

The analysis of the intra-day variability for five standard curves is given in Table I. The slope of the UV absorption–concentration line was 0.112 ± 0.001 (0.7%), with an r^2 value of 0.9998 ± 0.0002 (0.02%). Analysis of the inter-day variability for five daily standard curves is given in Table II. The slope of the UV absorption–concentration line was 0.118 ± 0.003 (2.9%), with an r^2 value of 0.9998 ± 0.0001 (0.01%).

Fig. 2 shows the serum concentration–time profile of antipyrine following a single intravenous injection (20 mg/kg) in 10 rats.

DISCUSSION

Recent reports have demonstrated highly efficient MECC assays for amphetamine and related compounds [17], pilocarpine, isopilocarpine, and their respective carpic acids [18], and catecholamines [19]. Absolute plate numbers ranged from 20 000 to greater than 200 000. In the present assay, absolute plate numbers were approximately 400 000, thus indicating that also antipyrine can be efficiently resolved using the present techniques. Furthermore, the variability in antipyrine migration times was low with a R.S.D. of 0.38%.

Several commonly used pharmaceutical compounds were investigated for possible interference with either antipyrine or the internal standard using the present assay method; these included: benzoic acid, caffeine, furosemide, gentamicin, indocyanine green, ketamine, lido-

TABLE II

INTER-DAY PRECISION OF SERUM ANTIPYRINE ANALYSIS ($n = 5$)

Added concentration (μg/ml)	Peak-height ratio	Measured concentration (μg/ml)	Analytical recovery (%)
0.5	0.057 ± 0.005 (8.1)	0.53 ± 0.05 (8.8)	89.5 ± 7.3 (8.1)
1	0.112 ± 0.008 (7.2)	1.00 ± 0.05 (4.8)	82.4 ± 5.9 (7.2)
2.5	0.278 ± 0.016 (5.9)	2.40 ± 0.10 (4.0)	86.6 ± 5.1 (5.9)
5	0.584 ± 0.010 (1.7)	5.00 ± 0.08 (1.6)	90.5 ± 1.5 (1.7)
10	1.187 ± 0.034 (2.8)	10.10 ± 0.21 (2.1)	105.0 ± 3.0 (2.8)
25	2.946 ± 0.087 (2.9)	24.97 ± 0.07 (0.3)	98.5 ± 2.9 (2.9)

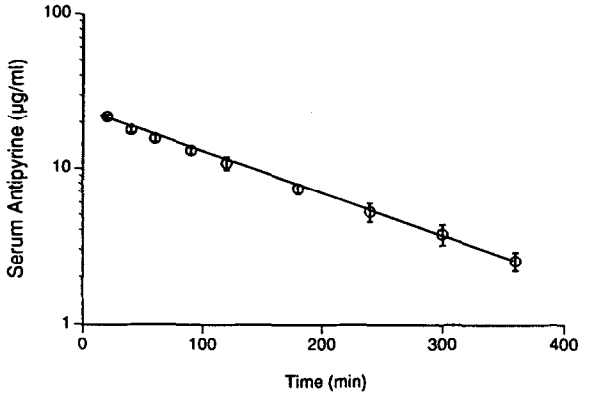

Fig. 2. Mean concentration–time profile of antipyrine in ten rats following a single intravenous injection (20 mg/kg). Samples were analyzed by MECC. Error bars indicate S.D.

caine, pentoxifylline, phenacetin, promethazine, riboflavin, sulfacetamide, sulfanilamide, theobromine, theophylline, triethanolamine, and xylazine. A number of these compounds could have been used as an internal standard, since the compounds could be resolved from antipyrine and the plasma components. Furthermore, sulfanilamide was used as an internal standard for the detection of antipyrine in human serum with the present assay method. When sulfanilamide was used for the analysis of rat serum, an additional serum component was found in the rat samples which interfered with the detection of sulfanilamide.

As can be seen from the results of the intra-day and inter-day analyses, the present assay provides reasonable precision and analytical recovery over the concentration range tested. Application of the present assay for the determination of serum antipyrine pharmacokinetics in rats is given in Fig. 1. The slope of the UV absorption–concentration curve showed little variability whether measured within each day or between days. This slope, which measures the relative response of the assay to changes in drug concentration, indicated the linearity of the present method. Furthermore, the limit of detection approaches that of liquid chromatographic methods for antipyrine. These results were obtained with a rapid, single-step deproteinization, not requiring further purification. Thus, capillary electrophoresis with a micellar buffer system can be used as a rapid, simple, and reproducible method for detecting antipyrine in serum.

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