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Short Communication

Determination of tris(hydroxymethyl)aminomethane (tromethamine) in human plasma and urine by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

Determination of tromethamine (2-amino-2-hydroxymethyl-1,3-propanediol) in human plasma and urine has been developed and validated. The method utilizes reversed-phase high-performance liquid chromatographic separation and fluorescence detection of a pre-column derivative. The assay was linear in the ranges of 1–200 μg/ml plasma and 5–500 μg/ml urine with accuracies (mean percent recovery) all within 10% of 100% and precisions (coefficient of variation) all less than 10%.

INTRODUCTION

Tromethamine (TRIS, Fig. 1), 2-amino-2-hydroxymethyl-1,3-propanediol, is a synthetic buffer commonly used to buffer aqueous solutions between pH 7 and 9 and is often used therapeutically as an alkalinizing agent for clinically manifest disorders rooted in systemic acidosis. The maximum recommended daily i.v. dose for a 70-kg adult is 970 ml of a 0.3 *M* solution (approximately 35 g). It is currently utilized in oral preparations of its gluconate at 500 mg per tablet for controlling gastric hyperacidity; it is also used experimentally as a buffering agent in various oral dosage forms to modify gastric acidity.

Tromethamine is not metabolized appreciably and is rapidly excreted preferentially in urine when administered intravenously [1]. In order to estimate its systemic absorption and elimination it was desired to conduct clinical studies to measure the plasma concentration–time profiles and urinary excretion of tromethamine. Previously published analytical methods [2–5] lacked the specificity required for urine matrices and the reliable sensitivity required for plasma samples.

A similar analytical method [6], published after the present work had been completed, demonstrated comparable sensitivity by HPLC–UV detection of the tetrabenzoylated derivative. However, the method reported here is more precise by a factor of 2, has a two order of

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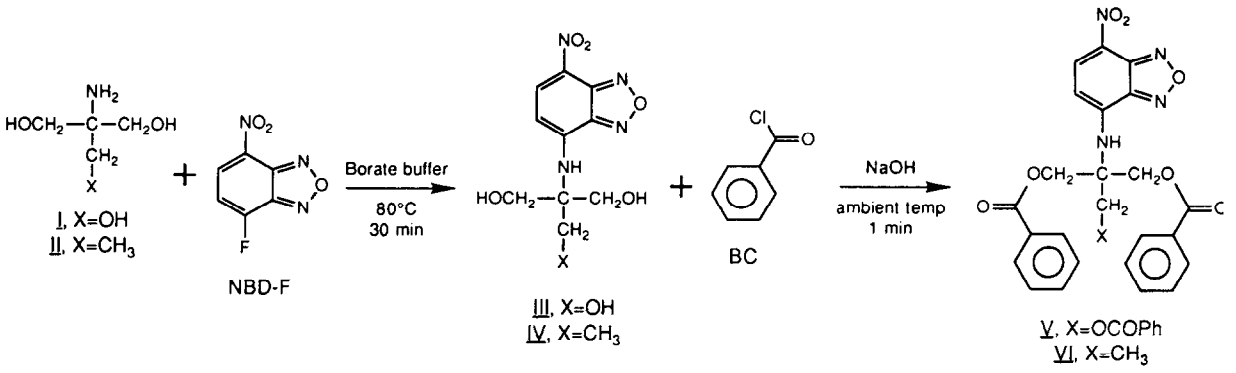

Fig. 1. Chemical structures and derivatization reaction for tromethamine and internal standard.

magnitude range of linearity, and has a shorter isocratic chromatographic run time. However, for these advantages an additional derivatization step and a fluorescence detector are required.

Preliminary results from our initial attempts in the development of an assay based on capillary gas chromatography (GC)–flame ionization detection of the tetrabenzoylated derivative of tromethamine showed that the derivative lacked sufficient thermal stability to allow quantitation below 25 μg/ml. They also demonstrated pronounced injector memory effects due to apparent adsorption of non-volatile residues from the sample matrix remaining in the injector insert. A high-performance liquid chromatographic (HPLC) method [7] using phenylboronic acid solid-phase extraction, cation-exchange separation and post-column *o*-phthalaldehyde derivatization and fluorometric detection was successful in separating the analyte and its internal standard from endogenous interferents in control urine. However, several urine samples contained interfering compounds which could not be resolved or removed.

The present method employs high-performance liquid chromatographic analysis and fluorescence detection of derivatized tromethamine extracted from plasma and urine. The derivatization method involved conversion of the amino group of tromethamine to the corresponding fluorphore (III, Fig. 1) using 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [8,9]. The intact hydroxyl groups were then exhaustively benzoylated with benzoyl chloride to the final derivative [5] (V, Fig. 1) to attain a higher specificity due to longer retention time in reversed-phase HPLC separation. 2-Amino-2-ethyl-1,3-propanediol (II, Fig. 1) was selected as internal standard (I.S.) because of its structural similarity. The method developed has been used for the quantification of tromethamine in human plasma and urine samples following oral intake of tromethamine with and without a therapeutic agent.

EXPERIMENTAL

Materials

All reagents were analytical-reagent grade. Chromatographic solvents were HPLC grade and all mobile phase solutions were filtered prior to use through Rainin 0.45-μm nylon membrane filters (Woburn, MA, USA). Standards were prepared in 20 ml plastic scintillation vials with Millipore double deionized and filtered Milli-Q water (Bedford, MA, USA). Samples were aliquoted into, derivatized, and extracted in Sarstedt polypropylene tubes (10 × 75 mm, Newton, NC, USA). Samples were placed in 2-ml Sun Brokers glass autosampler vials (Wilmington, NC, USA). Human control plasma was supplied by Sera Tec Biologicals (North Brunswick, NJ, USA) and human control urine by laboratory personnel. Benzoyl chloride (BC) was purchased from Aldrich (Milwaukee, WI, USA). NBD-F was obtained from Sigma (St. Louis, MO, USA) and used as a 4 mg/ml acetonitrile

solution. Both tromethamine and internal standard were obtained from Sigma.

Apparatus

Plasma and urine samples were vortex-mixed on a SMI Model 2600 multi-tube vortex-mixer (Emeryville, CA, USA), heated in a Lab Line Model 2097-6 multi-block heater (Melrose Park, IL, USA), and agitated during extraction on a Glas Col Model RD-350 multi-tube rotator (Terre Haute, IN, USA). The samples were centrifuged after extraction and the protein was precipitated in an IEC Model 7R refrigerated centrifuge (Needham Heights, MA, USA). The sample volume was reduced in a Speed Vac Vacuum evaporator Model SVC200H (Savant Instruments, Farmingdale, NY, USA).

Separation and quantification of tromethamine and internal standard (I.S.) derivatives was accomplished with a Perkin-Elmer Series 4 HPLC system (Norwalk, CT, USA) using a mixture of acetonitrile–0.01 *M* phosphoric acid (70:30, v/v) adjusted to pH 2.5 with 10 *M* potassium hydroxide. The flow-rate was 1.0 ml/min. The high organic mobile phase composition and low pH were required to elute both derivatives within a reasonable period of time. Shorter columns and lower organic modifier concentrations might be useful to other workers but were not examined here. A Perkin-Elmer ISS-100 autosampler, fitted with a 50-μl sample loop, filled the loop with 100 μl of sample and subsequently washed the sampling flow path with a solution of acetonitrile–0.01 *M* phosphoric acid (80:20, v/v). The chromatographic flow system consisted of a Rheodyne column inlet filter (0.5 μm, Cotati, CA, USA), a Supelco 2 cm × 4.6 mm I.D. Supelcosil octadecylsilane-bonded silica guard column (5 μm, Bellefonte, PA, USA), and a 25 cm × 4.6 mm Supelcosil octadecylsilane-bonded silica (5 μm) analytical column. Detection was performed with a Perking-Elmer Model 650–10S fluorescence spectrophotometer with the following settings: excitation at 460 nm with 9 nm slit, emission at 520 nm with a 9 nm slit. These wavelengths were found to be the maxima for excitation and emission in scans of each with the measuring cell statically loaded with sample solution. A Spectrum Model 1021A (Newark, DE, USA) noise filter/amplifier was used and set at a cut-off frequency of 0.002 and an output of 1. Raw data was collected, stored, manipulated and reported with a PE Nelson Turbochrom Data System (Cupertino, CA, USA) which samples a 1 V analog detector signal at 1 Hz. Tromethamine and I.S. derivatives were quantified by peak-height internal standardization at seven concentrations with first order linear regression curve fitting and reciprocal concentration weighing.

Sample preparation

Plasma. To a 12 × 75 mm polypropylene tube, 100 μl of plasma sample, an equal volume of working internal standard solution (400 μg/ml), and 7% perchloric acid (w/v) were added. Following vortex-mixing and centrifugation at 2000 *g* for 5 min , a 200-μl aliquot of the supernatant was withdrawn and added to 300 μl of 0.2 *M* borate buffer (pH 9.2). After brief vortex-mixing, 40 μl of NBD-F solution (4 mg/ml acetonitrile) was added and the solution was briefly vortex-mixed. The tubes with sample solution were capped and incubated in the multi-block heater for 30 min at 80°C. After cooling to room temperature in an agitated water bath for 10 min, 500 μl of 5 *M* sodium hydroxide were added, the tubes were vortex-mixed for 10 s, and 100 μl of benzoyl chloride were added followed by 1 min of vortex-mixing. The reaction mixture was then extracted with 2 ml of ethyl acetate–methanol (90:10, v/v) by rotation for 10 min and subsequently centrifuged at 2000 *g* for 5 min. The supernatant was aspirated and dried under vacuum in the Speed-Vac for one hour with compartment heating. The residue was dissolved in 1 ml of acetonitrile–0.01 *M* phosphoric acid (80:20, v/v) and 50 μl were injected on to the HPLC system.

Urine. To a 12 × 75 mm polypropylene test tube, 100 μl of urine sample was added and diluted with 400 μl of control urine. After vortex-mixing for 1 min, 200 μl of the diluted sample was withdrawn and placed in a clean tube, to which 100 μl of the working internal standard solution (200 μg/ml), 100 μl of water, and 100 μl of 0.01 *M* borate buffer (pH 8.5) were added, followed by 200 μl of the NBD-F

solution. This mixture was treated in the same way as described above for plasma.

RESULTS AND DISCUSSION

The above described method for quantification of tromethamine in human plasma and urine was optimized with regard to reaction conditions in a systematic fashion. The extent to which displacement of the fluorine from NBD-F by the primary amine of tromethamine occurs is a function of reaction-medium pH. Test samples containing 200 μl of water, 100 μl of 0.2 *M* borate buffer (pH 7–10), 200 μl of tromethamine standard (100 μg/ml water), and 20 μl of NBD-F (8 mg/ml acetonitrile) were allowed to react at 80°C for 60 min. The reaction was stopped by the addition of 50 μl of 1.0 *M* phosphoric acid followed by rapid cooling in a cryogenic acetone bath for 1 min. The formation of the intermediate product III (Fig. 1) was monitored by HPLC analysis utilizing chromatographic conditions (HPLC column and mobile phase) different from those described in the Experimental section for the final derivatives (V). A mobile phase consisting of 0.02 *M* sodium acetate–acetonitrile (70:30, v/v) was delivered through a Jones Chromatography 25 cm × 4.6 mm I.D. octadecylsilane-bonded silica column (5 μm, Denver, CO, USA). The results indicated that the maximum yield of reaction product was achieved at pH 8.5 (Fig. 2). Likewise, the effect of temperature and reaction time on the yield of

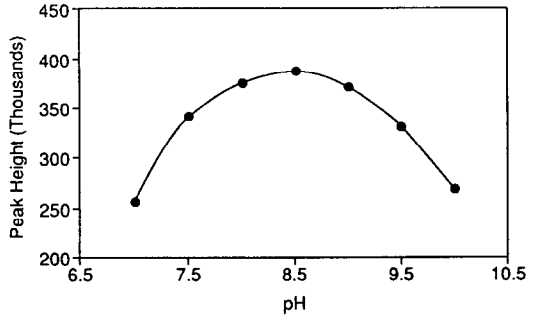

Fig. 2. pH Optimization of the reaction between tromethamine and NBD-F. The reaction mixture was prepared by adding 160 μg of NBD-F to 20 μg of tromethamine (molar ratio = 5.36:1) in 0.2 *M* borate buffer at different pH and heating at 80°C for 1 h (peak-height units are in μV).

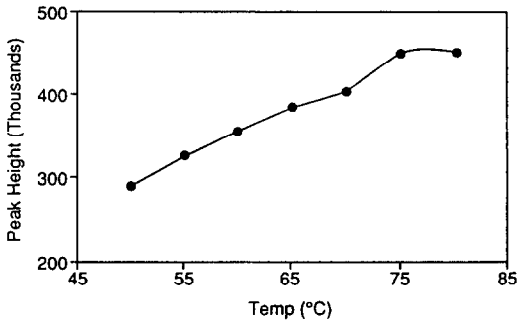

Fig. 3. Temperature optimization of the reaction between tromethamine and NBD-F. The reaction mixture was prepared by adding 160 μg of NBD-F to 20 μg of tromethamine (molar ratio = 5.36:1) in a 0.2 *M* borate buffer (pH = 8.5) and heating at different temperatures for 1 h (peak-height units are in μV).

the reaction product was studied at pH 8.5. The results given in Figs. 3 and 4 indicated that the reaction required 30 min at 80°C to be complete. Varying the molar ratio of NBD-F to tromethamine at pH 8.5 and 80°C for 30 min shows that a 5.36-fold molar excess of NBD-F is required to achieve the maximum yield (Fig. 5).

The extent to which the hydroxyl groups of tromethamine react with benzoyl chloride was optimized and checked by HPLC under the conditions described in the Experimental section. Sixty seconds at room temperature gave complete conversion and extraction of the final derivative into ethyl acetate–methanol (90:10, v/v). The volume of benzoyl chloride added

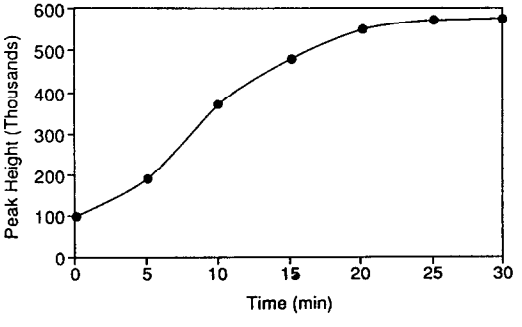

Fig. 4. Time optimization of the reaction between tromethamine and NBD-F. The reaction mixture was prepared by adding 160 μg of NBD-F to 20 μg of tromethamine (molar ratio = 5.36:1) in a 0.2 *M* borate buffer (pH 8.5) and heating at 80°C for different periods of time (peak-height units are in μV).

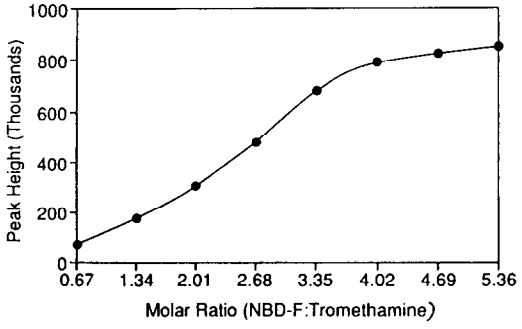

Fig. 5. Concentration optimization of the reaction between tromethamine and NBD-F. The reaction mixture was prepared by adding different molar ratios of NBD-F to 20 μg of tromethamine in 0.2 *M* borate buffer (pH 8.5) and heating at 80°C for thirty min (peak-height units are in μV).

was varied and addition of 100 μl was found to provide the highest response for tromethamine (20 μg) (Fig. 6). The decrease in detector response at benzoyl chloride volumes above 100 μl may appear somewhat surprising, departing from the expected titration-like curve. This is probably caused by hydrolysis of the excess reagent to benzoic acid giving a reduction of the basicity of the reaction medium. Interference or tailing solvent peaks due to the presence of unreacted NBD-F or its hydrolysis products were not observed. Therefore the mixture was directly injected into the HPLC system after derivatization.

Fig. 7A and B shows representative chromatograms from plasma and urine samples using the extraction and derivatization methods described above. Assay specificity was confirmed by assay-

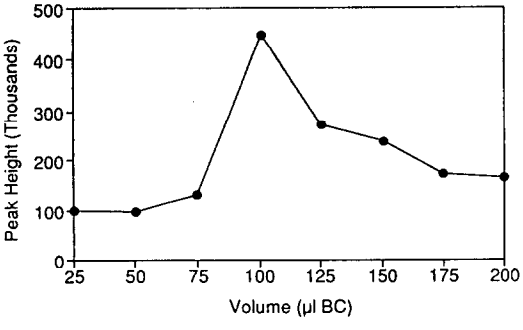

Fig. 6. Optimization of the reaction between NBD-derivatized tromethamine with different volumes of benzoyl chloride (BC) at ambient temperature for 1 min (peak-height units are in μV).

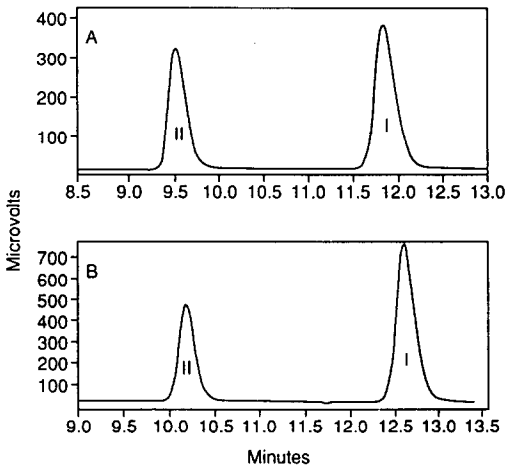

Fig. 7. Representative chromatograms of a human plasma sample (A) and a human urine sample (B) 1 h after oral administration of a 1000/1000 mg dose of tromethamine/diflunisal. Both plasma and urine samples were spiked with 10 and 20 μg of internal standard, respectively. Peaks: I = tromethamine derivative; II = internal standard derivative. Concentrations of tromethamine: *ca.* 200 μg/ml plasma and *ca.* 500 μg/ml urine.

ing the pre-dose plasma and urine samples which showed no interfering peaks eluting at the retention times of tromethamine and the I.S. derivative. The small difference in retention times of the components in the two chromatograms is due to mobile phase flow-rate differences between the two assays.

Standard curve ranges were 1–200 μg/ml in plasma and 5–500 μg/ml in urine. Intra-day and inter-day coefficients of variation (C.V., $n = 5$) were all <10% for each point of the curve. The intra-day regression statistics for both urine and plasma were determined as mean ± S.D. of five replicate curves each, and are shown in Table I. The accuracy and precision of the assay was established by preparing quality control (QC) samples at 7.5 μg and 150 μg/ml in plasma, and 45 μg and 450 μg/ml in urine. The QC samples were assayed and the calculated concentrations were 7.5 μg and 147.6 μg/ml in plasma and 43.9 μg and 444.3 μg/ml in urine, as shown in Table II.

The method described has been used to assay tromethamine concentrations in plasma and urine samples from human volunteers adminis-

TABLE I

REGRESSION STATISTICS FOR THE CALIBRATION CURVES FOR TROMETHAMINE IN HUMAN PLASMA AND URINE

Matrix	Range (μg/ml)	n	Slope	Intercept	Correlation coefficient	Standard error
Plasma	1–200	5	0.0499 ± 0.0053	0.0299 ± 0.0909	0.9986 ± 0.0022	0.1153 ± 0.0757
Urine	5–500	5	0.0202 ± 0.0006	0.0245 ± 0.0144	0.9998 ± 0.0002	0.0494 ± 0.0245

TABLE II

ACCURACY AND PRECISION FOR TROMETHAMINE IN HUMAN PLASMA AND URINE

Concentration added (μg/ml)	n	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Accuracy (mean recovery) (%)
Plasma				
7.5	5	7.5 ± 0.38	5.2	99.6
150.0	5	147.6 ± 14.55	9.9	98.4
Urine				
45.0	5	43.9 ± 0.99	2.2	97.6
450.0	5	444.3 ± 12.59	2.8	98.7

tered oral doses of tromethamine with and without a therapeutic agent (diflunical).

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