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High-performance liquid chromatographic method for the determination of tris(hydroxymethyl)aminomethane (tromethamine) in human plasma

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ABSTRACT

Determination of tris(hydroxymethyl)aminomethane (tromethamine) in human plasma involved derivatization of the amino and hydroxyl groups with a ultraviolet-absorbing chromophore followed by extraction into an organic phase. Reversed-phase high-performance liquid chromatography with gradient elution was used for the separation of the analyte from the internal standard (2,3-butanediol). The assay was linear in the range 1.0–1000.0 μg/ml of plasma and the coefficient of variation varied between 9.6 and 16.3%, whereas the accuracy varied between 90 and 108%. The limit of detection for the assay was 0.282 μg/ml. Stability of tris(hydroxymethyl)aminomethane in human plasma frozen at −20°C was studied over a period of three months and the data indicated no significant change.

INTRODUCTION

Tris(hydroxymethyl)aminomethane (THAM, Tris, tromethane, tromethamine) has been widely used as an alkalinizing agent in industry and chemistry. THAM has also found use in the correction of acid–base distortions of respiratory and metabolic origin [1–3]. Brasch *et al.* [4] studied the pharmacokinetics of THAM in healthy subjects and in patients with metabolic acidosis. Even though THAM has been used for the correction of acidosis in children and infants, to date there has been no report on the pharmacokinetics of THAM in newborn babies. Assay of THAM in plasma of neonates requires a sensitive method that uses a minimum amount of plasma.

THAM, a weak base, does not lend itself to the conventional methods of detection. The extremely hydrophilic nature of THAM does not favor its extraction from aqueous phase. Several methods for the analysis of THAM in human plasma have been reported. Some of the methods [5–8] are not sensitive enough and the others are not suitable [9] because of laborious double-extraction procedures. The method described here involves a known technique [9,10] of derivatization of the hydroxyl and amino groups with an acid chloride in the presence of a base to attach a UV-absorbing chromophore handle on the molecule to facilitate extraction and detection. However, the previously published high-performance liquid chromatographic (HPLC) method required a serum volume of 0.5 ml to give a sensitivity of 20 μg/ml [10]. A validated method for the assay of THAM using only 100 μl of human plasma and with greater sensitivity is presented here. Data indicating stability of THAM in spiked plasma

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stored at −20°C over a three-month period is also presented.

EXPERIMENTAL

Reagents and chemicals

THAM (Trizma base) and 2,3-butanediol were obtained from Sigma (St. Louis, MO, USA). Benzoyl chloride was obtained from J. T. Baker (Phillipsburg, NJ, USA). Sodium hydroxide was obtained from Fisher Scientific (Fair Lawn, NJ, USA). The solvents used were of HPLC grade and obtained from Burdick and Jackson Labs. (Muskegon, MI, USA).

Apparatus and chromatographic conditions

The HPLC apparatus was all from Shimadzu (Kyoto, Japan). A Model SCL-6B system controller was used to operate two Model LC-6A pumps, Model C-R3A integrator, Model SIL-6B autoinjector and Model SPD-6A ultraviolet spectrophotometric detector. An Omniscribe Series B-5000 strip chart recorder (Houston Instruments, Austin, TX, USA) with dual voltage pens was hooked onto the detector. The chromatographic column was an Ultrasphere octyl (100 mm × 4.6 mm I.D., particle size 5 μm) obtained from Beckman Instruments, Altex Division (San Ramon, CA, USA).

The mobile phase consisted of a constant flow-rate gradient of solution A (40:60 acetonitrile–25 m*M* potassium phosphate buffer, pH 6.5) and solution B (acetonitrile). The time program used for gradient elution is shown in the Fig. 1. The flow-rate was maintained at 3.0 ml/min. The detector wavelength was set at 237 nm and sensitivity at 0.64 a.u.f.s.

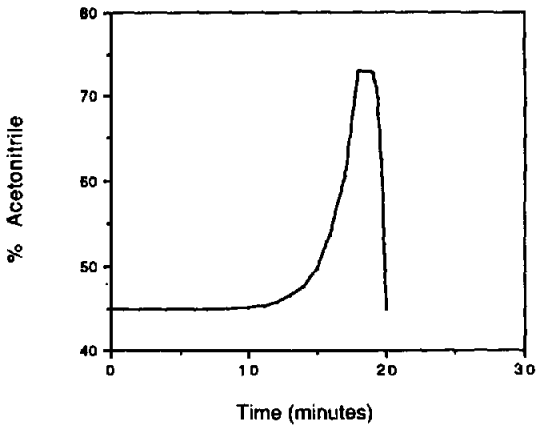

Fig. 1. Gradient elution pattern of the mobile phase.

Validation procedure

Validation of the analytical procedure included documentation of assay linearity, accuracy, precision and limit of detection. A minimum of six validation batches were run. Each batch contained a total of nine calibration standards, assayed singly per batch, at the following concentration: 0.0, 1.0, 5.0, 10.0, 25.0, 100.0, 500.0, 700.0 and 1000.0 μg THAM per ml of plasma. There were a total of six validation pools at different concentrations: low, medium and high. The validation pools had the following concentrations: 5.0, 10.0, 20.0, 50.0, 200.0 and 700.0 μg THAM per ml of plasma. The standards and validation samples were assayed in a random order. The linearity of the assay was assessed over a 1000-fold range (1.0–1000.0 μg/ml). The standard concentrations were regressed as two separate lines (1.0–25.0 and 25.0–1000.0 μg/ml), however, there was no difference in the processing of the standards and concentration of the internal standard added. A total of five standards were used for the construction of each regression line. A least-squares regression line, weighted by the inverse of peak-area ratio (THAM to internal standard) was used to predict the concentration of THAM in the validation pools. Accuracy and precision of the method were assessed from the validation pools. Percentage analytical recovery was used as a measure of accuracy and was defined as average concentration expressed as a percentage of the amount of analyte added. Precision was expressed in terms of percentage coefficient variation (C.V.), defined as standard deviation as a percentage of average computed concentration. The limit of detection was set at the lowest concentration that can be determined to be statistically significant from the analytical blank (0.0 μg/ml standard). The limit of detection was found by taking three times the standard de-

viation of the 0.0 μg/ml standard in the units of concentration.

Derivatization and extraction procedure

To 100 μl of plasma containing an appropriate concentration of THAM, a 50-μl aliquot of internal standard (2,3-butanediol; concentration 750 μg/ml of distilled water) was added. The samples were made alkaline by the addition of 200 μl of 4 *M* sodium hydroxide and vortex-mixed briefly. A 40-μl aliquot of benzoyl chloride was added to the sample and then vortex-mixed for 3 min. Extraction solvent (8 ml of 1% methanol in methyl *tert.*-butyl ether) was delivered to the samples from an oxford pipettor and extraction effected by vortex-mixing for 3 min. The tubes were then centrifuged at 1200 *g* for 5 min. The upper organic phase was transferred to clean conical tubes and evaporated to dryness in a vortex evaporator for 30 min at 55°C. The residue was reconstituted in 500 μl of methanol after equilibration to room temperature, and 10 μl of the sample were injected onto the HPLC system.

Stability study of THAM

Stability of THAM in human plasma stored at −20°C was studied at two different levels of concentration: low, 25.0 μg/ml; high, 500.0 μg/ml. Plasma samples spiked with the appropriate concentration of THAM were aliquoted into polystyrene tubes and stored along with blanks. At specified intervals of time (one, two, four, eight and twelve weeks), one set of stored spiked plasma samples was thawed and five replicates were analyzed along with replicates of identical concentrations of freshly prepared samples. The method for the analysis of THAM followed was as outlined above. Peak-area ratio (THAM to internal standard) obtained upon the analysis of freshly prepared and stored plasma samples was evaluated to assess relevant and statistically significant degradation [11].

RESULTS AND DISCUSSION

Fig. 2 shows the chromatograms of spiked plasma containing 1000.0 μg/ml THAM and internal standard (A), plasma sample spiked with 25.0 μg/ml THAM and internal standard (B) and blank plasma with no THAM or internal standard (C).

The regression statistics of the two curves generated are shown in Table I. Over the concentration range studied, *i.e.* 1.0–25.0 and 25.0–1000.0 μg/ml, the linearity was reproducibly demonstrated. It was considered necessary to regress the standard concentrations as two separate calibration curves because a single calibration curve constructed over the 1000-fold range would give very high variability at the lower concentrations and, therefore, lead to erroneous computed concentrations.

Accuracy and precision for the assay are presented in Table II. Precision, estimated as C.V.% for the spiked validation pools, ranged from 9.6 to 12.6% for the high curve and 11.4 to 16.4% for the low curve. Accuracy ranged from 90.4 to 108.0% for the high and low curve.

The limit of detection for the low curve was calculated to be 0.282 μg/ml and that for the high

TABLE I

REGRESSION STATISTICS FOR THE CALIBRATION CURVES FOR THAM IN HUMAN PLASMA

Range (μg/ml)	*n*	Slope	Intercept	Standard error of estimate	Correlation coefficient
Low curve					
1.0–25.0	7	40.47 ± 8.5	0.183 ± 0.094	0.528 ± 0.097	0.997 ± 0.001
High curve					
25.0–1000.0	7	40.15 ± 5.9	−0.607 ± 3.19	16.55 ± 9.56	0.999 ± 0.001

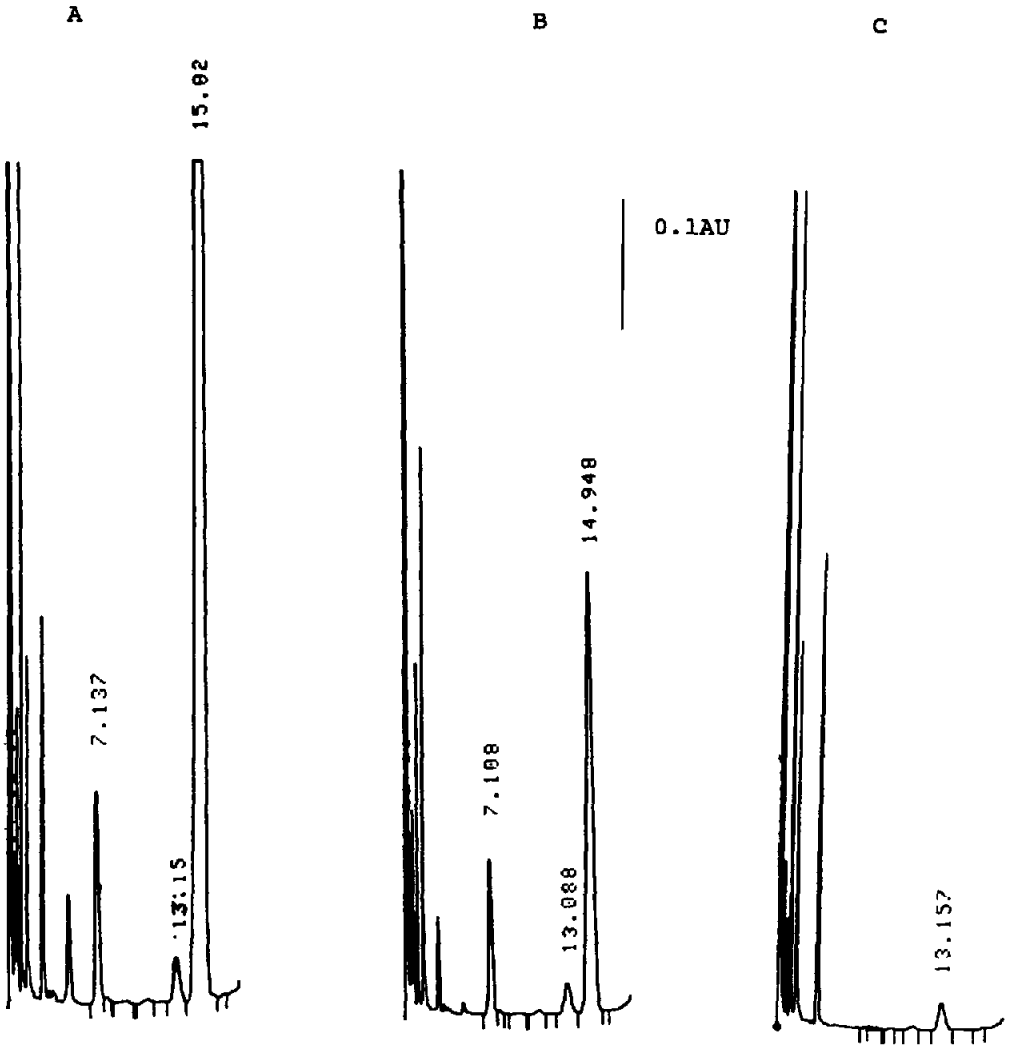

Fig. 2. Typical chromatograms of plasma spiked with (A) internal standard (7.1 min) and 1000.0 μg/ml THAM (15.0 min), (B) 25.0 μg/ml THAM and internal standard and (C) blank plasma.

TABLE II

ACCURACY AND PRECISION FOR THE LOW AND HIGH CURVE

Concentration added (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Analytical recovery (%)
Low curve				
5.0	21	4.52 ± 0.739	16.4	90.4
10.0	20	10.1 ± 1.65	16.3	101.2
20.0	24	19.4 ± 2.21	11.4	96.9
High curve				
50.0	21	45.6 ± 4.4	9.6	91.2
200.0	20	196.8 ± 24.8	12.6	98.4
700.0	18	756.7 ± 74.5	9.8	108.0

curve was 9.97 μg/ml of plasma. Therefore, the limit of detection for the assay was 0.282 μg/ml. Specificity of THAM and internal standard was demonstrated by the absence of any interfering peaks at their retention times in samples devoid of the analytes. Theoretical plates for the internal standard were 2357 (±261), whereas the capacity factor was 27.8. The same parameters for THAM could not be calculated because of the gradient elution system.

A gradient elution system was necessary because of the presence of some late-eluting peaks in the plasma. The pattern for gradient elution was optimized to obtain almost isocratic elution till THAM and internal standard had traversed approximately 90% of the column length and then the concentration of the organic phase in the mobile phase was increased to affect elution of the late-eluting peaks. An equilibrium time of 2 min was allowed before the subsequent injection.

Optimization of various steps of the reaction and extraction was carried out by varying one parameter at a time. The concentration of THAM used was 1000.0 μg/ml of plasma for most of the optimization procedures. Different amounts of benzoyl chloride were tried, and it was found that increase in benzoyl chloride from 40 to 100 μl did not change the peak height. An excess of benzoyl chloride (>200 μl) gave a white crystalline mass and completely overwhelmed the reaction. Previously, scavengers like glycine have been used to pick up the excess of benzoyl chloride in the reaction media after the completion of the reaction [10]. In this study it was not found to be necessary because sodium hydroxide reacts with benzoyl chloride to form sodium benzoate [12]. Different amounts of 4 *M* sodium hydroxide were added to the reaction media (devoid of THAM sample to represent a maximum excess of benzoyl chloride) and the absence of benzoyl chloride in the extraction media after the completion of the reaction was verified by comparison with a known peak of benzoyl chloride on HPLC. Optimization of the reaction and the extraction times was carried out from 1 to 5 min, and a 3-min reaction and extraction time was chosen as the optimum. Various extraction solvents were also evaluated (hexane, methylene chloride, methyl *tert.*-butyl ether) and did not give satisfactory results. Methanol (10, 2 and 1%), ethanol (1%), propanol (5%) in methyl *tert.*-butyl ether were also tried and 1% methanol in methyl *tert.*-butyl ether was selected to be the extraction solvent of choice based on the maximum recovery in the first extraction step.

Stability of THAM in plasma upon storage at −20°C was evaluated by the method proposed by Timm *et al.* [11]. The true percent change (Δ) in peak-area ratio over the storage period is enclosed by the lower limit and the upper limit of the confidence interval with a probability of 90%. A change in peak-area ratio during storage is statistically significant if the value of $\Delta = 0\%$ lies outside the confidence intervals. However, a significant degradation is important only when it reaches pharmacokinetically relevant proportions and a degradation of $\Delta = -10\%$ is considered to be relevant. A substance is donated as stable only if relevant degradation can be excluded with high certainty.

The summary of the plots for 90% confidence intervals over a twelve-week storage period are shown in Figs. 3 and 4. Data at one-week and two-week periods show a significant and possibly relevant decrease at a concentration of 25.0 μg/ml and a non-significant with possibly relevant decrease at 500.0 μg/ml. At the four-week period there is no significant decrease at 25.0 μg/ml with possibly relevant decrease but at 500.0 μg/ml

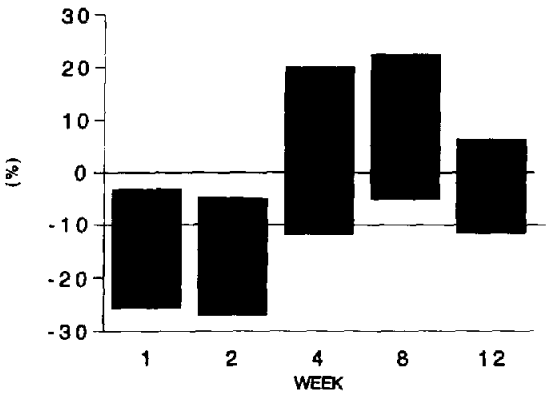

Fig. 3. Plot of 90% confidence intervals for the stability of THAM in plasma stored at −20°C. Concentration: 25.0 μg/ml.

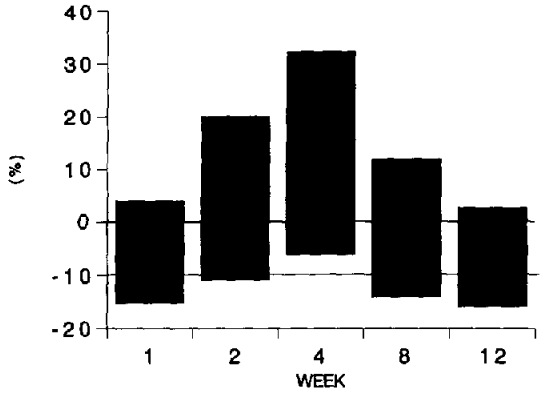

Fig. 4. Plot of 90% confidence intervals for the stability of THAM in plasma stored −20°C. Concentration: 500.0 μg/ml.

there is no significant or relevant decrease. At the eight-week period there is no significant or relevant decrease at 25.0 μg/ml and no significant decrease with possibly relevant decrease at 500.0 μg/ml. At the twelve-week storage period there is no significant decrease with possibly relevant degradation at both concentrations. Therefore, the 90% profile over a one- to twelve-week period is consistent with THAM in plasma being stable for at least twelve weeks when stored in polystyrene tubes.

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