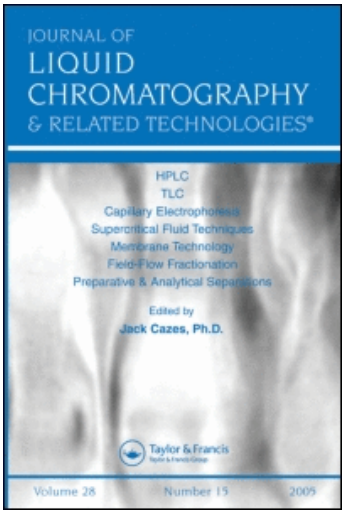

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Improved High Performance Liquid Chromatographic Method for the Determination of Tris(Hydroxy-Methyl)Aminomethane (THAM) in Human Plasma, Erythrocytes, and Whole Blood

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IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TRIS(HYDROXY-METHYL)AMINOMETHANE (THAM) IN HUMAN PLASMA, ERYTHROCYTES, AND WHOLE BLOOD

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ABSTRACT

A sensitive high performance liquid chromatographic (HPLC) assay was developed to quantitate the organic amine proton-acceptor, tris(hydroxymethyl)aminomethane (THAM) in human plasma, erythrocytes, and whole blood. An aliquot of the sample was heat evaporated (200°C for 2 h), derivatized, extracted, and then injected onto a symmetry C_8 column. The UV absorbance of the effluent was monitored at 237 nm. 2-Amino-2-methyl-1,3-propanediol was found to be an appropriate internal standard. The method has been applied to quantitate the samples from patients undergoing orthotopic liver transplantation. The assay has also been employed to assess the *in vitro* time of THAM-uptake into the erythrocytes from blood samples incubated at 37°C.

INTRODUCTION

On parenteral administration, tris(hydroxymethyl)aminomethane (THAM, alternatively referred as trisamine, tris or tris buffer), an organic amine proton-acceptor, becomes a transient component of the body buffering system. Pharmacodynamic advantage projected theoretically for THAM is, its ability to rapidly correct blood pH without increasing the sodium or carbon dioxide.[1] In practice, clinical use of THAM is recommended for correction of metabolic acidosis associated with cardiac by-pass surgery and cardiac arrest. THAM may be preferable to sodium bicarbonate for correcting severe metabolic acidosis in patients with restricted sodium or carbon dioxide elimination.[2] In patients with impaired renal function, THAM accumulation is assumed and hence, extreme caution is necessary if THAM is to be administered. However, this assumption regarding THAM accumulation remains to be substantiated by critical pharmacokinetic data. In fact, only very limited clinical pharmacokinetic data (in health and/or disease) on THAM is available.[3] Lack of suitable analytical method appears to be one of the reasons for paucity of pharmacokinetic data on THAM.[4,5]

In metabolic acidosis, hydrogen ion distributes through intracellular as well as extracellular fluid. However, significance of intracellular THAM levels to its therapeutic effect remains controversial.[3,6,7] The rate of intracellular accumulation of THAM may be very slow because, THAM is not bound to plasma proteins and has a low lipid solubility. Parallel measurements of the rate of intracellular THAM accumulation and pH may provide a better insight into the mechanism/s involved in therapeutic effects of THAM. To date, only very limited clinical data on intracellular kinetics of THAM is available from a study which used gas chromatography as the analytical technique to estimate THAM levels in erythrocytes.[3]

Being a base with extreme hydrophilic characteristic, THAM is not amenable for easy extraction and detection methods. Recently, reverse phase high-performance liquid chromatographic (HPLC) methods of THAM estimation have been developed.[4,5,8] The initial HPLC method used a C_8 column and spectrophotometric determination.[4] This assay was essentially an adoption of a known technique of derivatization of the hydroxyl and amino groups with an acid chloride in presence of a base to attach a UV-absorbing moiety on the molecule to facilitate extraction and detection. The latter modification of the method used C_{18} column and fluorescence detection to increase the precision of the method and involved an additional step of derivatization.[5] A recently described HPLC method for determination of THAM in blood involved a column which has both cation-exchange and reversed-phase retention properties (Dionex PAX-500) and pulsed

electrochemical detection.[8] This method has the advantage of minimal sample preparation. However, none of these HPLC methods were validated for assay of intracellular THAM levels.

In this paper, we describe an HPLC assay for THAM using a symmetry C_8 column and spectrophotometric UV detection. The present method involves the use of a new extraction protocol and internal standard for assay of THAM in biological samples. Besides plasma THAM level estimation, the method is also suitable for assay of THAM in erythrocytes and blood. The method is specific, reproducible, sensitive, and suitable for pharmacokinetic studies of THAM.

MATERIALS

All solvents used were HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA). Chemicals and standards were obtained from the following sources: THAM (Trizma base), 2,3-butanediol, 2-amino-2-methyl-1,3-propanediol, 2-amino-2-ethyl-1,3-propanediol, and benzoyl chloride (Sigma, St. Louis, MO, USA), sodium hydroxide, potassium phosphate monobasic (HPLC grade), and potassium phosphate dibasic (Fisher Scientific).

METHODS

Sample Preparation

The study protocol was approved by our Institution's ethical committee and informed consent was obtained from the participating patients. Blood samples were collected from patients undergoing orthotopic liver transplantation. After induction of anesthesia and tracheal intubation, patients were started on THAM infusion (0.6 mM/kg/hour) and the infusion was continued till 0.5 h after the reperfusion stage. Periodic blood samples were collected via an indwelling radial artery catheter into 4.5 mL vacutainer tubes containing 0.5 mL of 3.8 % sodium citrate.

The plasma and erythrocytes were separated by centrifugation (3500 g for 15 min). Sedimented erythrocytes were subjected to rapid freezing (on dry ice for at least 30 min) and thawing cycles for three times to rupture the membranes.[9] THAM levels in erythrocytes were expressed in μg/mL of whole blood.[10,11]

Derivatization and Extraction

The samples were processed using two methods, referred as Method-A and B. In Method-A, an aliquot of the sample (100 μL) was heat evaporated at 200°C for 2 hrs.[1,2] The dried samples were reconstituted in 100 μL of water. The samples were then mixed with an equal volume of internal standard (i.e. 100 μL of 4 mg/mL) and 200 μL of 4 M sodium hydroxide. In the next step, benzoyl chloride (50 μL) was added to the samples and then thoroughly mixed on a vortex-mixer (for 5 min). The samples were then extracted using 8 mL of 1 % methanol in methyl tert-butyl ether by vortex-mixing for 3 min. After extraction, the tubes were centrifuged at 2000 g for 10 min. The upper organic phase was transferred to clean tubes and evaporated to dryness in a water bath (55°C, over-night). The residue was reconstituted in 1 mL of methanol and 25 μL of the sample-extract was injected onto the HPLC system. In Method-B, the heat evaporation step was not followed and, but for this disparity, all other procedures were identical to that of the Method-A.

Chromatographic Conditions

HPLC was performed at ambient temperature of 22 ± 20°C using a multi-solvent delivery system (600 E, Waters Assoc., Milford, MA, USA). The set-up was equipped with an auto sampler (WISP, Waters Model 712), a tunable absorbance detector (Waters, Model 486), and a data module (Waters, Model 745). The mobile phase was composed of 25 mM potassium phosphate buffer pH 7.0 (solution A) and acetonitrile (solution B). The flow rate of the mobile phase was 1 mL/min. Before use, the mobile phase was filtered (0.45 μm membrane filter, Millipore, Bedford, MA, USA) and degassed. For degassing the mobile phase, vacuum, and ultrasonic power (Astrason Ultrasonic Cleaner, Model 7, Heat System Ultrasonics, Farmingdale, NY, USA) were applied simultaneously for about 30 seconds. Mobile phase components were kept degassed by continuous sparging with helium during chromatography. Chromatographic separation was achieved using a Waters symmetry C_8 column (100Å, 5μm, 3.9 x 150 mm) protected with Waters Sentry universal guard column. The UV absorbance detector was operated at a wavelength of 237 nm and sensitivity of 0.001 a.u.f.s.

Calibration Plot and Quantitation

Data for standard calibration plots were established by spiking blank samples with THAM at increasing concentrations and proceeding as described

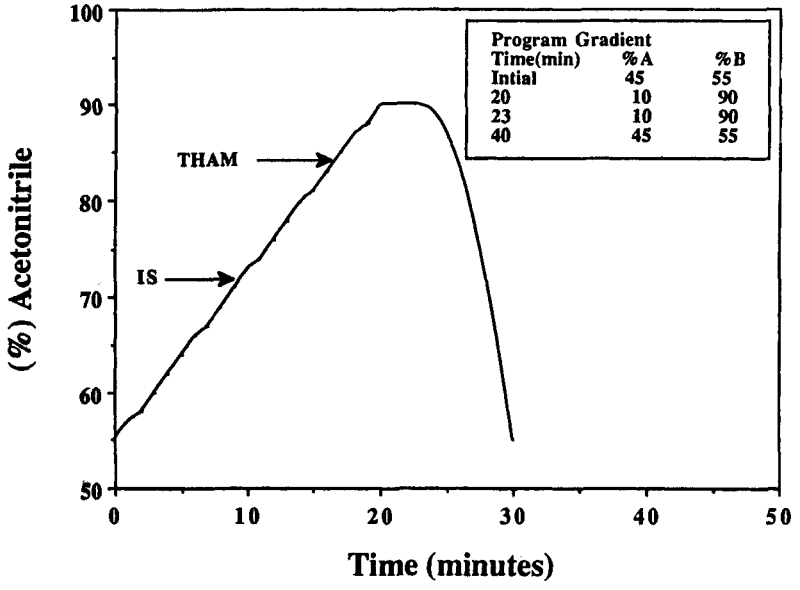

Figure 1. Gradient elution pattern of the mobile phase. The inset table gives the program gradient used for the mobile phase composed of 25 mM potassium phosphate buffer (A) and acetonitrile (B). Column was equilibrated for 20 min (with the initial condition) before injection of next sample. Arrows indicate the time of 2-amino-2-methyl-1,3-propanediol (IS) and THAM elution.

above. The plots were constructed by linear regression of, the peak-area ratio of the spiked concentration to internal standard (drug/IS). The limit of detection (LOD) for the assay was determined by replicate measurements of the corresponding blank matrix and calculating the concentration equivalent of 3 standard deviations for the analyte that can be distinguished from zero.[13] Calibration graphs were made on different days using freshly prepared mobile phases for testing the inter assay reproducibility from the replicate samples spiked with THAM.

For recovery experiments, blank samples (1 mL) were spiked with THAM (10, 20, 60 or 80 μg) and 400 μg of the internal standard, 2-amino-2-methyl-1,3-propanediol. These spiked samples were derivatized, extracted and assayed as described above.

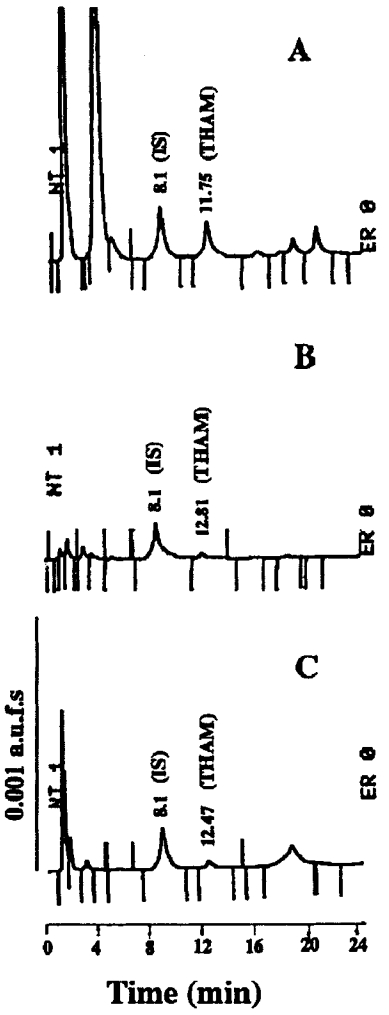

Figure 2. Representative chromatograms of plasma (A), erythrocyte (B) and blood (C) samples. Internal standard (IS): 2-amino-2-methyl-1,3-propanediol.

RESULTS AND DISCUSSION

Optimal separation of the THAM peak was achieved with a constant flow rate gradient of solution A (25 mM potassium phosphate buffer, pH 7.0) and acetonitrile (solution B) (Figure 1). Representative chromatograms obtained

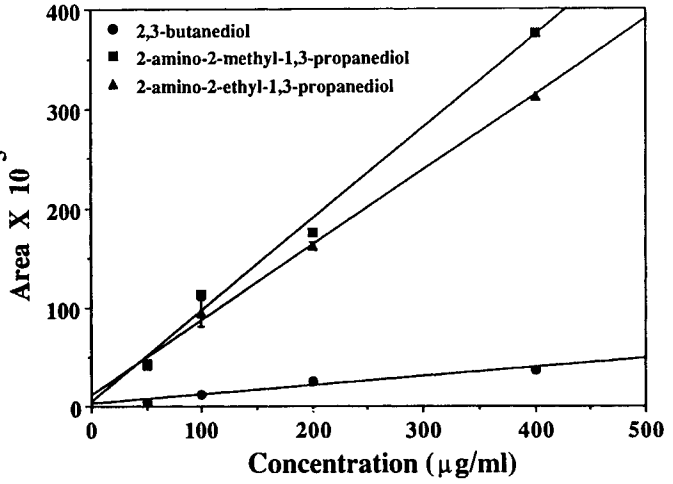

Figure 3. Plot of peak area as a function of the amount of internal standard added prior to derivatization. Note: higher peak area for 2-amino-2-methyl-1,3-propanediol compared to either 2,3 butanediol or 2-amino-2-ethyl-1,3-propanediol.

Table 1

Calibration Parameters of Standard Plots in the Analytical Range of 0.1 - 100 μg/mL of THAM Spiked Blood, Plasma, and Erythrocyte (n=3)

Method	Matrix	Quantitation Linearity (y - mx + c)	r2
Method -A	Plasma	y=0.0061 (±0.0001)x+0.0034 (±0.0029)	0.9991±0.0004
	Erythrocyte	y=0.0055 (±0.0001)x+0.0071 (±0.0013)	0.9952 ±0.0036
	Blood	y=0.0065 (±0.0002)x+0.0131 (±0.0003)	0.99476±0.0022
Method-B	Plasma	y=0.0035 (±0.0002)x+0.0121 (±0.0014)	0.9989±0.0004
	Erythrocyte	y=0.0036 (±0.0002)x-0.0046 (±0.0019)	0.9966±0.0023
	Blood	y=0.0040 (±0.0002)x+0.0108 (±0.0021)	0.9782±0.0169

y = Peak area ratio THAM/IS; x = amount injected; c = intercept with y-axis; m = integrator response factor. Standard deviation is given in parentheses.

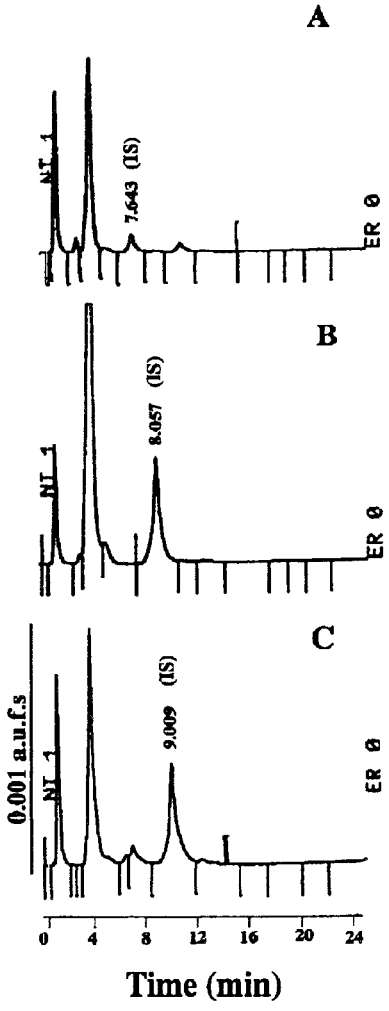

Figure 4. Typical chromatograms showing the retention time for 2,3 butanediol (A), amino-2-methyl-1,3-propanediol (B) and 2-amino-2-ethyl-1,3-propanediol (C) for the three putative internal standards (IS).

when derivatized extracts of plasma, erythrocyte and blood are injected is shown in Figure 2. The retention time of THAM in different matrices expressed in min (mean ± C.V. %, n = 16) was: plasma 11.75 ± 0.61, erythrocytes 12.81 ± 0.92 and blood 12.46 ± 1.2. Regardless of the matrix used, the average relative retention time of THAM to IS was ~ 1.5.

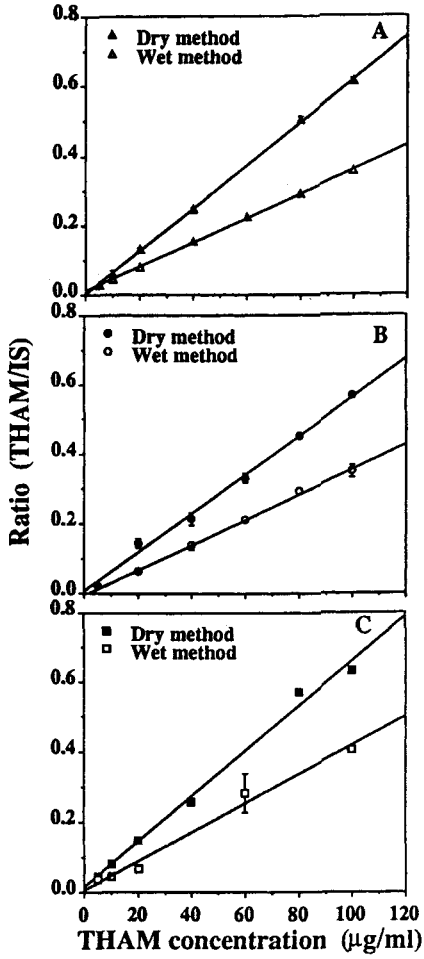

Figure 5. Calibration plots of the ratios of the area of THAM/ Internal standard for plasma (A), erythrocytes (B) and blood (C).

Based on the chemical structure of the analyte, the internal standards used in the earlier HPLC methods for THAM were, 2,3 butanediol[4] and 2-amino-2-ethyl-1,3-propanediol.[5] In the present experimental paradigm, both of these internal standards yielded a minor peak at the time point which could interfere with the THAM peak. Therefore, suitability of 2-amino-2-methyl-1,3-propanediol as an internal standard was evaluated. In the concentration range of 50 - 400 μg/mL, 2-amino-2-methyl-1,3-propanediol had a higher yield of

Table 2

Within-Day and Day-to-Day Reproducibility for the Determination of THAM Using Method-A for Spiked Plasma, Erythrocyte, and Blood Samples (n = 3)

Parameter	Matrix	Concentration Added (μg/mL)	% Recovery ± C.V %
Intra-assay	Plasma	20	102.3 ± 1.4
		80	101.3 ± 1.1
	Erythrocyte	20	113.5 ± 1.2
		80	100.6 ± 0.6
	Blood	20	103.2 ± 0.3
		80	107.9 ± 0.2
Inter-assay	Plasma	20	104.7 ± 2.6
		80	102.1 ± 1.1
	Erythrocyte	20	114.6 ± 2.0
		80	100.7 ± 1.7
		20	103.4 ± 0.3
	Blood	80	107.9 ± 0.1

derivatization compared to either 2,3 butanediol or 2-amino-2-ethyl-1,3-propanediol (Figure 3). The retention time in min (mean ± C.V.%, n=8) for these three compounds were, 7.64 ± 0.78 (2,3-butanediol), 8.06 ± 0.53 (2-amino-2-methyl-1,3-propanediol) and 9.01 ± 0.39 (2-amino-2-ethyl-1,3-propanediol) (Figure 4).

The decreasing yield of the ethyl versus methyl homologue of internal standard points to steric hindrance in the reaction. Based on these results, 2-amino-2-methyl-1,3-propanediol was considered as an appropriate internal standard.

The calibration plots were linear over the concentration range of 0.5 - 100 μg/mL and the regression equation showed correlation coefficients of greater than 0.9940 (Table 1). Calibration plots of Method-A yielded steeper slope than plots from Method-B (Figure 5). Using Method-A, LOD for THAM in different matrices expressed as μg/mL was as follows: blood 0.56, plasma 0.48

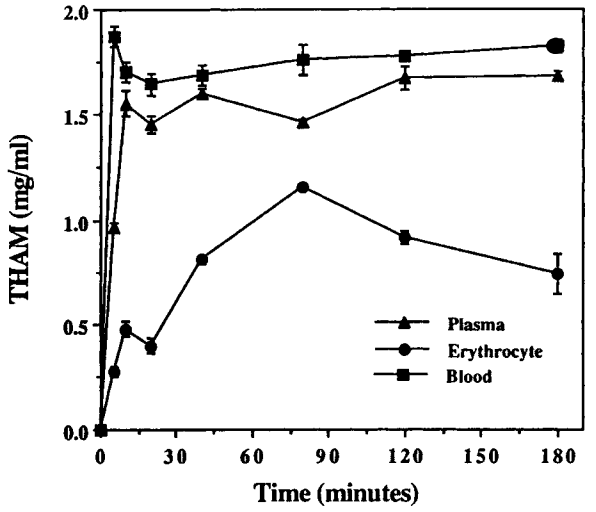

Figure 6. Distribution of THAM when the blood samples (n = 3) spiked (1.8 mg/ kg) and incubated *in vitro* at 37°C. At the time points shown in the figure, aliquot of samples were taken and THAM was assayed in all three matrices.

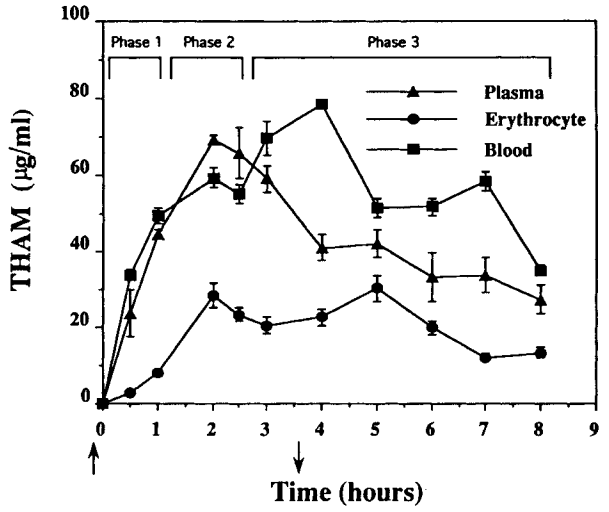

Figure 7. Mean THAM levels (± SD) in patients receiving THAM to correct acid base balance during orthostatic liver transplantation (n = 3). Arrows on the abscissa indicate the duration of THAM infusion. Phase 1-3 refers to the phases of the transplant surgery. Phase 1 is recipient hepatic dissection, Phase 2 is anhepatic (Veno-Veno bypass) and Phase 3 is the neo-hepatic (reperfusion) stage.

Table 3

Within-Day and Day-to-Day Reproducibility for the Determination of THAM Using Method-B for Spiked Plasma, Erythrocyte, and Blood samples (n = 3)

Parameter	Matrix	Concentration Added (μg/mL)	% Recovery ± C.V %
Intra-assay	Plasma	10	100.1 ± 0.3
		60	102.1 ± 0.1
	Erythrocyte	10	93.8 ± 3.9
		60	100.5 ± 1.2
	Blood	10	96.3 ± 13.1
		60	101.5 ± 9.4
Inter-assay	Plasma	10	97.7 ± 0.7
		60	102.0 ± 0.2
	Erythrocyte	10	88.0 ± 7.4
		60	101.0 ± 3.2
	Blood	10	87.4 ± 14.5
		60	98.1 ± 9.5

and erythrocytes 0.33. In Method-B, corresponding values for different matrices were, blood 0.77, plasma 0.81, and erythrocytes 0.89. The intra-assay precision (C.V.%) for 10 and 60 μg/mL THAM in Method-A ranged from 0.2 to 13.1 while the inter-assay precision was in the range of 0.1 - 14.5 (Table 2).

In Method-B, the intra-assay precision (C.V.%) for 20 and 80 μg/mL THAM was in the range of 0.2 - 2.6 while the inter-assay precision was in the range of 0.1 - 1.7 (Table 3).

The C.V.% of the analytical recovery for 10 and 60 μg/mL ranged from 0.2 to 4.3 in the Method-A while the range was 0.3 to 11.6 for Method-B (Table 4).

The assay method developed by us was employed to quantitate the THAM levels from clinical samples as well as from an *in vitro* study designed to assess the time course of THAM uptake into erythrocytes. When the *in vitro* sample of THAM in blood (1.8 mg/ mL) was incubated at 37°C, the uptake of THAM

Table 4

Recovery of THAM in Methods A and B for Spiked Plasma, Erythrocyte, and Blood Samples*

Method	Matrix	Concentration Added (μg/mL)	Analytical Recovery % ± C.V. %
Method-A	Plasma	20	103.8 ± 4.0
		80	101.6 ± 2.7
	Erythrocyte	20	114.3 ± 4.3
		80	100.6 ± 3.5
	Blood	20	103.2 ± 0.4
		80	107.8 ± 0.2
Method-B	Plasma	10	98.8 ± 4.3
		60	102.0 ± 0.3
	Erythrocyte	10	89.8 ± 11.6
		60	100.6 ± 6.4
	Blood	10	85.4 ± 9.9
		60	98.2 ± 8.4

* Values reported are mean ± C.V. % (n = 3). Coefficient of variation (Precision) = [SD/Mean] X 100. Analytical recovery (Accuracy) = [Found/Added] X 100.

by erythrocytes appeared to peak at 80 min (Figure 6). While analyzing the data from patient samples, we found the results could be made more reliable if the data of the individual patient is normalized by subtracting "basal" value obtained for that patient in the pre-THAM sample. Even though the reason/s for the "noise" and its wide variations is not apparent, it could be ascribed to concomitant multiple medications which these severely ill, anesthetized patients might have been receiving. Figure 7 illustrates the time course data from patients receiving THAM to correct acid base balance during orthostatic liver transplantation.

CONCLUSIONS

The analytical method reported here allow for the quantitative and reproducible analysis of THAM from plasma, erythrocytes, and whole blood samples. The method has sufficient sensitivity to characterize the pharmacokinetics of THAM.

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