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Note

Gas chromatographic method for the quantitative determination of tris(hydroxymethyl)aminomethane in plasma

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Tris(hydroxymethyl)aminoethane (THAM) is frequently used in the clinic as an alkalinizing agent. Pediatricians use THAM for the treatment of respiratory failure (respiratory distress syndrome) and certain other conditions accompanied by a low plasma pH and a large negative base excess [1]. Although THAM is a comparatively non-toxic agent, there are some dangers connected to the administration of large doses [2, 3]. A pharmacokinetic study of THAM in (often premature) infants would therefore be helpful in establishing a safe dosage regimen. A spectrophotometric method for the determination of THAM in plasma has been reported [4]. However, this method is not sensitive enough if the plasma samples are small (50—100 μl); nor are other analytical methods reported in the literature suitable [5—9]. In the proposed method the three hydroxyl groups and the primary amine group of the THAM molecule are benzoylated and the benzoylated product is extracted with an organic solvent mixture; after concentration by evaporation the organic phase is analyzed by gas chromatography. 1,2,6-Hexanetriol was found to be an appropriate internal standard.

MATERIALS AND METHODS

THAM (primary standard) was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). 1,2,6-Hexanetriol (HEX) was obtained from Carbide and Carbon Chemicals (New York, N.Y., U.S.A.) All of the other solvents and reagents used were of analytical grade.

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Gas chromatography

A Varian Aerograph Model 2700 gas chromatograph equipped with a flame ionization detector was used. The glass column (3 ft. × ¼ in. O.D.) was packed with 1% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The packed column was conditioned overnight at 300° (40 ml/min nitrogen flow), and silylated at 250° by injecting five portions of 10-μl Silyl 8 (Pierce, Rockford, Ill., U.S.A.). The operating conditions were: injection port temperature, 290°; column temperature, 255°; detector temperature, 290°; carrier gas (nitrogen) flow-rate, 40 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 350 ml/min.

Preparation of reference compounds

The benzoylated products of THAM (B_4-THAM) and of HEX (B_3-HEX) were prepared using the method as described by Bighley et al. [10] for the preparation of the reaction product of pentaerythritol with *p*-methoxybenzoyl chloride.

B_4-THAM.

B_4-THAM was prepared by reacting 0.025 mole THAM in 50 ml pyridine with 0.10 mole benzoyl chloride at room temperature for 16 h. The reaction mixture was poured into 200 ml of ice-water and extracted with two successive 200-ml portions of ethyl acetate. The combined ethyl acetate extract was washed successively with 5% sodium bicarbonate, 10% hydrochloric acid, water and saturated sodium chloride solution, and then dried over anhydrous sodium sulphate. The ethyl acetate extract was reduced to dryness in vacuo. The oily residue was washed with warm hexane; after cooling the mixture the hexane was decanted and the residue dried at room temperature under a nitrogen flow. Upon standing the oil turned into a white, crystalline solid (yield 96%), m.p. 109—111°; the nuclear magnetic resonance (C^2HCl_3) spectrum was consistent with the expected structure of B_4-THAM, δ 7.1—8.0 (m, 21H, aromatic and amino), 4.8—5.1 (s, 6H, CH_2O); single spot at R_F = 0.66 on a 0.25-mm silica gel GF_{254} plate (New England Nuclear, Boston, Mass., U.S.A.) developed with benzene—methanol (9:1).

Analysis. Calculated for $C_{32}H_{27}O_7N$: C, 71.50; H, 5.06; O, 20.83; N, 2.61. Found: C, 71.27; H, 4.98; O, 20.91; N, 2.55.

B_3-HEX.

B_3-HEX was prepared by reacting 0.025 mole HEX with 0.075 mole benzoyl chloride and following the procedure as described for the preparation of B_4-THAM. After several days the oily product turned into a white crystalline solid (yield, 96%); m.p. 55—57°; the nuclear magnetic resonance (C^2HCl_3) spectrum was consistent with the expected structure of B_3-HEX, δ 7.1—8.2 (m, 15H, aromatic), 5.2—5.8 (m, 1H, CHO), 4.1—4.7 (m, 4H, CH_2O), 1.4—2.2 (m, 6H, CH_2); single spot at R_F = 0.76 (chromatographic system, see under B_4-THAM).

Analysis. Calculated for $C_{27}H_{26}O_6$: C, 72.63; H, 5.87; O, 21.50. Found: C, 72.61; H, 5.79; O, 21.34.

Procedure

A 100-μl volume of plasma in a centrifuge tube was mixed with 2 ml 0.003% HEX in water and 0.8 ml 10% trichloroacetic acid and centrifuged at 5000 *g* for 15 min. The supernatant was decanted into a 15-ml tube closed with a PTFE-lined screw cap; 0.8 ml 10% sodium hydroxide solution and 200 μl benzoyl chloride were added, and the contents of the tube were mixed for 2.5 min with a vortex-type mixer. A 2-ml volume of hexane—chloroform (3:2.05) was added and the mixture was vortexed for 1.5 min. After addition of 0.6 ml 10% sodium hydroxide solution and mixing for 15 sec the tube was centrifuged for 3 min (5000 *g*). The organic (upper) phase was transferred to a weighed centrifuge tube and the hexane and chloroform were evaporated under a nitrogen stream at 40°. The residue, consisting mainly of benzoyl chloride (15—30 mg) and the benzoylated products, was mixed with 60—150 μl hexane—chloroform (3:2.05). A 2.5-μl portion of the resulting solution, containing approximately 20% benzoyl chloride, was injected into the gas chromatograph.

Calibration curve

Pooled plasma samples (100 μl) were spiked with 0.5—10.0 μg THAM and treated as indicated under *Procedure*. Following chromatography the heights of the peak corresponding to B_4-THAM and to B_3-HEX were measured. The peak-height ratio was plotted against the amount of THAM in the sample.

RESULTS AND DISCUSSION

THAM has very low oil—water partition coefficients in organic solvent—water systems and can not be extracted as such from the plasma. A derivatization method similar to that described by Bighley et al. [10] was therefore developed. HEX was selected as an internal standard because it also is derivatized to yield a product with increased oil—water partition coefficient.

Fig. 1 shows typical chromatograms obtained with blank plasma, plasma to which THAM and HEX were added, and plasma analyzed as described under *Procedure* from a patient treated with THAM. The reaction between THAM and benzoyl chloride was virtually complete under the experimental conditions; the yield of B_4-THAM did not change when the reaction mixture was shaken for 30 min or 1 h at 40°. The yield of B_3-HEX was slightly decreased after shaking for 1 h at 40°. Precipitation of the plasma proteins with trichloroacetic acid was necessary because without such treatment a plasma peak appeared in the chromatogram, interfering with the B_3-HEX peak.

After mixing the reaction mixture for 2.5 min the contents of the tube were acidic and alkali had to be added in order to prevent the extraction of disturbingly large amounts of benzoic acid into the organic phase. The amount of benzoyl chloride used in the reaction proved to be critical. After completion of the reaction procedure the organic phase must contain a slight excess of benzoyl chloride, which is needed to prevent degradation of the benzoylated products; however, too much benzoyl chloride would result in a large final volume, because the injected sample should not contain more than 20% ben-

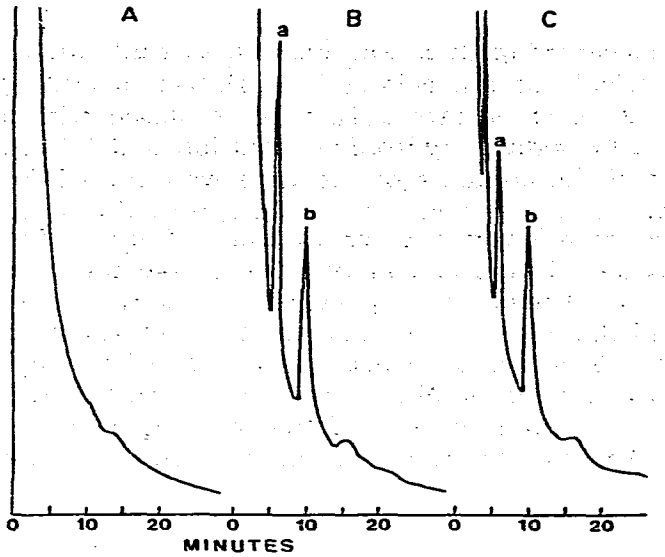

Fig.1. Chromatograms obtained from an extract of blank plasma (A), an extract of blank plasma with THAM (a) and HEX (b) (B), and an extract of plasma from a patient who received THAM (a) to which HEX (b) has been added (C). Attenuation, $128 \cdot 10^{-12}$.

zoyl chloride, higher concentrations yielding too broad a solvent front. The concentration of benzoyl chloride in the injected sample influenced the peak-height ratio of the B_4-THAM and the B_3-HEX peaks, without a concomitant change in the retention times. The ratio (B_4-THAM/B_3-HEX) increased with increasing benzoyl chloride concentrations (0—10%) in the injected solution. At higher concentrations of benzoyl chloride (10—20%) the peak-height ratio remained fairly constant. This change in the peak-height ratio was mainly the result of a decrease in the height of the B_3-HEX peak, possibly caused by an on column decomposition of a part of the injected B_3-HEX. The B_4-THAM peak was much less affected, and was somewhat higher in the presence of benzoyl chloride, owing to a decrease in the extent of tailing.

Before injection of the samples two 5-μl injections were done with a concentrated solution of B_4-THAM (10 mg/ml) and B_3-HEX (20 mg/ml) in hexane—chloroform (3:2.05), containing 20% benzoyl chloride. The time between injection of the samples was 30 minutes.

The recovery of the compounds from plasma as compared with water was 89% (coefficient of variation 8%, $n = 7$) for THAM and 62% (coefficient of variation, 12%, $n = 7$) for HEX. After a single extraction of the reaction mixture with hexane—chloroform (3:2.05), no B_4-THAM and B_3-HEX could be detected in the aqueous phase. The peak-height ratios at different concentrations and the standard deviations are presented in Table I.

The sensitivity of the method was sufficient for the determination of 0.3—0.5 μg THAM in 100 μl plasma. The plasma levels of an infant (boy, 2860 g) who had received an intravenous bolus dose of 109 mg THAM was found to be 430 μg after 30 min and 86 μg/ml after 4.5 h. The method appears to be quite sufficiently sensitive for the quantitative determination of therapeutic levels of THAM in small amounts of plasma.

TABLE I

PEAK-HEIGHT RATIO OF B_4-THAM TO B_3-HEX, STANDARD DEVIATION AND COEFFICIENT OF VARIATION OBTAINED WITH PLASMA SAMPLES (100 µl) CONTAINING 5—100 µg/ml THAM

Concentration (µg/ml)	Peak-height ratio (mean)	Number of determinations	Standard deviation	Coefficient of variation (%)
5	0.091	6	0.021	23.5
25	0.488	6	0.042	8.6
50	0.973	6	0.065	6.7
75	1.465	6	0.088	6.0
100	2.008	6	0.090	4.5

REFERENCES

1 J. Strauss, Pediatrics, 41 (1968) 667.
2 G.G. Nahas, Clin. Pharmacol. Ther., 4 (1963) 784.
3 N.R.C. Roberton, Arch. Dis. Child., 45 (1970) 206.
4 A. Kanarek and M. Tal, Anal. Biochem., 57 (1974) 78.
5 H. Rosen, Ann. N.Y. Acad. Sci., 92 (1961) 414.
6 S. Linn and M. Roberts, Ann. N.Y. Acad. Sci., 92 (1961) 419.
7 L.C. Clark, Jr., Ann. N.Y. Acad. Sci., 92 (1961) 687.
8 B. Zaar and A. Grönwall, Scand. J. Clin. Lab. Invest., 13 (1961) 588.
9 J. Strauss, K. Bernath and S.A. Kaplan, Proc. Soc. Exp. Biol. Med., 113 (1963) 58.
10 L.D. Bighley, D.E. Wurster, D. Cruden-Loeb and R.V. Smith, J. Chromatogr., 110 (1975) 375.