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# DETERMINATION OF PLASMA TRANEXAMIC ACID USING CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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## SUMMARY

A procedure is described for the determination of plasma tranexamic acid concentrations using cation exchange high-performance liquid chromatography with fluorescence detection following post-column derivatisation with *o*-phthalaldehyde. The chromatographic conditions were optimised with respect to detector performance and the method applied to measuring the plasma tranexamic acid levels of patients in a double-blind trial.

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## INTRODUCTION

Tranexamic acid (TXA), the *trans*-structure of 4-aminomethyl cyclohexane carboxylic acid, is a potent inhibitor of plasminogen activation to plasmin and, consequently, an effective antifibrinolytic agent [1, 2].

A major problem in the treatment of patients who have suffered a subarachnoid haemorrhage is the likelihood of a rebleed [3]. Recently there has been a resurgence of interest in using TXA to treat patients who have suffered a subarachnoid haemorrhage in an effort to prevent the fibrinolysis of the vascular clot and hence a rebleed [4, 5]. The effectiveness of TXA in this context is disputed, however, because of a number of contradictory reports [6–10].

Overall, different groups are seen to employ a variety of TXA doses by various routes of administration in patient treatment [4]. This may account for the differences reported by the groups in the effectiveness of TXA to prevent rebleeding.

Plasma levels of TXA, rather than quantities of TXA given, may therefore be more useful in assessing the efficiency of TXA in preventing a rebleed. Methods exist for the determination of plasma TXA by electron-capture gas–liquid chromatography (GLC) [11], high voltage paper electrophoresis [12] and high-performance liquid chromatography (HPLC) of fluorescamine derivatives [13]. We report here the measurement of TXA by utilising HPLC with post-column derivatisation to fluorescent products and illustrate the optimisation of the method. The technique is simple and suitable for the routine monitoring of TXA over the range of 5 to 100 mg/l of patients plasma, a range which occurs during the treatment of subarachnoid haemorrhage [4].

## EXPERIMENTAL

### *Chemicals*

Tranexamic acid was supplied by Aldrich (Gillingham, U.K.) while the *o*-phthalaldehyde was purchased from Sigma London (Poole, U.K.). Helium was obtained from British Oxygen (Harlow, U.K.). The 4-aminomethyl bicyclo-(2,2,2)octane-1-carboxylic acid (AMBOC) was donated by KabiVitrum (Uxbridge, U.K.). All other chemicals were obtained from BDH (Poole, U.K.).

### *Apparatus*

The HPLC system consisted of a Pye LC3 high pressure pump (Pye Unicam, Cambridge, U.K.), a Rheodyne Model 7010 injection valve fitted with a 100-$\mu$l sample loop (Rheodyne, Berkeley, CA, U.S.A.) and an analytical column of 250 mm × 4.6 mm I.D. stainless-steel packed with 10-$\mu$m diameter Nucleosil SA (HPLC Technology, Macclesfield, U.K.) preceded by a Brownlee guard column with 10-$\mu$m cation-exchange resin contained in a 30 mm × 4.6 mm I.D. cartridge (HPLC Technology). All other chromatography components were supplied by Anachem (Luton, U.K.). Derivatisation reagent was delivered by a Watson Marlow 18 pump (Watson Marlow, Falmouth, U.K.) with 1.29 mm I.D. tubing (Elkay Laboratory Products, Basingstoke, U.K.) via a 12-ml I.V. air column damper. Derivatives were detected by a Perkin-Elmer MPF 3

fluorimeter (Perkin-Elmer, Beaconsfield, U.K.) using 410-nm excitation and 450-nm emission wavelengths at a bandpass of 14 nm, and a 200-$\mu$l flow-cell.

*Solvents and reagents*

The mobile phase consisted of 0.1 *M* trisodium citrate (adjusted to pH 4 with hydrochloric acid)- methanol (98:2). Caprylic acid was added (0.1 ml/l) as a bactericidal agent. The derivatisation reagent [14, 15] consisted of 0.7 *M* potassium borate buffer (adjusted to pH 9.5 with potassium hydroxide) containing 2 g/l EDTA and to which was added 800 mg/l *o*-phthalaldehyde in 10 ml methanol and 2 ml/l mercaptoethanol. Prior to the addition of the mercaptoethanol both mobile phase and derivatisation reagent were filtered through 0.45-$\mu$m pore diameter membrane filters (Millipore, London, U.K.) before use. Mobile phase was degassed with helium prior to use after which it was usable for up to one week. Water used in the preparation of the mobile phase and derivatisation reagent was deionised and doubly glass-distilled.

*Standards*

Stock solutions of 250 mg/l internal standard, AMBOC and 1 g/l TXA were prepared in deionised, doubly glass-distilled water.

*Plasma extraction and chromatography*

Plasma was obtained from whole blood containing 3.13 g/l citrate. Plasma containing TXA was stored at −20°C [12, 16]. We also found it to be stable for 24 h at 4°C prior to use. To extract TXA from plasma, 0.1 ml internal standard and 0.7 ml distilled water were added to 1 ml plasma. Then 0.2 ml 4 *M* perchloric acid was added and after vigorous shaking the mixture was allowed to stand at room temperature for 10 min, again, with occasional shaking, followed by centrifugation at 3000 *g* for 5 min. The supernatant liquid was then injected into the HPLC system.

Mobile phase flow-rate was maintained at 1.4 ml/min to give a pressure of 140 bar at a column temperature of 26°C. Derivatisation of eluted compounds was achieved by the immediate mixing of the column eluent with derivatisation reagent delivered at 0.7 ml/min (free flow). This occurred across the head of a T piece (0.8 mm I.D.) followed by 1 m × 0.3 mm I.D. coiled tubing [14] carrying the mixture to the fluorimeter.

Standard curves of 0—100 mg/l TXA in plasma were prepared from standard TXA concentrations in plasma against the ratio of TXA standard peak height to internal standard (25 mg/l) peak height. In all cases the volume of TXA standard, internal standard and distilled water added to 1 ml pooled plasma (from patients known not to have been treated with TXA) totalled 0.8 ml. The quantity of TXA in a treated patient's plasma was obtained by calculating the ratio of the peak height of TXA to internal standard and reading off the corresponding TXA concentration.

## RESULTS

Separation of the TXA and AMBOC was directly affected by changes in the solvent pH with both compounds eluting more rapidly as the pH increased. A

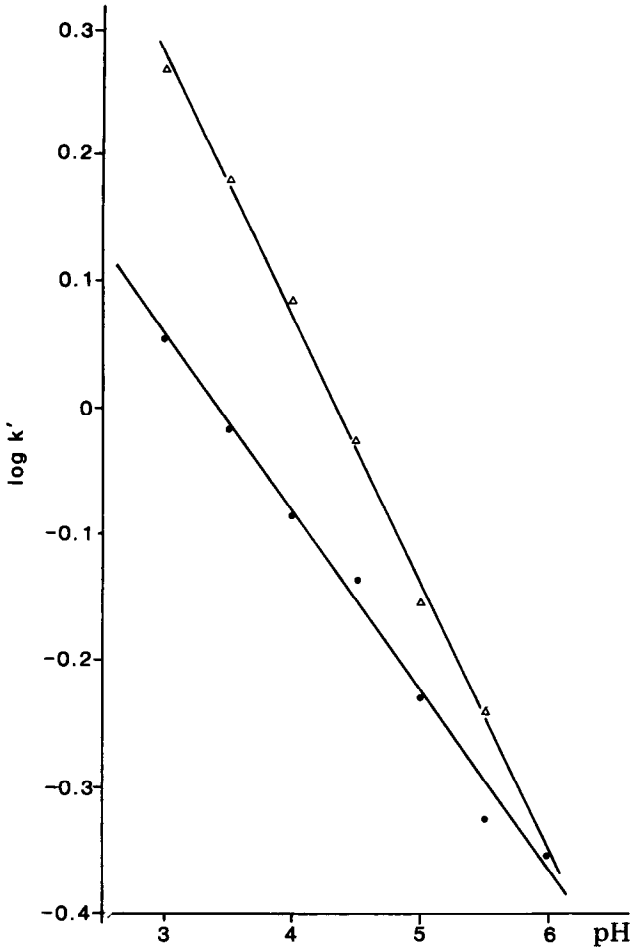


Fig. 1. Retention of TXA (•) and AMBOC (△) on a 250 mm × 4.6 mm Nucleosil 10 SA column with the Brownlee guard column 30 mm × 4.6 mm cation cartridge versus solvent pH. Solvent consisted of 0.1 *M* trisodium citrate adjusted to required pH with hydrochloric acid. No methanol was added.

comparison of the log capacity ratio ($k'$) against pH (Fig. 1) showed that the change in elution times increased more rapidly for AMBOC than with TXA. The optimum pH was taken as 4. Although better separation appears to occur at pH 3 total separation of the two compounds was achieved at pH 4 with a resolution factor (*Rs*) of 2.4 and shorter retention times. Considerably greater peak heights can be achieved by increasing the pH of the solvent but resolution between TXA and AMBOC is rapidly lost as the pH increases.

Increasing the concentration of methanol in the solvent (pH 4) decreased the retention times of TXA and AMBOC with the capacity ratios for AMBOC falling slightly more rapidly than those of TXA (Fig. 2). A methanol concentration of 2% was used hereafter as this was found to give a resolution factor (*Rs*) of 2.2, good peak height and a short retention period of 7 min free from interference by substances carried at the solvent front.

The linearity of the detection system was demonstrated by injecting known

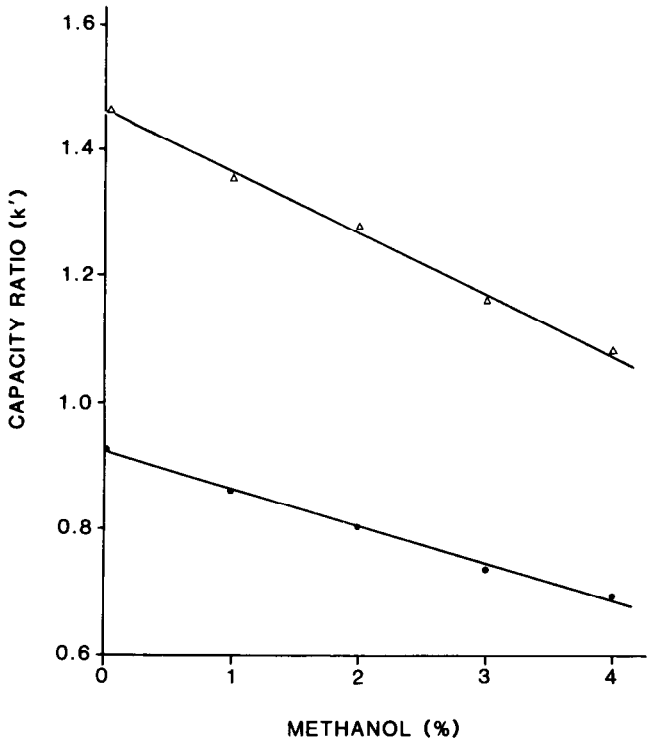


Fig. 2. Retention of TXA (•) and AMBOC (△) on a 250 mm × 4.6 mm Nucleosil 10 SA column with the Brownlee guard column 30 mm × 4.6 mm cation cartridge versus methanol concentration. Solvent consisted of 0.1 *M* trisodium citrate, pH 4.

amounts of TXA with 25 mg/l AMBOC into the liquid chromatograph. Response, determined as peak height ratios, was shown to be linear over the range 1—100 mg/l TXA investigated with a correlation coefficient ($r$) of 0.9997. Under these optimal conditions and with detector sensitivity increased to give noise levels of 3% full scale deflection (f.s.d.), TXA levels as low as 10 μg/l were detected at a signal-to-noise ratio of 10:1.

The linearity of the extraction procedure and HPLC was verified by extracting known amounts of TXA together with 25 mg/l AMBOC from pooled plasma and injecting the supernatant fluid into the liquid chromatograph. Peak height ratios, when determined, were shown to be linear over the range 1—100 mg/l TXA with a correlation coefficient ($r$) of 0.996. Mean recoveries of 72.8% for TXA and 48% for AMBOC were calculated. Precision was evaluated for both intra- and interassays (Table I) as the coefficient of variation (C.V.) for each concentration of TXA added.

TXA resolved from all the common amino acids which, with the exception of arginine, eluted from the column before TXA (Table II). Most of the amino acids, it appeared, would form part of the peak associated with the solvent front. Histidine had the lowest resolution factor (1.71) against TXA but was still completely separated. Arginine resolved from TXA but did overlap AMBOC against which it had a resolution factor of 1.1. While the two components were readily distinguishable, any large quantity of arginine in the

TABLE I

INTER- AND INTRA-ASSAY PRECISION FOR THE DETERMINATION OF TXA BY HPLC

Coefficients of variation were calculated from the peak height ratios given between known TXA concentrations and 25 mg/l AMBOC.

| Concentration TXA acid (mg/l) | Intra-assay | | Inter-assay | |
|---|---|---|---|---|
| | *n* | C.V. (%) | *n* | C.V. (%) |
| 1 | 6 | 9.49 | — | — |
| 5 | — | — | 7 | 10.39 |
| 10 | 6 | 3.23 | 15 | 9.96 |
| 20 | 6 | 10.00 | 15 | 7.05 |
| 30 | 6 | 9.25 | 15 | 7.28 |
| 40 | 6 | 3.88 | 15 | 6.67 |
| 50 | 6 | 4.74 | 15 | 6.85 |
| 60 | 6 | 6.57 | 5 | 7.34 |
| 70 | 6 | 6.01 | 5 | 6.56 |
| 80 | 6 | 2.24 | 5 | 9.60 |
| 90 | 6 | 6.01 | 5 | 7.75 |
| 100 | 6 | 3.72 | 5 | 8.29 |

TABLE II

RESOLUTION FACTORS ($R_s$) FOR AMINO ACID AGAINST TXA

Resolution factors were obtained by comparing equal peak heights of the compounds.

| Amino acid | Elution time (min) | Resolution factor ($R_s$) |
|---|---|---|
| Glutamic acid | 4.0 | 4.71 |
| Cysteic acid | 4.05 | 4.57 |
| Aspartic acid | 4.1 | 4.43 |
| Glutamine | 4.15 | 4.29 |
| Threonine | 4.2 | 4.14 |
| Serine | 4.2 | 4.14 |
| Tryptophan | 4.2 | 4.14 |
| Asparagine | 4.25 | 4.0 |
| Lysine | 4.3 | 3.86 |
| Alanine | 4.3 | 3.86 |
| Glycine | 4.3 | 3.86 |
| Tyrosine | 4.4 | 3.57 |
| Valine | 4.4 | 3.57 |
| Methionine | 4.5 | 3.29 |
| Leucine | 4.65 | 2.86 |
| Isoleucine | 4.65 | 2.86 |
| Ornithine | 4.7 | 2.71 |
| Phenyl alanine | 4.75 | 2.57 |
| Histidine | 5.05 | 1.71 |
| Tranexamic acid | 5.65 | — |
| Internal standard | 6.65 | 2.22 |
| Arginine | 7.3 | 3.0 |

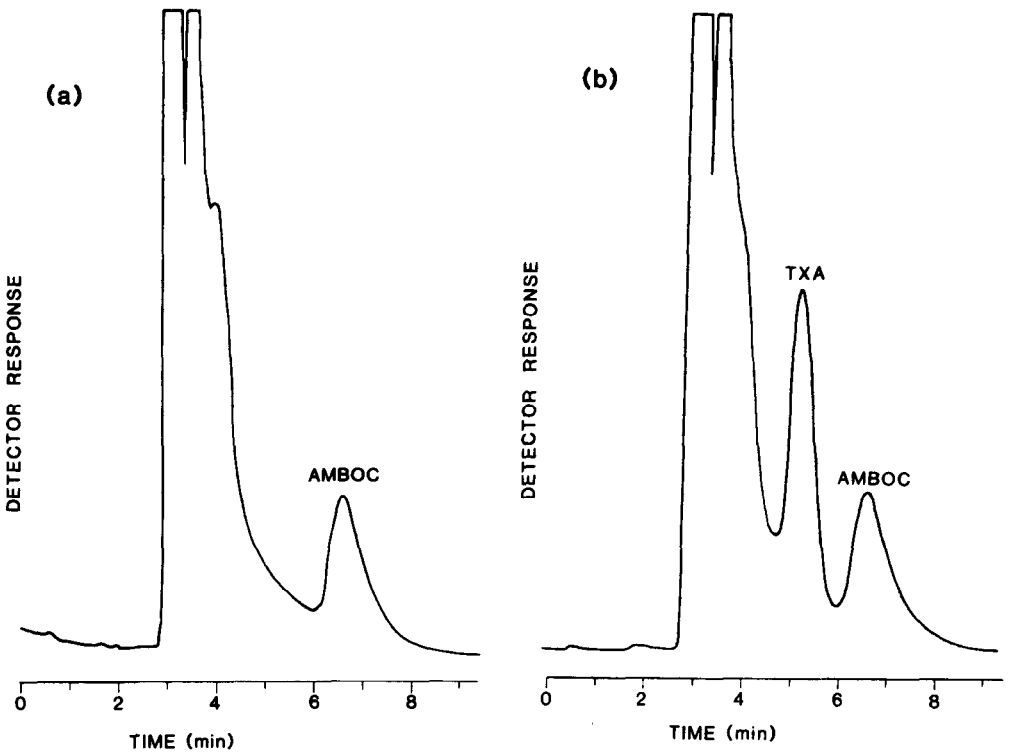


Fig. 3. Chromatograms of HPLC assay of patients plasma to which 25 $\mu$g AMBOC had been added to 1 ml of plasma. (a) Before the administration of TXA; (b) 2 h after the administration of 1.5 g TXA given orally 4-hourly showing plasma levels of 24.5 mg/l TXA. Solvent consisted of 0.1 *M* trisodium citrate, pH 4, containing 2% methanol, flow-rate 1.4 ml/min on a 250 mm × 4.6 mm Nucleosil 10 SA column with the Brownlee guard column 30 mm × 4.6 mm cation cartridge.

plasma would have interfered with the internal standard. However, the linearity of the extraction procedure and HPLC suggested that any possible interfering substances were not present in sufficient amounts to affect the assay.

In a double-blind trial of TXA in 100 patients all had a plasma sample taken before being given either TXA or placebo (Fig. 3). It was subsequently shown that none of these samples had detectable levels of TXA present. Of those samples obtained after the administration of TXA or placebo the method was 100% correct in determining which patients had been given TXA, levels varying from 6.4 to 89 mg/l plasma of TXA depending on the point reached in the trial.

## DISCUSSION

Improving the retention times and peak shapes of amino acids by the manipulation of solvent pH or methanol concentration has been previously reported [17—19]. The optimal conditions reported here for solvent pH and methanol concentration give an elution order for amino acids which is very similar to those reported by other authors [18, 19]. More importantly, good

selectivity between TXA and AMBOC is obtained and between these and most amino acids. An exception is the partial interference of arginine with the AMBOC peak. This, together with the relatively poor recovery of AMBOC, suggests that although AMBOC is a good internal standard in the GLC determination of TXA [11], using an alternative internal standard for the HPLC assay could be beneficial. Indeed, the internal standard ratio method, having a correlation coefficient of 0.996, appears to offer few advantages against a direct comparison of peak height to standard curve with a correlation coefficient of 0.991.

The linearity and precision of the detection system and extraction procedure give an assay for a range of TXA plasma levels that is very reproducible. Certainly the performance of this post-column derivatisation method compares with that of the reversed-phase separation of TXA—fluorescamine derivatives described by Lacroix et al. [13]. The two methods employ similar working ranges of TXA concentrations in plasma with comparable intra-day precision and linear detector response. The sensitivity of the detection system following post-column derivatisation corresponds to that previously reported [14], is considerably greater than that of pre-column derivatisation systems [13] and is far in excess of requirements for plasma TXA measurements giving an opportunity for much smaller sample volumes to be employed and longer column life to be obtained. Inter-day precision values for the pre-column method [13], obtained over a ten-day period, appear to surpass those of the post-column system reported here. The latter, however, were obtained at intervals over a twelve-month period of plasma TXA determinations and consequently the values should not be related.

A previous problem encountered using the *o*-phthalaldehyde derivatising reagent was the gradual deterioration of the buffered mixture of a period of one week with an accompanying increase of a brown colouration [14]. Adding EDTA during the preparation procedure decreases the base-line noise and appears to stabilise the reagent such that it can be stored refrigerated for several months if necessary.

Fodstad et al. [4] suggested that the contradictory reports of the effectiveness of TXA in preventing aneurysmal rebleeding may be due to the different doses and different routes of administration employed. Following the drug regime employed by Fodstad et al. [4] a comparable range of plasma TXA levels was determined. Consequently, the simple but sensitive, selective and reproducible method reported here may be of use in correlating plasma levels of TXA with its putative clinical actions.

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