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Note

Rapid chromatographic technique for the determination of ε-aminocaproic acid in physiological fluids

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ε-Aminocaproic acid (EACA) is an antifibrinolytic agent, whose chemistry, mechanism of action and clinical properties have been reviewed recently[1]. During therapy with EACA, it is useful to have a rapid, accurate assay for the determination of EACA levels in plasma, urine and cerebrospinal fluid. Although a number of analytical methods for EACA determination have been reported, all require considerable time and effort, and a more practical method is required for determinations in the various physiological fluids.

Initial studies in this laboratory indicated that EACA was assayed readily in plasma, cerebrospinal fluid, urine or tissue extracts using the single-column Efron buffer system[2] on the Technicon NC-1 amino acid analyzer. Since this procedure requires 21 h for complete separation, we developed a rapid short-column method which involves separation of EACA from the other amino acids present in physiological fluid samples, using ninhydrin reactivity for detection. The method requires less than 2 h for analysis and has sufficient precision to follow EACA levels in blood, urine, cerebrospinal fluid and erythrocytes during a course of therapy.

MATERIALS AND METHODS

The standard ε-aminocaproic acid used in this study was obtained from Lederle, Pearl River, N.Y., U.S.A., (Amicar®), while other amino acid standards were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). No other ninhydrin-positive material was noted in the EACA samples.

Plasma samples were deproteinized by the addition of 50 mg solid sulfosalicylic acid per milliliter of plasma, followed by removal of the precipitated protein by centrifugation. Urine was deproteinized by the addition of 30 mg solid sulfosalicylic acid per milliliter of urine, and any precipitate formed was removed by centrifugation. Cerebrospinal fluid was deproteinized by the addition of 3 mg solid sulfosalicylic acid per milliliter of cerebrospinal fluid. Erythrocyte extracts were prepared by the method of Levy and Barkin[3]. In routine assays, 0.4 ml of deproteinized plasma, 0.005 to 0.010 ml of urine, 1.0 ml of cerebrospinal fluid, and 1 ml of red cell extract are placed on the column.

A jacketed 35×0.6 cm column (Metaloglass, Boston, Mass., U.S.A.) packed

with Technicon Chromobeads B (Technicon, Tarrytown, N.Y., U.S.A.) was used in this procedure. The column temperature was maintained at 65°, with a buffer flow-rate of 0.5 ml per minute maintained by a high-pressure pump (Milton Roy, Philadelphia, Pa., U.S.A.). The column eluate was reacted with ninhydrin reagent[4] using a standard NC-1 amino acid analyzer detection system (shown schematically in Fig. 1). This system includes a 20-min dwell-time in the 95° heating bath, and a colorimeter set at 570 nm with a 15-mm flow-cell.

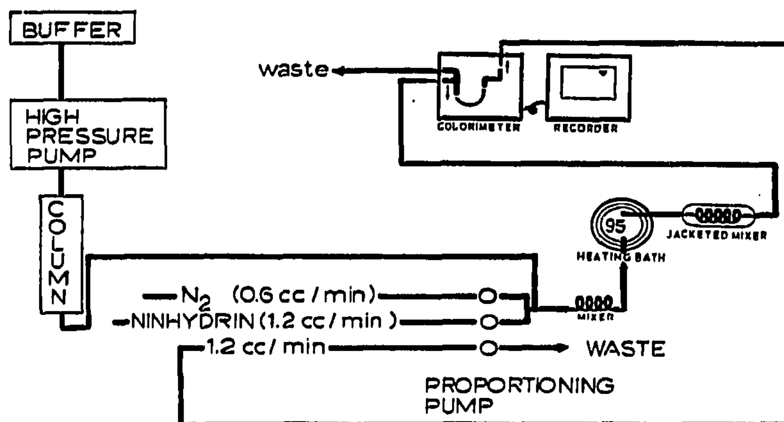

Fig. 1. Schematic diagram showing the ninhydrin detection system of the NC-1 analyzer used.

Samples were applied to the column in a solution containing a final concentration of 3.5% sulfosalicylic acid, and the EACA and other amino acids were eluted with 1:2 mixture of 0.05 *M* citrate buffer, pH 2.875, and 0.267 *M* sodium citrate buffer, pH 4.74. These were the initial and final buffers described by Efron[2] for the analysis of physiological fluids on the NC-1 analyzer. Other analyzer resins such as Spherix xx8-60-0 (Phoenix, Gardiner, N.Y., U.S.A.) were also satisfactory. Upon elution of EACA, the column is immediately regenerated by the addition of 2 ml of 2 *N* NaOH to the column, followed by the eluting buffer.

Since the analytical system has a dwell-time of 20 min in the 95° heating bath, the procedure may be shortened by beginning the column regeneration as soon as the EACA peak begins to appear on the recorder. A fresh sample may be applied to the column as soon as the starting buffer has traveled two-thirds of the way down the column (noted by the color change in the resin).

RESULTS AND DISCUSSION

The column system exhibited optimal resolution at a column temperature of 65°, although adequate resolution can be obtained from 50°–70°. The buffer flow-rate through the column may be increased to 0.75 to 1.0 ml/min if rapidity is required, although some skewing of peaks may result at increased flow-rates. The resolution and typical elution patterns obtained in the urine and plasma of subjects treated with EACA are shown in Fig. 2. The calibration curves obtained when standard amounts of EACA were added either to plasma and urine samples and then sub-

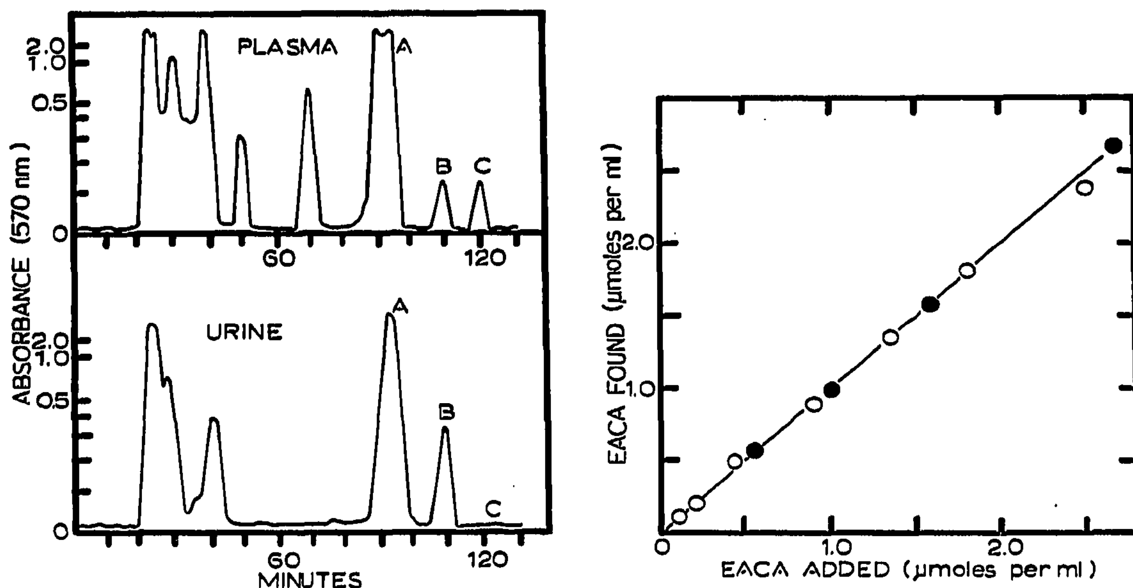

Fig. 2. Typical elution profiles of plasma and urine samples from a patient undergoing EACA therapy. Peak A contains ammonium ions, ornithine, lysine and histidine; peak B, EACA; and peak C, arginine. Arginine is not detected in urine due to the extremely low levels present in this volume of urine.

Fig. 3. Recovery of EACA added to physiological fluid samples. ○, Plasma; ●, urine.

jected to the entire deproteinization and analytical procedure are shown in Fig. 3, demonstrating excellent recovery of EACA.

Since EACA is not an α-amino acid, the color constant obtained after reaction with ninhydrin varies considerably with changes in the precise reaction time. Thus, in contrast to color constants obtained for α-amino acids, substantially different constants were obtained for EACA on each of our four NC-1 amino acid analyzer detection systems. This was true even when the same batch of buffer and ninhydrin was used for all analysis. Thus differences in color constants resulted from slight differences in the flow-rate through the heating bath of each analyzer. The color constant obtained on each analyzer, however, was highly reproducible, and once the appropriate color constants had been obtained, replicate analyses using different analyzers agreed well.

No detectable quantities of EACA were found in plasma, red cells, urine or cerebrospinal fluid samples of subjects not treated with EACA. Subjects undergoing EACA therapy had plasma levels in the range of 20 to 200 µmoles/dl, cerebrospinal fluid levels of 0.6 to 25 µmoles/dl, and red cell levels of 5 to 75 µmoles/dl. The EACA levels in 24-h urine samples obtained were extremely high, indicating that most EACA may be excreted unchanged in the urine.

Although a variety of analytical procedures for EACA have been reported, all have a number of disadvantages. Buyske and Colucci[5] use a method requiring diazomethane, a toxic and explosive material. The high-voltage electrophoretic method of Sjoerdsma and Hanson[6] does not readily resolve EACA from other amino acids.

The method of McNicol *et al.*[7] depends upon resolution of EACA from other amino acids on ion-exchange resin-loaded paper, but is time consuming and lacks the precision of the present method. Skoza and Johnson[8] use a method requiring removal of α-amino acids by chelation with copper, removal of amines by ion exchange, followed by reaction of EACA with dinitrofluorobenzene and spectrophotometric determination of the dinitrophenylated product. Methods based on antifibrinolytic properties of EACA are sensitive[9], but may be subject to errors on the part of the operator as well as interference from naturally occurring antifibrinolytic agents. The IR-120 column chromatographic method of Takada *et al.*[10] neither lists experimental conditions nor resolves EACA from other amino acids. Moreover, sample preparation is time consuming and requires numerous steps subject to sample loss. Stegemann[11] has reported a column chromatographic procedure requiring three days. EACA has also been determined by electrophoresis on aluminium oxide thin-layer plates followed by conductimetric titration[12], by thin-layer chromatography using silica gel G[13], and by paper chromatography[14]. Gas chromatographic methods necessitating considerable sample preparation time[15,16] have also been reported. No method approaches the one described in ease of sample preparation, sensitivity, reliability and rapidity of assay. The method described requires 2 h for analysis after receipt of the physiological fluid sample, and has sufficient precision to follow EACA levels in blood, urine, cerebrospinal fluid and erythrocyte extracts during a course of EACA therapy.

The value of short-column methods for the determination of specific compounds in a busy clinical laboratory has been well documented[17,18]. We have found that the short-column method described is easily carried out during the time interval required for the regeneration of the long column used for complete amino acid analysis of physiological fluid samples on the NC-1 analyzer. This permits us to perform a number of EACA determinations per day on our NC-1 analyzers in addition to physiological fluid amino acid analysis. This greatly expands the capacity of our present analyzer.

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