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Note

Analysis of γ-carboxyglutamate

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γ-Carboxyglutamate (GLA) has been found to be present in many proteins, including prothrombin[1]. Although acid labile, GLA has been determined by using either cation- or anion-exchange resins[2–5]. The existing analytical procedures, however, have certain deficiencies; the cation-exchange procedure allows GLA to come in contact with low-pH buffers[2] and the anion-exchange method[5] is prone to reagent degassing in the analyzer flowlines. Although the Technicon AutoAnalyzer used in the present work is equipped with a de-bubbler for the removal of gas before entry into the colorimeter cuvette, it cannot cope with the excess gas generated with the reagent formulations used in the anion-exchange procedure.

This communication describes modifications of the anion-exchange method to give a procedure satisfactory for use with a Technicon AutoAnalyzer. The method is capable of separating GLA from all the other amino acids except taurine.

EXPERIMENTAL

The Technicon AutoAnalyzer, Model AAA-1, had been modified to allow the column eluent to go direct to the reaction coil[6], eliminating the eluent aliquot sampling procedure in the original instrument. The reaction bath was replaced by a reaction chamber[7], set at 68°, a lower temperature than customary for amino acid analysis.

A column of Aminex A-28 (0.9 × 17 cm) anion-exchange resin (CH_3COO^-) was used and operated at 30 or 35°. The elution buffer was 0.3 *M* sodium acetate at pH 4.7. At a rate of 60 ml/h, the overall analysis time was 75 min. No column regeneration was attempted between analyses.

The ninhydrin reagent contained 3 l of methyl cellosolve and 1 l of deionized water to which 60 g of ninhydrin and 10 ml of titanous chloride solution were added in order. The reagent was prepared and stored under a nitrogen atmosphere.

Standard amino acid solutions and GLA solutions for application to the column were prepared either in pH 2.2 sodium citrate or in pH 4.7 sodium acetate buffer. The prothrombin alkaline hydrolysis (20 h at 110°) was carried out *in vacuo* using 0.2 ml of 15% sodium hydroxide solution for 0.6 mg of prothrombin. The hydrolyzates were adjusted to pH 4.7 by the addition of 0.5 ml of 1.8 *M* acetic acid before analysis[5].

The sample of ammonium γ-carboxyglutamate was kindly supplied by Dr. John

Suttie of the University of Wisconsin and was obtained from Dr. I. G. Young, Australian National University, Canberra. Plasma, barium eluate, containing prothrombin was obtained from Sigma (St. Louis, Mo., U.S.A.).

RESULTS AND DISCUSSION

Initially the γ-carboxyglutamate determinations were carried out using the buffered ninhydrin reagent of Spackman *et al.*[8]. The column operating temperature was 30°. Fig. 1a shows a chromatogram obtained with this reagent. The baseline printout is unstable and difficulty is experienced in obtaining the correct baseline at the commencement of the analysis. This instability is brought about by reagent degassing in the analyzer flowline. Although the analyzer is equipped with a debubbler, more gas is generated than can be effectively removed. The temperature of the reaction chamber was lowered to 68° to further minimize gas production in the flowline.

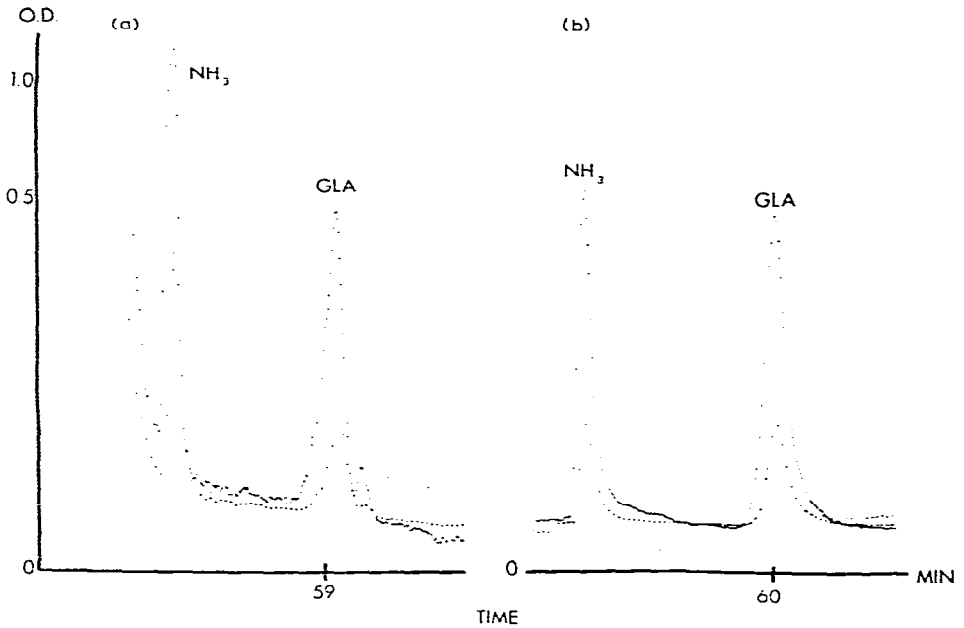


Fig. 1. Analysis of GLA using 4 *M* acetate buffer in the ninhydrin reagent (a), and without acetate buffer in the ninhydrin reagent (b).

It has been demonstrated that for the ninhydrin–amino acid reaction to give optimum colour intensity, a pH of 4.7 is required[9]. The pH of the column elution buffer used for the GLA determination is at 4.7, hence the buffer in the ninhydrin reagent could be removed and water substituted in its place.

Fig. 1b shows a GLA determination carried out with un-buffered ninhydrin reagent. The baseline is no longer erratic. The first peak is due to ammonia, the peak representing GLA is located at 59 min on the chromatogram. The column operating temperature was 35°.

Fig. 2a shows the chromatogram obtained when glutamic acid, cysteic acid

and GLA were present in the sample. The colour yield of γ-carboxyglutamate was found to be 33% of that found with glutamate. Fig. 2b is the same mixture with aspartic acid included. The procedure does not resolve aspartic and glutamic acid, but these amino acids can be estimated by using conventional cation exchange amino acid analysis. Fig. 2c shows the analysis of GLA when contained in a complex

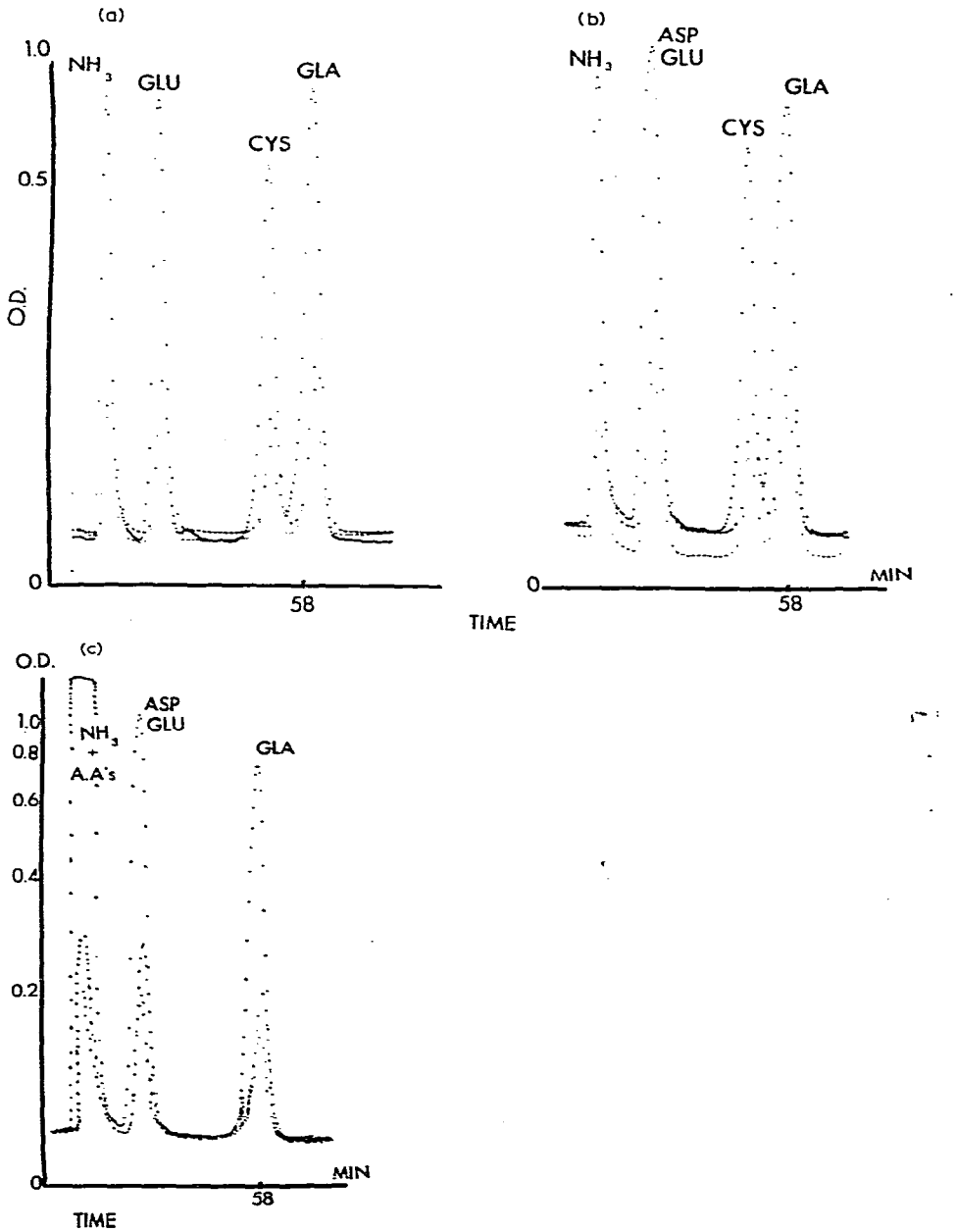
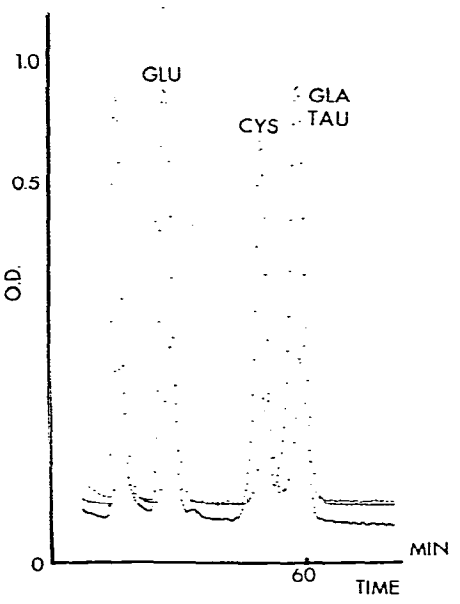


Fig. 2. Analyses of mixtures containing (a) glutamic acid (GLU), cysteic acid (CYS) and GLA; (b) GLU, aspartic acid (ASP), CYS and GLA; and (c) a standard calibration mixture plus GLA.

synthetic mixture of amino acids (Beckman Calibration Standard 312220). The chromatogram is similar to that shown in Fig. 2b. Cysteic acid was not contained in the synthetic mixture. All the neutral, aromatic and basic amino acids are eluted with ammonia at the commencement of the analysis. Glutamic and aspartic acids were eluted as a single peak, preceding the elution of GLA by *ca.* 30 min.

The presence of taurine in an analysis mixture can interfere with the quantitative determination of GLA by the proposed method (Fig. 3). This may prove to be of significance in the analysis of physiological fluids.

Fig. 4 shows a chromatogram of an alkaline hydrolyzate of prothrombin. In this analysis the peak attributed to ammonia is poorly resolved from the bulk of the amino acids in the sample. The peak located between the combined aspartate and glutamate peak and the GLA peak has not been identified. GLA was eluted at 60 min.



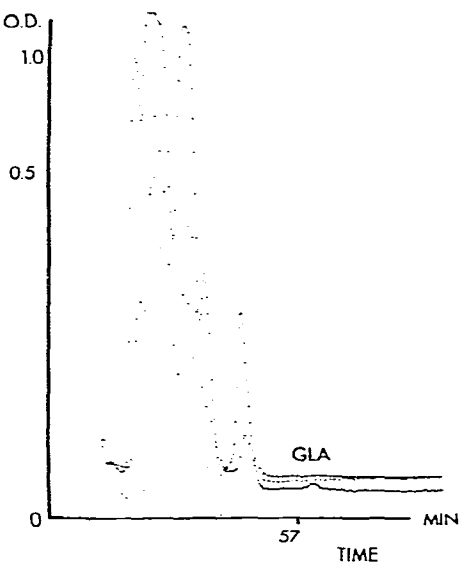


Fig. 3. Analysis of mixture containing GLU, CYS, taurine (TAU) and GLA.

Fig. 4. Analysis of alkaline hydrolyzate of prothrombin.

Although column regeneration between analyses was not carried out it was found that the anion-exchange resin tended to pack down quickly. Back pressure from the resin was registering 800 p.s.i. after ten determinations had been carried out. It should be pointed out that the acetate buffer proved to be highly corrosive to metal fittings; flushing the system out with water is recommended if the analyzer is to be shut-down for any length of time.

Summarizing, the modifications to be made to the usual amino acid analysis procedure performed with the Technicon AutoAnalyzer are:

(1) use of acetate buffer in place of citrate for column elution;

(2) lowering of the temperature in the reaction chamber to minimize degassing of reagents;

(3) replacement of the acetate buffer in the ninhydrin reagent with water.

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