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Note

Post-column reaction of amino acids with the pentane-2,4-dione–formaldehyde system for their automated analysis

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The Hantzsch reaction between one molecule each of an aldehyde and an amine and two molecules of β-diketone is a useful reaction for the fluorimetric analysis of the compounds involved. The analysis of the aldehyde, especially formaldehyde, has been the most thoroughly studied, and an excellent method by Nash[1] is available for its micro-determination. This method has been extended to the indirect analysis of compounds that form formaldehyde by appropriate pre-treatment. Periodate oxidation of carbohydrates[2] is a typical example of such pre-treatment.

The primary amine is the second target of fluorimetric analysis. A sensitive method for detecting aliphatic amines on thin-layer plates was reported by Sawicki and Carnes[3]. Hexosamines were also fluorimetrically post-column reacted for high-performance liquid chromatography (HPLC)[4]. However, there are no papers dealing with the HPLC of amino acids by using the Hantzsch reaction. Therefore, we examined the applicability of this reaction to the post-column reaction of amino acid.

EXPERIMENTAL

Chemicals

Analytical-reagent grade samples of pentane-2,4-dione and formalin (the source of formaldehyde) were purchased from Kishida Kagaku (Osaka, Japan) and used without further purification. All other chemicals and authentic specimens of amino acids were also of analytical-reagent grade.

Measurement of fluorescence intensities

An aqueous 10^{-3} *M* solution (1.00 ml) of a sample of an amino acid in 0.05 *M* hydrochloric acid, formalin diluted with 1.00 *M* acetate buffer (1.00 ml) with one of the pH values shown in Fig. 1 to contain 9% of formaldehyde and a 6% solution (1.00 ml) of pentane-2,4-dione in the same buffer were mixed, and the mixture was heated for 10 min on a boiling water-bath. The solution was cooled to room temperature and the fluorescence spectrum was recorded on a Hitachi 850 spectrofluorimeter using a 1-cm quartz cell. To obtain the reagent blank a parallel experiment was performed by replacing the sample solution with 0.05 *M* hydrochloric acid.

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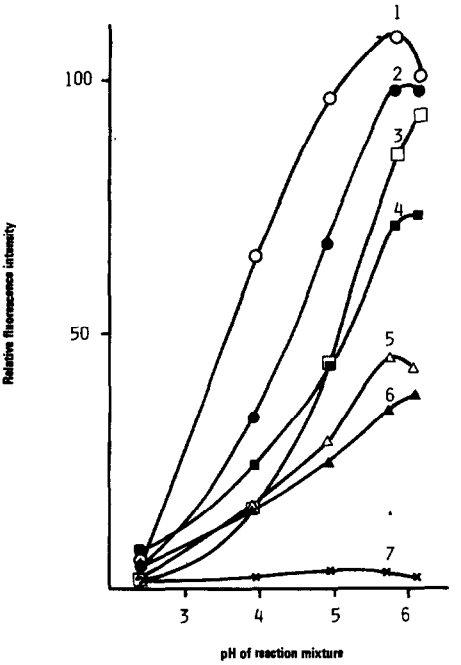

Fig. 1. pH dependence of fluorescence generation from representative amino acids. Concentration of pentane-2,4-dione, 6%; concentration of formaldehyde, 9%; concentration of acetate buffer, 1.00 *M*; reaction temperature, 100°C; reaction time, 10 min; detection, 417 nm (excitation), 476 nm (emission). 1 = Lysine; 2 = glycine; 3 = serine; 4 = phenylalanine; 5 = aspartic acid; 6 = valine; 7 = blank.

Automated analysis of amino acids

A Hitachi 034 apparatus was used for the automated analysis of amino acids. A jacketed glass column (40 cm × 9 mm I.D.) packed with Hitachi 2611 resin was eluted first with eluent I (0.20 *M* citrate buffer, pH 3.25), then the eluent was changed automatically to II (0.20 *M* citrate buffer, pH 4.25) at 80 min and finally to III (1.20 *M* citrate buffer, pH 5.28) at 135 min. The flow-rate was constant (1.0 ml/min) throughout the analysis. Mixtures of amino acids were introduced on to the column via an injector carrying a 500-μl loop. To the eluate was added, via a Y-shaped PTFE connector, a 6% solution of pentane-2,4-dione in 1.0 *M* acetate buffer (pH 6.22), which was supplied from one pump head of Atto 2396 twin-piston pumps at a flow-rate of 0.5 ml/min. To the mixture was further added, in a similar manner, a solution of formalin diluted with the same buffer to contain 9% formaldehyde from the other pump head at the same flow-rate. The effluent was allowed to react in a PTFE coil (30 m × 0.5 mm), immersed in a glycerol bath thermostated at 95 ± 1°C, then led into a Hitachi 650-10-LC fluoromonitor equipped with a 90-μl quartz flow cell. Peaks were monitored at 476 nm with excitation at 417 nm.

RESULTS AND DISCUSSION

Fluorescence spectra

At pH 6.2 all amino acids other than tyrosine, proline and hydroxyproline

gave excitation and emission maxima at the common wavelengths of 417 and 476 nm, respectively, where hexosamines also have maxima[4]. The excitation maximum of tyrosine was at 407 nm, slightly shifted to shorter wavelength, whereas the emission maximum was at a longer wavelength, 519 nm. Both the excitation and emission maxima of proline underwent hypsochromic shifts to 328 and 372 nm, respectively. Hydroxyproline gave excitation and emission maxima at the same wavelengths as those for proline. The relative fluorescence intensities of tyrosine, proline and hydroxyproline at 417 nm (excitation) and 476 nm (emission) were 36.4, 14.5 and 4.6%, respectively, of those at their maxima.

Optimization of fluorescence generation

The most influential factor affecting fluorescence generation is the acidity of the reaction media. As shown in Fig 1, representative amino acids had maximal fluorescence intensity at pH 5.5–6.0 when acetate buffer was used. In order to obtain a pH in this range it is convenient to mix a sample solution and the two reagent (pentane-2,4-dione and formaldehyde) solutions in 1 *M* acetate buffer of pH 6.22, all in equal proportions. The concentration of the buffer salt had only a slight influence on fluorescence generation, as can be seen from Fig. 2a, which was obtained for glycine.

On the other hand, fluorescence generation was strongly affected by the concentrations of the reagents, as indicated in Fig. 2b and c. The fluorescence intensity increased with increasing concentration of the reagents, in the same manner as in the determination of hexosamines[4], giving plateaux at pentane-2,4-dione and formaldehyde concentrations of 6 and 9%, respectively. Under the established conditions (6% pentane-2,4-dione and 9% formaldehyde in 1.00 *M* acetate buffer, pH 6.22), the relative molar responses of amino acids at 417 nm (excitation) and 476 nm (emission)

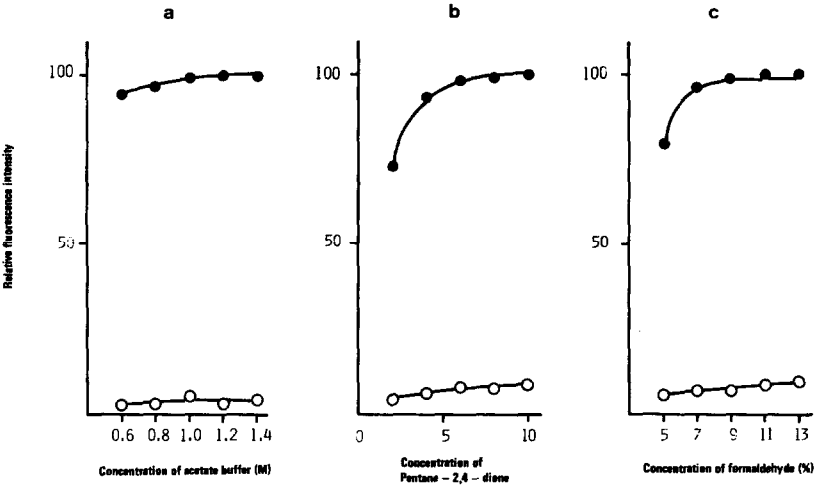

Fig. 2. Effect of buffer and reagent concentrations on fluorescence generation. (a) Concentration of buffer salt. pH of acetate buffer, 6.22; concentration of acetate buffer, 0.60, 0.80, 1.00, 1.20 and 1.40 *M*. Other conditions as in Fig. 1. (b) Concentration of pentane-2,4-dione. pH of acetate buffer, 6.22; concentration of pentane-2,4-dione, 2.00, 4.00, 6.00, 8.00 and 10.0%. Other conditions as in Fig. 1. (c) Concentration of formaldehyde. pH of acetate buffer, 6.22; concentration of formaldehyde, 5.00, 7.00, 9.00, 11.0 and 13.0%. Other conditions as in Fig. 1. ●, Values obtained for glycine; ○, reagent blank.

were as follows: hydroxyproline, 0.0; aspartic acid, 35; threonine, 36; serine, 98; glutamic acid, 30; proline, 1.3; glycine, 100; alanine, 30; cysteine, 50; valine, 14; methionine, 46; isoleucine, 28; leucine, 46; tyrosine, 3.0; phenylalanine, 45; lysine 125; ammonia, 99; histidine, 30; and arginine, 43.

Application to post-column reactions of amino acids

As the amino acid analyser used in this work was operated by stepwise elution with three citrate buffers having pH values ranging from 3.25 to 5.28, the difference in acidity may affect fluorescence generation. However, examination of the pH of a mixture of one of the eluents with reagent solutions having the same pH value of 6.22 indicated that the decrease in the pH of the reaction medium was less than 0.8, owing to the compensating effect of the reagent buffer. Hence the pH range of the reaction media was *ca.* 5.2–5.7, the depression of fluorescence intensity caused by the eluents being relatively small.

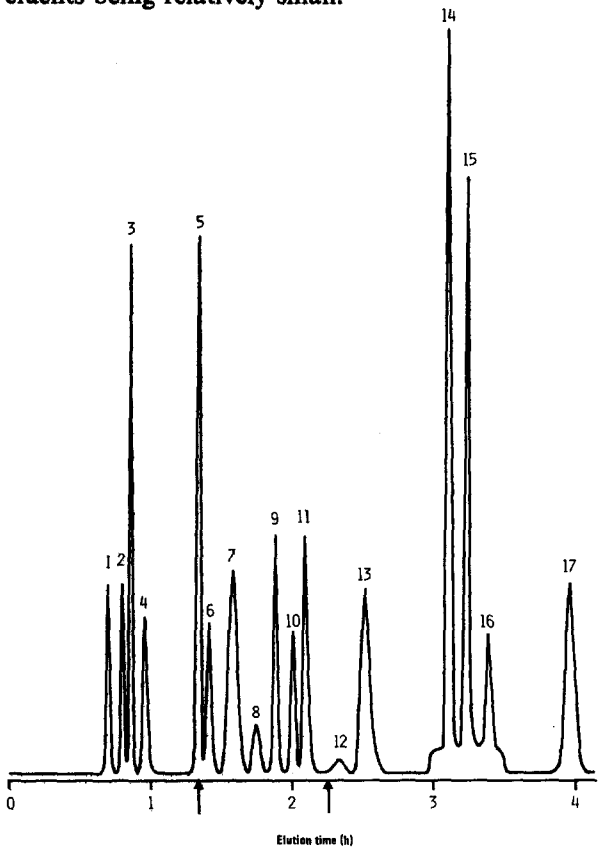

Fig. 3. Automated analysis of amino acids by HPLC, as detected fluorimetrically after post-column reaction with the pentane-2,4-dione–formaldehyde system. Arrows indicate the times of buffer change. pH of acetate buffer, 6.22; other conditions as in Fig. 1. Sample, 125 nmol each. Peak assignment: 1 = aspartic acid; 2 = threonine; 3 = serine; 4 = glutamic acid; 5 = glycine; 6 = alanine; 7 = cysteine; 8 = valine; 9 = methionine; 10 = isoleucine; 11 = leucine; 12 = tyrosine; 13 = phenylalanine; 14 = lysine; 15 = ammonia; 16 = histidine; 17 = arginine. The sample solutions contained proline and hydroxyproline, but they were not detected under these conditions.

Fig. 3 shows a chromatogram obtained for an equimolar mixture of amino acids. Analysis of smaller amounts of samples indicated that the detection limits of, *e.g.*, glycine and lysine at a signal-to-noise ratio of 2 were 342 and 267 pmol, respectively. These sensitivities were approximately five and four times higher than those for the ninhydrin method obtained under the same separation conditions.

The sensitivity of the present method is approximately one order of magnitude lower than that of the *o*-phthalaldehyde method, although variations among amino acid species should be considered. However, the *o*-phthalaldehyde method requires expensive reagents, and the reagent solution is not so stable. Dansylation is another fluorimetric method, but it is not suitable for post-column reactions.

The linearity ranges of the calibration graphs obtained by the present method are wide. For example, those of serine, glycine and lysine covered sample amounts ranging from 5 to 500 nmol. Less sensitive amino acids had higher linearity ranges.

The coefficients of variation for the determination of sensitive amino acids having molar responses greater than 30 were less than 5% at the 50 nmol level.

Fig. 4a–d show examples of amino acid analyses by the present method. The results indicate that a commercial nutritional drink (Fig. 4a) contained threonine, isoleucine and phenylalanine, together with small amounts of glutamic acid and ly-

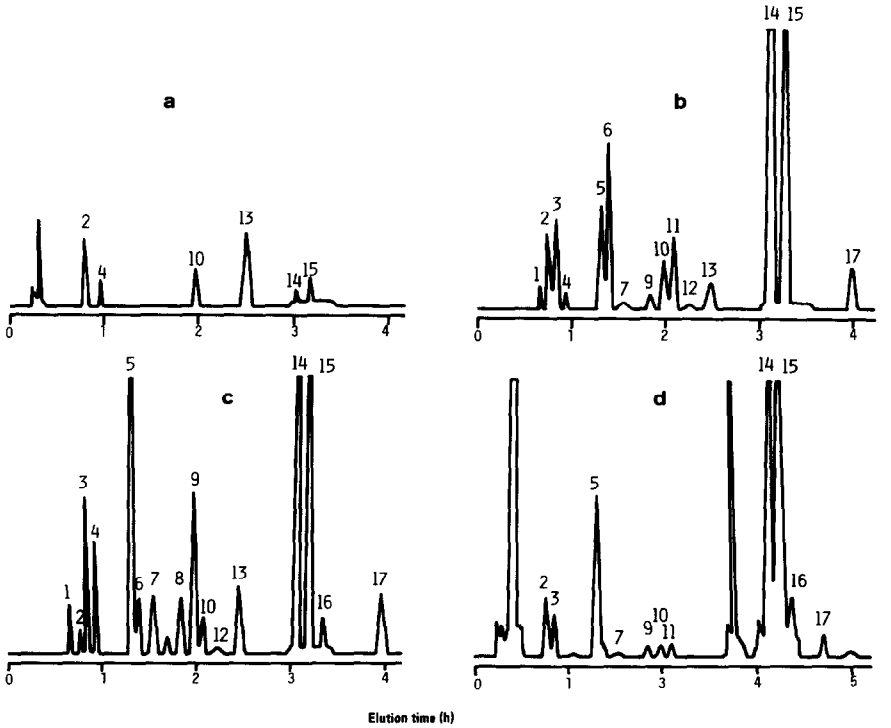

Fig. 4. Analysis of amino acids in (a) a nutritional drink, (b) an acid hydrolysate of chymotrypsinogen, (c) an acid hydrolysate of insulin and (d) a normal human urine. Samples: nutritional drink, 500 μl; chymotrypsinogen, 111 μg; insulin, 106 μg; urine, 20 μl. Other conditions as in Fig. 3. The protein samples were hydrolysed in 6 *M* hydrochloric acid for 20 h at 110°C, the hydrolysate was evaporated to dryness and the residue was dissolved in 0.05 *M* hydrochloric acid (5.00 ml). Portions of 500 μl of the solutions were injected on to the column. Peaks as in Fig. 3.

sine. Both protein samples of chymotrypsinogen and insulin (Fig. 4b and c) contained fourteen amino acids (aspartic acid, threonine, serine, glutamic acid, glycine, alanine, cysteine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine and arginine). The chymotrypsinogen sample had small amounts of two additional amino acids (valine and histidine). All amino acid contents were consistent with the reported values. The normal urine sample (Fig. 4d) showed the presence of a few amino acids, such as threonine, serine, glycine and lysine, together with several minor amino acids.

The above results indicate that post-column reaction with the pentane-2,4-dione–formaldehyde system produces fluorescence with most amino acids that allows their sensitive detection in HPLC. Although the molar responses vary over a wide range, a number of amino acids could be detected more sensitively than by the conventional ninhydrin method. The reagent solutions used in the present work were stable for at least 1 week without the need for particular precautions such as handling in an atmosphere of nitrogen, and they are economical. However, proline and hydroxyproline were difficult to detect, as in the ninhydrin method. The use of the wavelengths for excitation and emission maxima may improve the sensitivity, but it will be still low compared with those for other amino acids.

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