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SIMPLIFIED PROCEDURE FOR SPECIFIC DETERMINATION OF IMINO ACIDS IN HUMAN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

After deproteinization with 70% aqueous ethanol and evaporation to dryness, a plasma sample was subjected to the formol reaction. Then Dns derivatization of imino acids was carried out for 20 min at 37°C. An aliquot of the resulting solution was subjected to reversed-phase chromatography using continuous gradient elution with acetonitrile—50 m*M* acetate buffer (pH 4.0) as solvent system. This method is selective for imino acids. Hydroxyproline and proline levels could be determined using 30 μl of plasma.

INTRODUCTION

The diseases hyperprolinaemia and hyperhydroxyprolinaemia are metabolic disorders of imino acids [1], and the analysis of proline or hydroxyproline is very important in their diagnosis.

Proline and hydroxyproline are also the characteristic imino acids of the group of proteins known as collagens. 4-Hydroxyproline is a marker for all

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types of collagen, and 3-hydroxyproline for basement membrane collagen [2]. The analysis of hydroxyprolines can offer useful information in the diagnosis or biochemical analysis of collagen disorders [3].

The simultaneous detection of amino acids including imino acids was attempted by converting secondary amino groups to primary ones using sodium hypochlorite solution prior to reaction with *o*-phthalaldehyde—2-mercaptoethanol reagent [4]. Although this additional post-column reaction made it possible to detect imino acids, the sensitivity for the detection of amino acids was markedly reduced. Several reports attempting to analyse proline or hydroxyproline have been published [4,5—8]. In spite of their titles, the methods were not so specific for imino acids. Furthermore, they were time-consuming and involved a high cost of analysis, complexity of operation, etc.

It is well known that compounds containing primary amino groups react with formaldehyde to reduce their basicity, and formol titration has been generally used for the determination of amino acids using this reaction [9].

It is considered that imino acids might be selectively detected if a sample was previously subjected to the formol reaction, followed by Dns derivatization of the imino acids. This paper describes the specific determination of free proline and hydroxyproline in human blood plasma using high-performance liquid chromatography.

MATERIALS AND METHODS

Chemicals

Imino and amino acids were purchased from Ajinomoto (Tokyo, Japan). A standard solution was prepared by dissolving them in 0.1 *M* hydrochloric acid solution to a concentration of 0.4 mg/ml. This solution was used after being neutralized with 0.1 *M* sodium hydroxide solution and then appropriately diluted with 0.1 *M* phosphate buffer (pH 6.8). Aldehydes and Dns chloride were obtained from Wako (Osaka, Japan). Dns chloride was dissolved in acetone to a concentration of 2 mg/ml after recrystallization from *n*-hexane. Acetonitrile and water were of liquid chromatographic grade (Wako). Other reagents were of analytical grade.

Apparatus

A high-performance liquid chromatograph consisting of a Model KHD-26 DX mini-micropump, a Model KD-1 damper, and a Model KHP-UI-130 universal injector was used. These parts were purchased from Kyowa Seimitsu (Tokyo, Japan). The separation was performed on a 250 × 4.0 mm stainless-steel column packed with LiChrosorb RP-18, particle size 5 μm (Merck, Darmstadt, F.R.G.), which was prepared by a slurry packing technique in our laboratory. The eluted Dns derivatives were detected with a Model FP-110 fluorescence spectrofluorometer (Japan Spectroscopic, Tokyo, Japan). Detailed operating conditions are shown in Fig. 3.

Determination of imino acids in human blood plasma

A 500-μl volume of 70% aqueous ethanol solution was added to 30 μl of plasma, and the mixture was vigorously shaken. After centrifugation (7000 *g*

for 15 min), the supernatant was removed and evaporated to dryness. The residue was dissolved in 100 μl of 0.1 *M* phosphate buffer (pH 6.8) containing 10% formaldehyde by sonicating for 2 min. In this step, the reaction of the compounds containing primary amino groups with formaldehyde was performed simultaneously with dissolution of the residue. After that, Dns derivatization of imino acids was carried out based on the method of Airhart et al. [10] as follows. A 50-μl volume of 0.5 *M* sodium bicarbonate solution and 100 μl of Dns chloride acetone solution (2 mg/ml) were added to this solution, and the mixture was left for 20 min at 37°C in the dark. After Dns derivatization, an aliquot (50 μl) of the resulting solution was injected directly onto the column. When insoluble materials were observed in the solution, the supernatant was injected after centrifugation.

The determination of proline and hydroxyproline was carried out based on a calibration graph prepared by treating standard solutions of these compounds in a similar manner.

RESULTS AND DISCUSSION

Dns derivatization of proline and hydroxyproline proceeded relatively smoothly even in the presence of formaldehyde. When imino acids were Dns-derivatized in 0.1 *M* phosphate buffer containing 15% formaldehyde at 37°C, peak heights of their Dns derivatives varied with reaction time, as shown in Fig. 1. Peak heights of both imino acids became constant within 20 min. Higher reaction temperatures gave similar results, though peak heights tended to be increased somewhat. Unknown interfering peaks, however, appeared on the chromatogram with the rise in temperature. So, Dns derivatization of

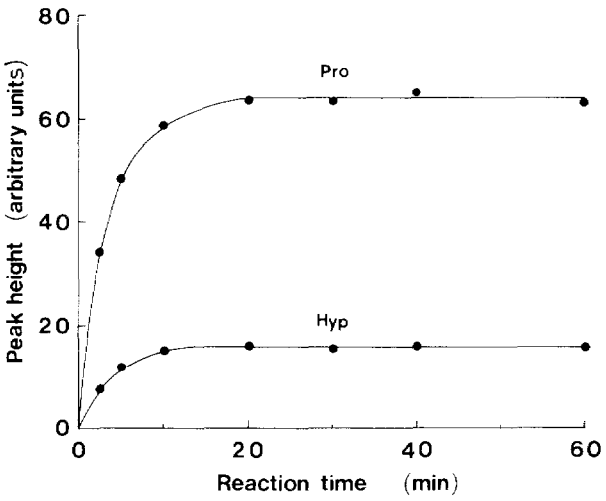

Fig. 1. Dns derivatization of imino acids in the presence of formaldehyde. Proline (Pro) and hydroxyproline (Hyp) (20 μg/ml of each) were Dns-derivatized at 37°C in 100 μl of 0.1 *M* phosphate buffer (pH 6.8) containing 15% formaldehyde by adding 50 μl of 0.5 *M* sodium bicarbonate solution and 100 μl of Dns chloride acetone solution (2 mg/ml). An aliquot of the resulting solutions was subjected to HPLC. Each point was obtained from a single experiment.

imino acids in the presence of formaldehyde was carried out for 20 min at 37°C.

The optimal conditions for the formol reaction with amino acids were investigated using several amino acids dissolved in phosphate buffer containing formaldehyde of various concentrations (to 15%). Dns derivatization was carried out after standing at room temperature. The results showed that the reaction with all amino acids was completed within several minutes at every concentration of formaldehyde tested. The formol reaction at higher temperature also showed the necessity of several minutes for completion of the reaction. Phosphate buffer of pH 6.8 was the most suitable for the formol reaction with amino acids. From these results, the formol reaction was performed for 2 min at room temperature in 0.1 *M* phosphate buffer (pH 6.8) before Dns derivatization.

The influence of the concentration of formaldehyde on peak heights of imino and amino acids (0.02 mg/ml of each was used) is shown in Fig. 2. The ratios of peak heights of amino acids at different concentrations of formaldehyde to those in the absence of formaldehyde markedly decreased with increasing formaldehyde concentration. There was a significant difference between variations of peak height ratios of imino acids and those of amino acids. At a formaldehyde concentration of 10%, almost all peaks of amino acids disappeared. On the other hand, proline gave a peak height of about 60% of that found in the absence of formaldehyde, and hydroxyproline a peak height of about 20% of that in the absence of formaldehyde.

Fig. 3 shows the chromatograms obtained from a standard mixture of imino

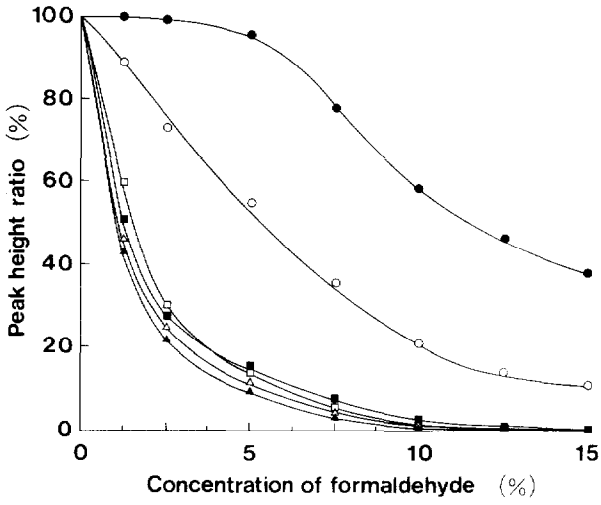

Fig. 2. Effect of the concentration of formaldehyde in phosphate buffer on peak height ratios of imino and amino acids. Imino and amino acids (20 μg/ml of each) were subjected to the formol reaction in 0.1 *M* phosphate buffer (pH 6.8) containing various concentrations of formaldehyde and then Dns-derivatized in the same manner as described in Fig. 1. Peak height ratios are indicated as the ratios of peak heights of Dns derivatives in the presence of formaldehyde to those in the absence of it. Each point was obtained from a single experiment. (●) Proline; (○) hydroxyproline; (▲) alanine; (△) methionine; (■) phenylalanine; (□) leucine.

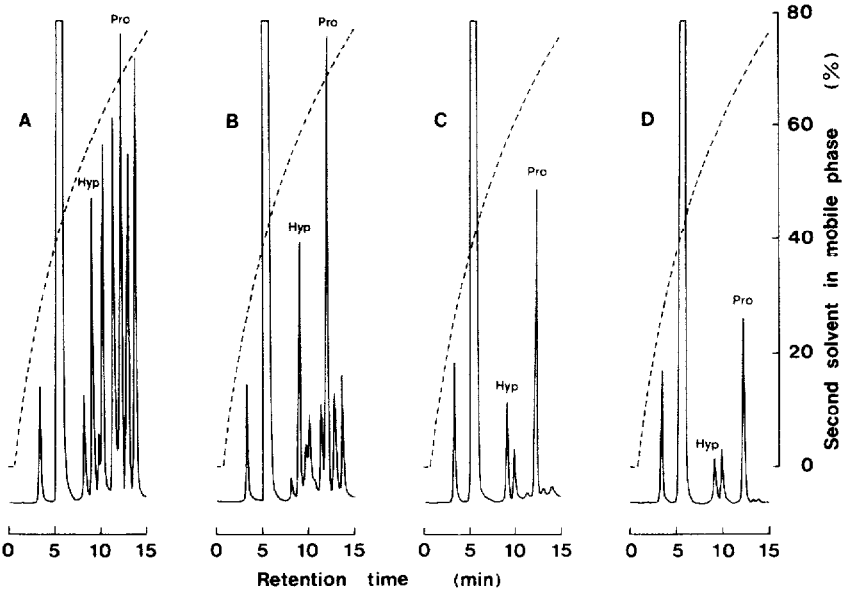

Fig. 3. High-performance liquid chromatograms of standard mixtures of imino and amino acids. Imino and amino acids (20 μg/ml of each) were Dns-derivatized after the formol reaction in 100 μl of 0.1 *M* phosphate buffer (pH 6.8) containing 0% (A), 2.5% (B), 10% (C), or 15% (D) formaldehyde. Operating conditions: column, 250 × 4.0 mm LiChrosorb RP-18 (5 μm); mobile phase, first solvent (10% acetonitrile in 50 m*M* acetate buffer, pH 4.0)—second solvent (70% acetonitrile in water). The gradient was prepared by adding the second solvent to the first solvent as shown by the dotted lines; flow-rate, 1.0 ml/min; column temperature, 50° C; fluorescence detector, excitation 340 nm, emission 510 nm.

and amino acids Dns-derivatized after the formol reaction using several concentrations of formaldehyde. Operating conditions shown in the legend of Fig. 3 were employed after different kinds of conditions were investigated. While the alkaline solution (approximately pH 8) after Dns derivatization was injected directly onto the column, no problem such as reduced column efficiency occurred at least during two months of continuous operation. The addition of 2.5% formaldehyde markedly reduced peaks of amino acids such as aspartic acid, alanine, methionine, phenylalanine, and leucine, etc. (Fig. 3B), which gave major peaks when the formol reaction was omitted (Fig. 3A). Nevertheless, peaks of imino acids were not reduced at all. When formaldehyde at concentrations higher than 10% was added (Fig. 3C and D), peaks of amino acids disappeared almost completely. Though there was no peak corresponding to amino acids at a formaldehyde concentration of 15% (Fig. 3D), peaks of imino acids were also considerably reduced. Therefore, the formaldehyde concentration of 10% was chosen for the formol reaction prior to Dns derivatization.

The investigation using aldehydes other than formaldehyde showed that acetaldehyde and propionaldehyde could also reduce peaks of amino acids. Ratios of peak heights of alanine, methionine, phenylalanine, and leucine (0.02 mg/ml of each was used) to peak heights in the absence of aldehydes decreased with increasing aldehyde concentration as shown in Fig. 4. The effects of these aldehydes were, however, inferior to that of formaldehyde.

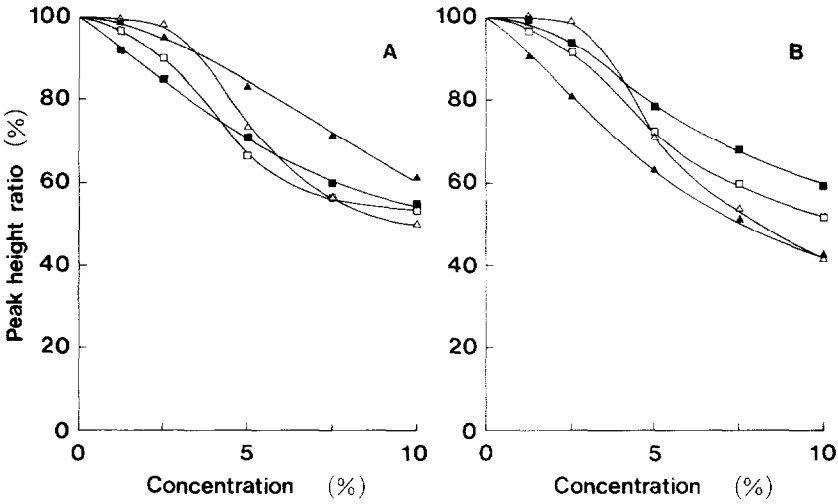

Fig. 4. Effect of the concentration of aldehydes in phosphate buffer on peak height ratios of amino acids. Amino acids were treated according to the method described in Fig. 2. Each point was obtained from a single experiment. (A) Acetaldehyde; (B) propionaldehyde. (▲) Alanine; (△) methionine; (■) phenylalanine; (□) leucine.

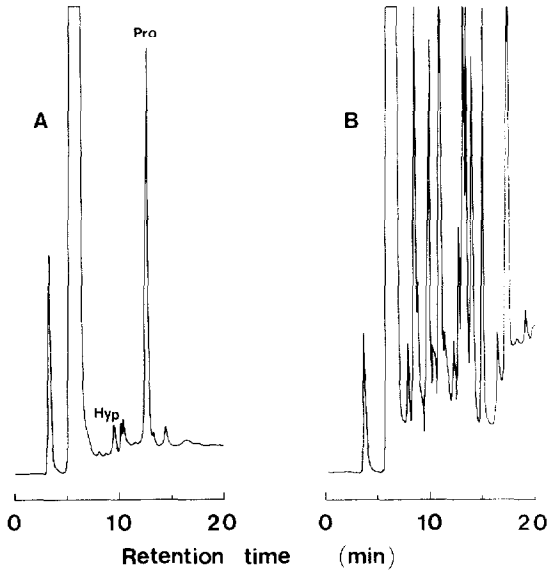

Fig. 5. High-performance liquid chromatograms obtained from human blood plasma. (A) Dns derivatization after the formol reaction; (B) Dns derivatization without the formol reaction. Operating conditions as in Fig. 3.

The procedure for the determination of free imino acids in blood plasma was fixed as described in Materials and methods after the conditions for pre-treatments were investigated. The calibration graphs obtained from standard solutions of proline and hydroxyproline showed good linearity at least in the concentration range of sample solution from 0.001 to 0.1 mg/ml of both imino acids.

The chromatograms in Fig. 5 were obtained from normal human plasma. There was almost no major peak other than those of imino acids on the chromatogram (Fig. 5A), which was obtained from a plasma sample Dns-derivatized after the formol reaction. On the other hand, a number of other major peaks were observed on the chromatogram (Fig. 5B) from the sample which was not treated with formaldehyde. As is apparent by comparing the two chromatograms, the formol reaction prior to Dns derivatization made it possible to selectively detect imino acids in plasma containing many other amino acids.

When recovery and reproducibility of the determination were estimated using plasma samples to which standard proline and hydroxyproline were added (the concentration of each imino acid was 0.01 mg/ml), percentage recoveries and coefficients of variation (n=6) were 108% and 5.5% for proline and 95% and 6.6% for hydroxyproline, respectively.

Amino acids containing primary amino groups react with formaldehyde to produce Schiff bases [9] and consequently are reduced in their basicity. The principle of the present method is considered as follows. Amino acids readily react with formaldehyde to produce Schiff bases, thus losing their reactivity to Dns chloride. On the other hand, imino acids can react with Dns chloride to produce fluorescent derivatives because the secondary amino groups in them are not so reactive to formaldehyde as the primary amino groups in amino acids. Therefore, imino acids can be selectively detected even from a mixture of imino and amino acids.

Fluorescent responses of imino acids decreased with increasing formaldehyde concentration. This phenomenon may be due to the reaction of imino acids with formaldehyde to produce hydroxymethyl derivatives [11] which cannot react with Dns chloride.

Anyhow, it is speculated that there is a considerable difference between imino and amino acids concerning the reaction rates of Schiff base production and hydroxymethylation. Investigation into the structures of derivatives was not carried out in the present study.

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