Journal of Chromatography, 233 (1982) 313—316
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1432

Note

Rapid, simultaneous and sensitive determination of free hydroxyproline and proline in human serum by high-performance liquid chromatography

KAZUHARU NAKAZAWA, HARUMI TANAKA* and MASATAKA ARIMA

Division of Child Neurology, National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawa-Higashi-Machi, Kodaira, Tokyo (Japan)

(Received May 12th, 1982)

Automated amino acid analysis with ninhydrin reaction gives a low sensitivity for imino acids. Fluorogenic amine reagents such as fluorescamine and *o*-phthalaldehyde yield a high fluorescence intensity with primary amino acids except imino acids [1—3]. Recently, 4-chloro-7-nitrobenzofurazan has been used for the assay of primary amino acids and imino acids [4,5]. Böhlen and Mellet [6] developed a method of determining hydroxyproline and proline using the combination of *o*-phthalaldehyde and alkaline sodium hypochlorite, which is an oxidant that converts imino acids into primary amino acids. The present paper describes a rapid, simultaneous and micro assay for free hydroxyproline and proline in human serum by high-performance liquid chromatography using the post-labeled method with *o*-phthalaldehyde and sodium hypochlorite.

EXPERIMENTAL

Reagents

Lithium citrate and the standard solution of amino acids for an amino acid autoanalyzer, *o*-phthalaldehyde of biochemical grade, and all the other reagents of analytical grade were obtained from Wako Pure Chemical Industries (Osaka, Japan). Commercial 10% sodium hypochlorite (NaOCl) was purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan).

Mobile phase

One-thirtieth *M* lithium citrate solution was adjusted to pH 3.15 by perchloric acid. Lithium hydroxide (0.2 *M*) was made as a regeneration solution. These solutions were degassed under reduced pressure for 20 min.

0378-4347/82/0000—0000/$02.75

Reaction buffer

NaOCl solution was prepared by adding 2.0 ml of the 10% sodium hypochlorite to 0.3 *M* borate buffer; it was titrated with 4 *M* sodium hydroxide to pH 10.5, and made up to 1000 ml with distilled water for liquid chromatography. *o*-Phthalaldehyde solution was prepared as follows: 4 g of *o*-phthalaldehyde and 1 ml of 2-mercaptoethanol were dissolved in 30 ml of ethanol. After 0.3 *M* borate buffer containing 0.5 g of Brij 35 was added to the ethanol solution, the solution was adjusted to pH 10.5 by 4 *M* sodium hydroxide, and was made up to 1000 ml with distilled water for liquid chromatography.

Apparatus

The following equipment was purchased from Shimadzu (Kyoto, Japan): Model LC-3A high-performance liquid chromatograph; Model FLD-1 fluorescence detector with a mercury source lamp (excitation wavelength 360 nm; emission wavelength 450 nm); data analyzer C-R1A Chromatopac connected to the detector for identification and integration of peaks; and a 15 cm × 4 mm column filled with sulfonated polystyrene cation-exchange resin (Shimadzu Gel ISC-07/S1504, particle size, 7 μm). The column oven temperature was set at 55°C. The flow-rate of the mobile phase was set at 0.3 ml/min, yielding an operating pressure of 20 kg/cm^2. The flow-rate of the NaOCl solution and the o-phthalaldehyde solution was set at 0.25 ml/min, and the lengths of the oxidative reaction coil and the fluorogenic reaction coil were 1 m and 2 m, respectively. The reaction temperature was set at 55°C.

Sample preparation procedure

Determination of the concentration of hydroxyproline and proline was performed by the standard addition method on the basis of the peak area ratios against the internal standard. One-tenth milliliter of human serum was transferred into each of the four micro test tubes, to which the standard hydroxyproline and proline solutions were added as follows. To the first sample, 0.05 ml of distilled water was added. To the second, 0.05 ml of a solution of 10 μmol/l hydroxyproline and 100 μmol/l proline was added. To the third, 0.05 ml of a solution of 20 μmol/l hydroxyproline and 200 μmol/l proline was added. To the fourth, 0.05 ml of a solution of 40 μmol/l hydroxyproline and 400 μmol/l proline was added. Since the chemical properties of N-methylglycine (sarcosine) are similar to those of imino acids and it is rarely detected in human serum, 0.05 ml of 8% sulfosalicylic acid solution containing 1 m*M* of N-methylglycine as the internal standard was added to each sample. These samples were vortex-mixed for 30 sec and then centrifuged at 12,800 *g* for 1 min. One-hundredth of the deproteinized serum supernatant was injected into the high-performance liquid chromatograph.

RESULTS AND DISCUSSION

The NaOCl reagent dramatically attenuated the generation of fluorescence from primary amino acids (Fig. 1A and B). There was a good linear correlation between the peak area ratios and the sera containing the standard imino acids, with correlation coefficients of 0.997 and 0.999 (Fig. 2). The free hydroxy-

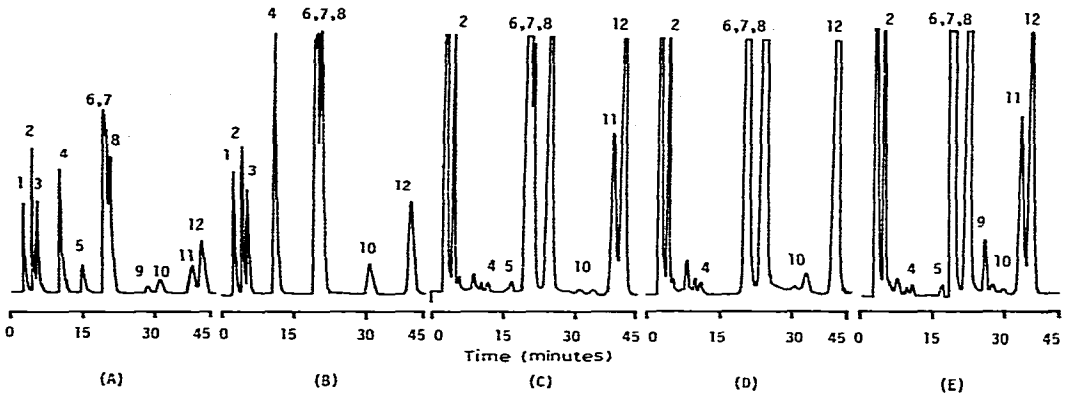

Fig. 1. Chromatograms of amino acid analysis. (A) Standard amino acids: 0.1 nmol of phosphoserine (1), taurine (2) and phosphoethanolamine (3); 0.2 nmol of aspartic acid (4), hydroxyproline (5), threonine (6), serine (7), glutamic acid (8), proline (11) and glycine (12); 0.5 nmol of N-methylglycine (9) and 0.05 nmol of L-amino adipic acid (10). (B) Standard amino acids analysed using *o*-phthalaldehyde solution without NaOCl solution. (C) Analysis of free amino acids in human serum. (D) Free amino acids in human serum analysed using *o*-phthalaldehyde solution alone. (E) Free amino acids in human serum mixed with the internal standard of N-methylglycine. The analytical condition is similar to that of (C).

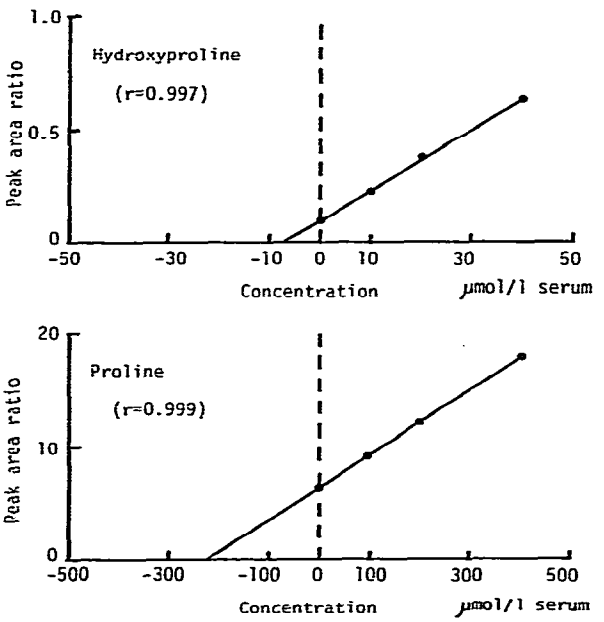

Fig. 2. Standard addition curves for determination of free hydroxyproline and proline in serum from a healthy subject. The concentrations of free hydroxyproline and proline in serum were 6.7 μmol/l and 225.3 μmol/l, respectively. The equation of the line was determined by the least-sqaures method.

proline and proline concentration in the sera from ten healthy subjects measured by the present method was 6.2 ± 1.3 μmol/l for hydroxyproline and 212.0 ± 16.0 μmol/l for proline. The limit of detection was found to be 1.0 μmol/l for hydroxyproline and 10.0 μmol/l for proline in human serum. It is

possible to determine the imino acids using 0.05 ml of serum when the method with standard calibration curves is adopted.

There have been few clinical reports on hypersarcosinemia [7], and sarcosine cannot be detected in normal human serum under the present conditions (Fig. 1C, D and E). Thus sarcosine is a convenient internal standard to assay imino acids in human serum. The present procedure is suitable for the routine determination of free hydroxyproline and proline in human serum at the picomole level by modification of Böhlen and Mellet's method [6]. This method may be useful for the detection of abnormalities in imino acid metabolism and collagen synthesis.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. Kenji Yamada (Department of Pharmacology, Tokyo College of Pharmacy) for his valuable advice. This study was partly supported by Grant No. 81-12-05 from the National Center for Nervous, Mental and Muscular Disorders (NCNMMD) of the Ministry of Health and Welfare, Japan.

REFERENCES

1 J.R. Benson and P.E. Hare, Proc. Nat. Acad. Sci. U.S., 72 (1975) 619.
2 M. Roth, Anal. Chem., 43 (1971) 880.
3 M. Roth and A. Hampaï, J. Chromatogr., 83 (1973) 353.
4 M. Roth, Clin. Chim. Acta, 83 (1978) 273.
5 M. Ahnoff, I. Grundevik, A. Arfwidsson, J. Fonselius and B. Persson, Anal. Chem., 53 (1981) 485.
6 P. Böhlen and M. Mellet, Anal. Biochem., 94 (1979) 313.
7 T. Gerritsen and H.A. Waisman, in J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 4th ed., 1978, p. 514.