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Determination of citrulline and homocitrulline by high-performance liquid chromatography with post-column derivatization

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ABSTRACT

A high-performance liquid chromatographic method was developed for the determination of citrulline and homocitrulline using a post-column colorimetric reaction with *o*-phthalaldehyde and N-(1-naphthyl)-ethylenediamine Citrulline and homocitrulline were determined with no interferences from protein amino acids. The results show that the level of citrulline in the plasma of patients with uremia on intermittent hemodialysis is higher than that in healthy human plasma, and that homocitrulline is excreted into the urine of healthy adults

INTRODUCTION

Both citrulline and homocitrulline are non-protein amino acids with an N-carbamyl group. Citrulline is not only an intermediate in the urea cycle [1] (ornithine cycle) but also a precursor for biosynthesis of arginine [2]. Most of the citrulline released into blood by the liver is taken up by the kidney for the biosynthesis of arginine [3]. Homocitrulline is present in the urine of infants [4] and patients with hyperornithinemia [5]. However, it is unclear whether homocitrulline is biosynthesized from lysine and carbamyl phosphate [6] or is a metabolite of proteins, in which some lysine residues are N-carbamylated by cyanate ion (a spontaneous degradation product of urea) [7].

An amino acid analyzer has been used to determine citrulline and homocitrulline [8–11]. However, various protein amino acids interfere in this determination in biological materials. For the selective detection of N-carbamyl compounds, a colorimetric reaction with *o*-phthalaldehyde and N-(1-naphthyl)ethylenediamine in sulphuric acid (OPA/NED reaction) has been reported [12]. In the reaction using dilute sulphuric acid (modified method) [13], the colour developed was unstable and the absorbance of the reagent blank increased with time. However, this modified method is applicable to post-column reactions in high-performance liquid chromatography (HPLC), because it is possible to control the reaction conditions carefully.

This paper describes a post-column derivatization with OPA/NED. Further-

more, we tried to apply the method to the determination of citrulline in the plasma of healthy subjects and patients with uremia and to the determination of homocitrulline in the urine of healthy adults.

EXPERIMENTAL

Reagents and materials

Homocitrulline was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Citrulline and the other amino acids were purchased from Sigma (St. Louis, MO, U.S.A). OPA, NED and trichloroacetic acid (TCA) were purchased from Wako (Osaka, Japan). All other chemicals were of reagent grade. Reagent solutions were prepared by dissolving solutes in deionized and distilled water. TSK gel SCX was purchased from Tosoh (Tokyo, Japan).

Subjects

Plasma samples were obtained from nearby hospitals and promptly assayed. Normal plasma was collected from ten healthy men and six women, aged 22 to 31 years, with normal weight for their age and sex. Patients with chronic renal failure, aged 35 to 50 years, were on long-term dialysis. The plasma of uremic patients was collected before hemodialysis. Urine samples were collected from eight healthy men, aged 22 to 31 years, early in the morning.

Apparatus and chromatographic conditions

The post-column reaction system consisted of a double reciprocating pump (PSU-2.5NP, Shimamura Instrument, Tokyo, Japan) and a dry reaction bath (DB-3, Shimamura Instrument). The flow-cell of the VIS spectrophotometric detector used (UVILOG-7V, Oyo-Bunko Kiki, Tokyo, Japan) was resistant to acidic solution. The established chromatographic conditions are shown in Fig. 1.
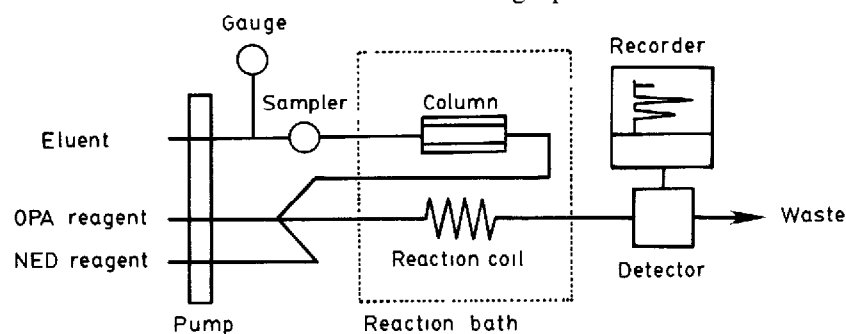

Fig 1 Schematic representation of the HPLC equipment used for the determination of citrulline and homocitrulline. Column, TSK gel SCX (150 mm × 4 mm I D); eluent, 50 mM citrate buffer containing 0 3 M sodium chloride (pH 3 5), OPA reagent, 1 0 M H$_2$SO$_4$ containing 0 1% Brij 35 and 1 5 g/l OPA; NED reagent, 1 0 M H$_2$SO$_4$ containing 0.1% Brij 35, 5 g/l boric acid and 0 8 g/l NED, flow-rate of eluent, 0.4 ml/min; flow-rate of reagent, 0 2 ml/min, reaction coil, 10 m × 0 5 mm I.D ; reaction bath, 50°C, detection, 520 nm; sample size, 80 μl

Determination of citrulline in plasma

A 100-μl aliquot of plasma was mixed with 200 μl of 10% (w/v) TCA. After standing for 10 min, the solution was centrifuged at 10 000 g for 3 min and 80 μl of the supernatant were analysed by HPLC.

Determination of homocitrulline in urine

A 4-ml volume of urine, the pH of which was adjusted to *ca.* 2 by 8 *M* hydrochloric acid, was applied to a column (20 mm × 5 mm I.D.) of Amberlite CG-120 (H$^+$ form). The column was washed with 2.0 ml of water, and then homocitrulline was eluted with 0.1 *M* Tris-HCl buffer containing 1.0 *M* sodium chloride (pH 7.6); the first 1.0 ml was discarded and the next 1.0 ml was collected. To the eluate, 10 μl of 8 *M* hydrochloric acid were added and a 80-μl portion of the solution was analysed by HPLC.

Determination of urea in plasma

A 10-μl portion of plasma was mixed with 500 μl of 1 mg/10 ml urease solution. The solution was kept for 10 min at 50°C. To the reaction solution were added 1.5 ml of water, 1.0 ml of 2% phenol containing 0.01% sodium nitroprusside (SNP) and 1.0 ml of 1.0% sodium hydroxide containing 0.2% sodium hypochlorate. The reaction mixture was kept for 10 min at 50°C, and its absorbance was measured at 635 nm against a blank solution.

RESULTS AND DISCUSSION

Detection of citrulline and homocitrulline using the OPA/NED reaction

In the OPA/NED reaction reported by Jung *et al.* [12], the sample solution is mixed with an OPA reagent and a NED reagent, successively. For post-column
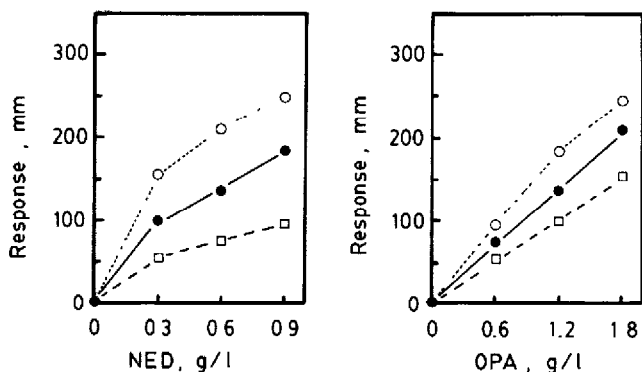

Fig. 2. Effects of OPA and NED levels on the OPA/NED reaction. (Left) OPA reagent, 1 0 *M* H$_2$SO$_4$ containing 0.1% Brij 35 and OPA (○, 1 8 g/l, ●, 1 2 g/l, □, 0.6 g/l); NED reagent, 1 0 *M* H$_2$SO$_4$ containing 0.1% Brij 35 and NED. (Right) OPA reagent, 1.0 *M* H$_2$SO$_4$ containing 0.1% Brij 35 and OPA, NED reagent, 1 0 *M* H$_2$SO$_4$ containing 0.1% Brij 35 and NED (○, 0 9 g/l, ●, 0.6 g/l, □, 0.3 g/l). Other conditions as in Fig 1.

HPLC, the reaction conditions were restricted as follows: the concentration of sulphuric acid was 0.5 M, the flow-rate of eluent was 0.4 ml/min, the flow-rate of each reagent was 0.2 ml/min, and the reaction time was 150 s (10 m × 0.5 mm I.D., PTFE tube). In the flow injection analytical system, the effects of the OPA and NED concentrations on this reaction are shown in Fig. 2. The sensitivity increased with the OPA concentration, although the absorbance of the reagent blank at 520 nm increased.

The effect of the reaction temperature is shown in Fig. 3. A noise peak became greater as the temperature was raised above 50°C. The flow diagram of the recommended post-column HPLC is shown in Fig. 1.
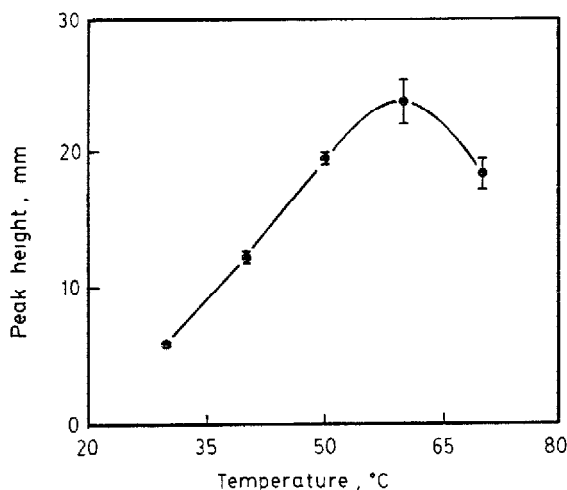

Fig 3 Effect of temperature on the OPA/NED reaction for N-carbamyl compound detection The temperature of the reaction bath in the flow injection analytical system was varied in the range 30–70°C. Sample, standard mixture solution of citrulline and homocitrulline (each concentration 250 μM); sample size, 80 μl, reaction coil, 10 m × 0 5 mm I D ; detection, 520 nm (0 16 a u f s) Other conditions as in Fig 1.

HPLC separation of citrulline and homocitrulline

Citrulline, homocitrulline and urea were separated by HPLC with a cation-exchange resin column (TSK gel SCX, 150 mm × 4 mm I.D.). Citrate buffer solution, which did not interfere with the OPA/NED reaction, was used as the eluent. Typical chromatograms of citrulline and homocitrulline in the presence of a large amount of urea and protein amino acids are shown in Fig. 4.

When the urea concentration was below 30 mM it did not interfere with the assay of citrulline and homocitrulline, as it was eluted with the solvent front and λ_{max} of the reaction product of urea differed from those of citrulline and homocitrulline [14]. The protein amino acids were not detected

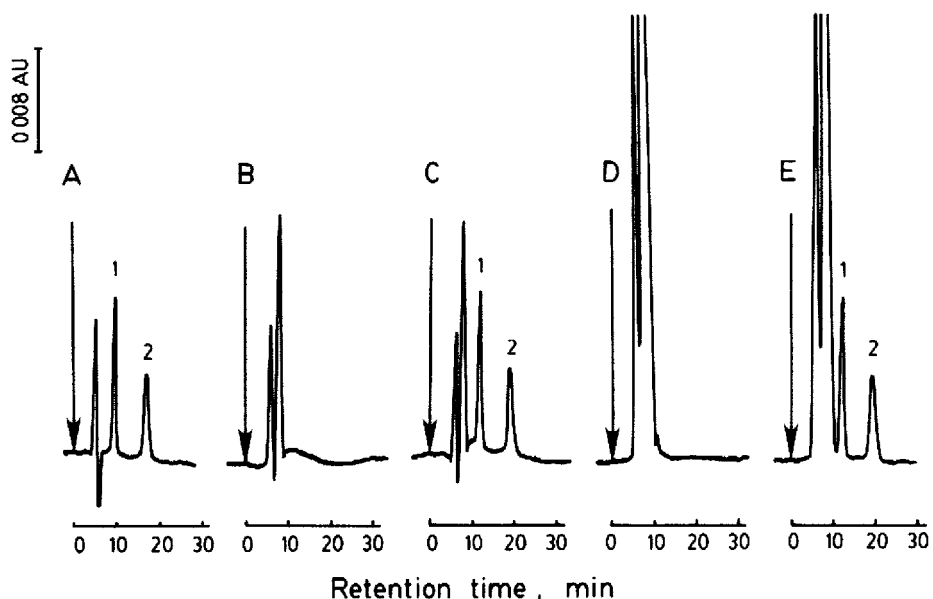The calibration curve for citrulline was linear in the range 2.6–500 μM and that

Fig 4 Separation of citrulline and homocitrulline in the presence of amino acids and urea (A) Standard solution mixture of citrulline (33 μM) and homocitrulline (33 μM), (B) solution mixture of eighteen amino acids (each amino acid, 2.5 mM); (C) solution mixture of eighteen amino acids (each amino acid, 2.5 mM), citrulline (33 μM) and homocitrulline (33 μM); (D) urea solution (33 mM); (E) solution mixture of urea (33 mM), citrulline (33 μM) and homocitrulline (33 μM). Peaks 1 = citrulline, 2 = homocitrulline

for homocitrulline was linear in the range 4.3–500 μM (sample volume, 80 μl). When 20 and 200 μM citrulline and homocitrulline were determined ten times by the recommended procedure, the relative standard deviations were below 2.0%.

Determination of citrulline in plasma

Arginine is biosynthesized in the mammalian liver. However, the liver cannot supply arginine to the other organs because of the extremely high level of arginase activity [3]. The kidney may play an important role in the supply of arginine to the other organs [3]. Thus, the liver synthesizes citrulline and releases it into blood, and it is taken up by the kidney and transformed into arginine. This phenomenon indicates that the citrulline is present in the plasma, and its level in the plasma of patients with renal failure is higher than that in the plasma of healthy subjects. Analytical results for citrulline in the plasma of patients with uremia on intermittent hemodialysis and of healthy subjects are shown in Fig. 5. The citrulline level in the plasma of patients is higher than that in the plasma of healthy subjects and is dependent on that of urea (r = 0.821). Homocitrulline was not detected in either the plasma of healthy subjects or the plasma of patients with uremia.
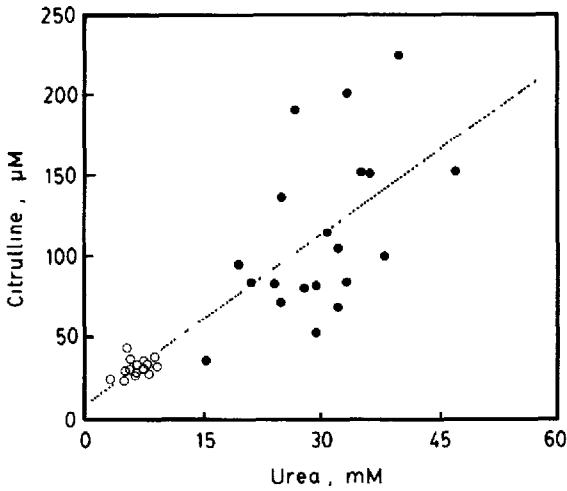
Fig 5 Correlation between urea levels and citrulline levels in the plasma of healthy subjects (○) and uremic patients on intermittent hemodialysis (●)

TABLE I

RECOVERY OF CITRULLINE AND HOMOCITRULLINE FROM HUMAN URINE

Each value represents the mean ± S.D. of three analyses

Compound	Added (μM)	Found (μM)	Recovery (%)
Citrulline	—	4.78 ± 0 42	—
	10 0	11.9 ± 0.78	70 9 ± 7.8
	20.0	19 7 ± 0.53	59 6 ± 2 1
Homocitrulline	—	24 2 ± 0.77	—
	10 0	33.3 ± 0 75	91.2 ± 7.7
	20.0	47 2 ± 1 88	91.9 ± 7 5

TABLE II

ANALYTICAL RESULTS FOR HOMOCITRULLINE IN HUMAN URINE

Each value represents the mean ± S.D. of samples from eight men (22–23 years).

Compound	Concentration
Creatinine	1 75 ± 1.21 g/l
Homocitrulline	7 20 ± 2 27 μmol/l
	5 05 ± 1 73 μmol/g of creatinine

Determination of homocitrulline in human urine

Homocitrulline is present in the urine of infants [4] and in the urine of patients with hyperornithinemia [5]. It is thought that homocitrulline may be synthesized by the lysine cycle. Whether homocitrulline is present in human adult urine has not been elucidated

The procedure for the pretreatment of urine using a cation-exchange resin is described in Experimental. The results of recovery tests from human adult urine are shown in Table I. The recoveries of homocitrulline varied from 91 to 92%. However, the recoveries of citrulline are not good because of its weak affinity for a cation-exchange resin. The analytical results for homocitrulline in the urine of human adults (22–31 years) are shown in Table II, which shows that a small amount of homocitrulline is excreted into healthy adult urine.

HPLC using the OPA/NED reaction for post-column detection is applicable to the analysis of citrulline and homocitrulline in biological materials containing a large amount of protein amino acids. Furthermore, it may be possible to determine citrulline and homocitrulline in protein hydrolysates.

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