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Short communication

Precise analysis of primary amino acids in urine by an automated high-performance liquid chromatography method: comparison with ion-exchange chromatography

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Abstract

A precise, simple and rapid method for the quantitative determination of primary amino acids in urine based on high-performance liquid chromatography and *o*-phthaldialdehyde pre-column derivatization is described. All primary urinary amino acids could be determined within 49 min (injection to injection). Amino acid concentrations in 40 urinary samples were measured by this method and the results were compared with those measured by ion-exchange chromatography. The correlation coefficient for the common amino acids was greater than 0.90. This is the first study in which such a detailed comparison has been made on urine samples. It appeared that the method described is an excellent alternative to the classical ion-exchange method for the quantitation of urinary amino acids.

Keywords: Amino acids

1. Introduction

Many reversed-phase high-performance liquid chromatography (HPLC) methods for the analysis of amino acids in urine have been described in the literature [1–9]. However, overall resolution was mostly poor which resulted in unreliable amino acid values due to the existence of much more interfering peaks in urine than in plasma samples. Moreover, in none of these studies have satisfactory comparisons between HPLC and classical ion-exchange procedures been made. Although in the study of Qureshi et al. [3] quantitative amino acid data are given, no correlations between amino acids measured by both methods have been given. Important issues for the analysis of urinary amino acids are: (1) a method exhibiting a very good resolution for over 40 amino acids because of the complex sample matrix; (2) a very sensitive technique because of the low concentrations of some amino acids occurring in urine; (3) low within-laboratory variabilities for the amino acids measured [10]. Furthermore, the method should be fast, simple and fully automated. Therefore, we developed a relatively rapid, simple and precise HPLC method for the quantitation of urinary amino acids, by using automated pre-column derivatization with *o*-phthaldialdehyde (OPA). Moreover, we com-

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pared the absolute values of many amino acids obtained with our HPLC method with an established ion-exchange method in 40 urine samples. To our knowledge this is the first study in which such thorough comparisons have been made on urine samples.

2. Experimental

2.1. Equipment

The HPLC system, the separation column (5-μm Spherisorb ODS 2 cartridge, 125×3 mm) and the chromatographic conditions were essentially the same as described earlier [11]. The only difference was the detector used, which was in this study a Jasco Model FP-920 fluorescence detector (B&L Systems, Maarssen, The Netherlands) equipped with a 150-W xenon lamp and a 16-μl flow cell. The ion-exchange method made use of the Biochrom 20 chromatography system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), equipped with 5 Ultropac buffers and a PEEK column, and post-column detection with ninhydrin [12]. Both the 440- and 570-nm signals were used for quantitation of the amino acids by measuring areas relative to the internal standard norleucine; total run time was 161 min.

2.2. Reagents, solvents and derivatization procedure

These were also the same as described earlier [11], except for a minor modification in the derivatization reagent. This reagent was prepared by dissolving 50 mg of OPA in 1 ml of methanol, adding 18 ml of deoxygenated (30 min bubbling with nitrogen) sodium borate buffer (0.2 mol/l, pH 10.4), 50 μl of 2-mercaptoethanol, 10 mg of nitrilotriacetic acid (Sigma–Aldrich, St. Louis, MO, USA) and 50 μl of Brij 35. The addition of nitrilotriacetic acid markedly improved the stability of this reagent by acting as a metal chelating agent [13]. For analysis the OPA-reagent was used directly without further dilution.

2.3. Sample preparation

Urine was obtained from 40 randomly selected patients (26 men and 14 women; age range, 0–53 years) and stored frozen at −24°C. For the HPLC analysis, urine samples (500 μl) were deproteinized with 50 μl of 12% (w/v) 5-sulfosalicylic acid, containing 1 m*M* norvaline and homoserine. After immediate vortex-mixing and standing for 15 min at 4°C, the precipitate was spun down (15 min at 18 400 g_{max}). Next, 400 μl of the supernatant was transferred to a 0.8-ml polypropylene vial and was mixed with 100 μl of 0.3 *M* LiOH in order to adjust the pH to 2.5. For analysis with the ion-exchange method, the deproteinization of 500 μl of urine was performed with 500 μl of 5% (w/v) 5-sulfosalicylic acid, containing 0.5 m*M* norleucine. After immediate vortex-mixing, the precipitate was spun down (10 min at 10 000 g_{max}) and the supernatant was filtered through a 0.2-μm membrane filter.

2.4. Assay performance

Quality of the analytical data was assessed by assaying 40 urine samples by the HPLC and the ion-exchange method and compare the results of both methods.

3. Results and discussion

Linearity and limit of detection of the HPLC method were the same as described earlier for the plasma method [11]. The precision and accuracy of the urine HPLC method was only determined for some amino acids, because this method—although applied to a different matrix—is essentially the same as the plasma method. The precision (reported as relative standard deviation) was below 5%. The accuracy (calculated as percentage of mean deviation from actual concentration) was found to be well within the acceptable limits of between −8 and +9%. Because the ion-exchange method made use of a commercially available amino acid analyzer, no validation data have been created for this method.

The correlation between both procedures was for most amino acids higher than 0.90 (Table 1). An

Table 1
Correlation of HPLC and ion-exchange analysis of urinary amino acids

Amino acid	*n*	*r*	*P*	Slope	Concentration range (μmol/l)
α-Aminoadipic acid	36	0.8283	<0.0001	0.5489	3–121
α-Aminobutyric acid	25	0.7803	<0.0001	0.5987	3–35
Alanine	38	0.9743	<0.0001	0.9796	46–394
Arginine	23	0.2593	0.2322	0.4526	3–66
Asparagine	36	0.9870	<0.0001	0.9883	11–165
Aspartic acid	39	0.5962	<0.0001	0.2409	1–183
β-Alanine	11	0.6498	0.0305	0.6937	5–114
β-Aminoisobutyric acid	33	0.8112	<0.0001	0.7391	12–687
Carnosine	17	0.3038	0.2358	0.0839	13–831
Citrulline	17	0.9784	<0.0001	1.0930	1–75
Cystathionine	18	0.8767	<0.0001	0.5667	13–123
Ethanolamine	7	0.9919	<0.0001	1.0940	55–348
Glutamic acid	31	0.8752	<0.0001	1.8030	2–68
Glutamine	40	0.9760	<0.0001	1.0360	35–1280
Glycine	38	0.9891	<0.0001	1.1300	157–3064
Histidine	39	0.9876	<0.0001	1.1370	33–1194
Hydroxylysine	37	0.4552	0.0046	0.2664	5–86
Isoleucine	27	0.8770	<0.0001	0.7925	3–43
Leucine	34	0.8909	<0.0001	0.8281	5–121
Lysine	38	0.9635	<0.0001	1.0360	11–568
1-Methylhistidine	15	0.9813	<0.0001	1.1590	40–739
3-Methylhistidine	40	0.9775	<0.0001	1.1420	11–185
Ornithine	34	0.9772	<0.0001	0.9056	4–227
Phenylalanine	38	0.9671	<0.0001	1.0090	3–75
Serine	39	0.9907	<0.0001	1.0440	14–820
Taurine	40	0.9832	<0.0001	1.0960	8–1564
Threonine	39	0.9959	<0.0001	0.9577	9–980
Tyrosine	38	0.9663	<0.0001	1.1200	7–126
Valine	19	0.7270	0.0004	0.7605	3–128

example for the amino acids alanine and lysine is shown in Fig. 1. This figure also shows that, even at very high concentrations, correlation is excellent. The correlation coefficient was only lower for amino acids occurring at rather low concentrations in the urinary samples: α-aminoadipic acid (0.828), α-aminobutyric acid (0.780), arginine (0.259), β-alanine (0.650), β-aminoisobutyric acid (0.811), carnosine (0.304), cystathionine (0.877), glutamic acid (0.875), hydroxylysine (0.455), isoleucine (0.877), leucine (0.891) and valine (0.727; Table 1). The only exception was aspartic acid ($r=0.596$), the concentration of which was consistently higher with the ion-exchange method (resulting in a very low slope, see Table 1). When the urinary aspartic acid concentrations were expressed as mmol/mol creatinine, the data obtained by the HPLC procedure (0.2–29.2 mmol aspartic acid/mol creatinine) were in the normal range (2–12 mmol aspartic acid/mol creatinine), while the ion-exchange data were much higher (11.7–103.8 mmol aspartic acid/mol creatinine). This indicates that, with the ion-exchange method, at least with the operation conditions of the Biochrom 20 chromatography system used in this study, some unknown compound coelutes completely with aspartic acid.

On the other hand, the HPLC method slightly overestimated the glutamic acid level in urine because of the presence of an unknown interfering compound (high slope, see Table 1). Another problem of this method is that sometimes ammonia interferes with the determination of valine and methionine (Fig. 2A). To eliminate this interference, urine specimens containing extremely high concen-

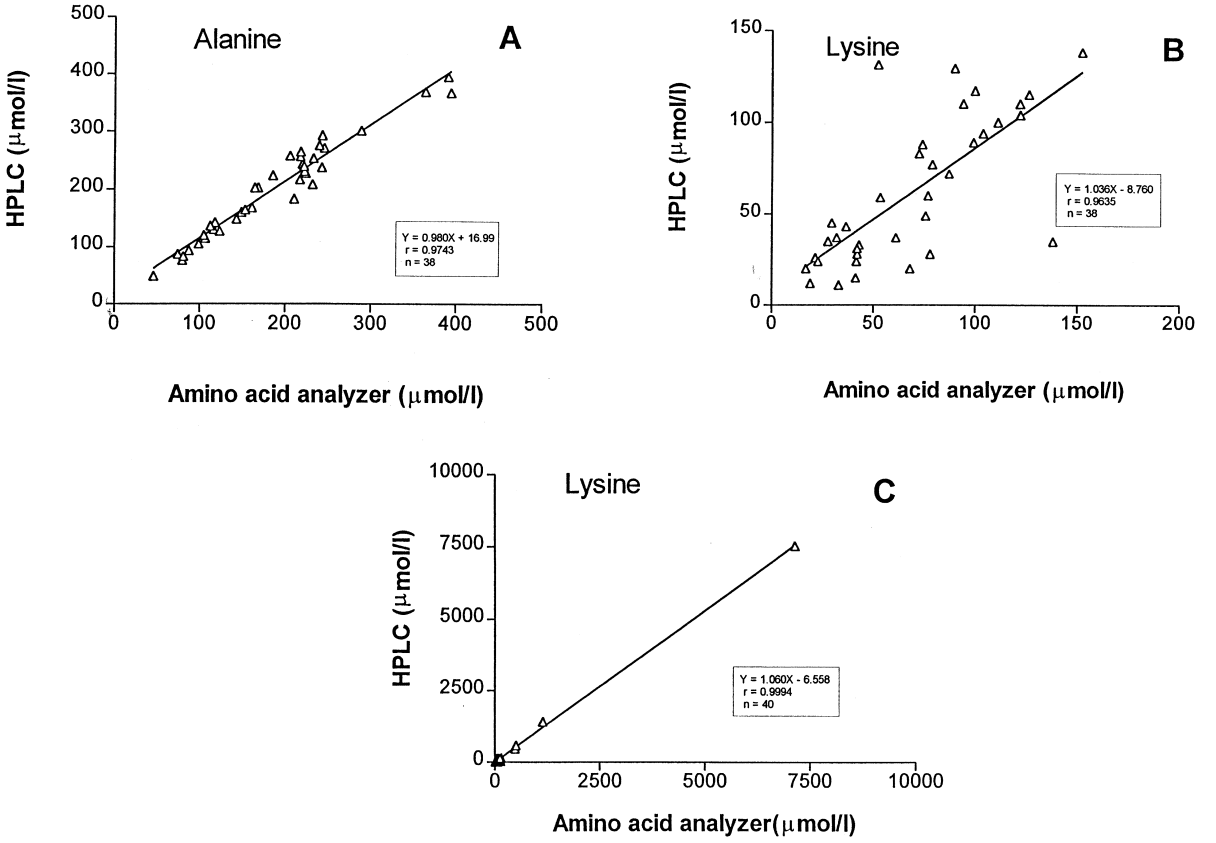

Fig. 1. Correlation between the HPLC and amino acid analyzer method for the determination of (A) 0–500 μ*M* alanine, (B) 0–200 μ*M* lysine and (C) 0–8000 μ*M* lysine in a urine sample. The HPLC method is based on reversed-phase HPLC and automated pre-column derivatization with *o*-phthaldialdehyde, and makes use of a 5-μm Spherisorb ODS 2 column (125×3 mm). A ternary gradient system and a flow-rate of 1.0 ml/min were employed and quantitation was done by measuring peak heights relative to the internal standard norvaline. Total run time was 49 min. The amino acid analyzer method is based on ion-exchange chromatography and makes use of the Biochrom 20 chromatography system, equipped with 5 Ultropac buffers and a PEEK column, and post-column detection with ninhydrin. Both the 440- and 570-nm signals were used for quantitation of the amino acids by measuring areas relative to the internal standard norleucine; total run time was 161 min.

trations of ammonia were pretreated with tetraphenylboron before the derivatization procedure [14]. In this manner the ammonia peak was significantly reduced, enabling the quantitative determination of both valine and methionine without any problem. It is, however, not recommended to pretreat the amino acid standard solution with tetraphenylboron because of a time-dependent decrease in histidine, the methylhistidines, arginine, cystathionine, ornithine, lysine, carnosine and anserine.

In case of the ion-exchange method, the quantitative determination of some amino acids was hampered by the presence of interfering peaks caused by medication. The presence of (metabolites of) antibiotics, for instance, interfered with the determination of tyrosine or phenylalanine. Methionine and homocitrulline can coelute (Fig. 2B), however, by changing some chromatographic conditions (column temperature, buffer times) these compounds can be resolved. Due to its higher sensitivity, the measurement of very low amino acid levels was more precise in the HPLC method. An example of an amino acid chromatogram obtained by both methods is shown in Fig. 2. It can be seen that more peaks appear in the case of the HPLC method. However, most peaks are

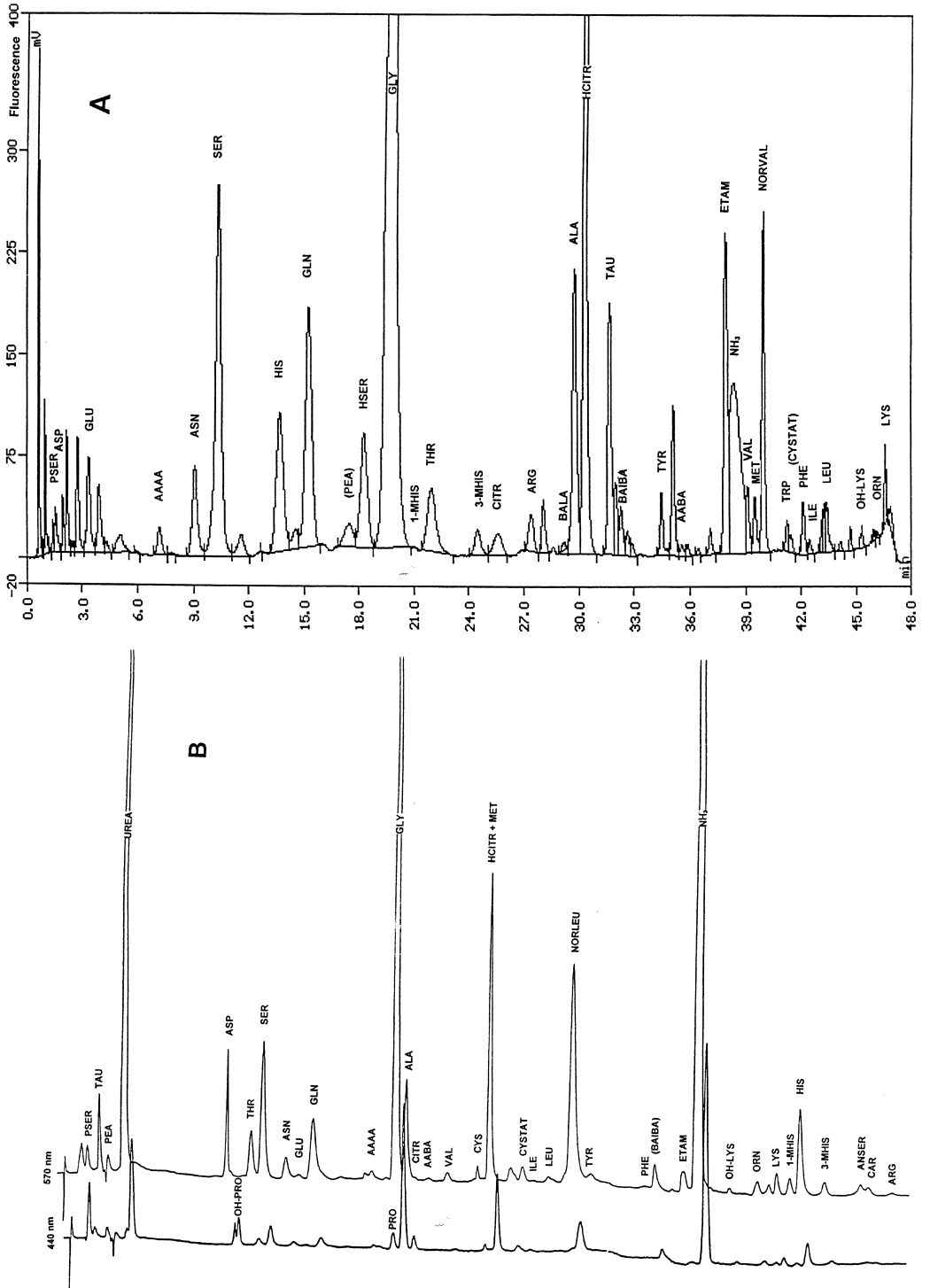

Fig. 2. Amino acid chromatograms of a human urine sample measured by (A) the HPLC method and (B) the ion-exchange method. The chromatographic conditions are the same as described in the legend to Fig. 1.

fully resolved through which interference is minimal and, therefore, reliable amino acid values are obtained with this method.

In conclusion, one might say that the high sensitivity, accuracy, speed and relative insensitivity for exogenous factors make this HPLC method an excellent alternative to the classical ion-exchange method. A disadvantage of the HPLC method is that secondary amino acids and cystine/homocystine cannot be measured. In addition, the presence of high levels of ammonia interfere with the determination of valine and require pretreatment of the sample.

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