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Note

Determination of *m*-tyrosine and *o*-tyrosine in human serum by high-performance liquid chromatography with fluorimetric detection

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L-Phenylalanine is metabolized largely by conversion to *p*-tyrosine in mammals [1]. In addition, many authors have reported the presence of *m*-tyramine (3-hydroxyphenylethylamine) [2, 3] and *o*-tyramine (4-hydroxyphenylethylamine) [4—6] in human urine, and it has been demonstrated that all these compounds are derived from *m*-tyrosine (3-hydroxyphenylalanine) and *o*-tyrosine (4-hydroxyphenylalanine) in the human body [7—9]. Thus, it seems probable that *m*-tyrosine and *o*-tyrosine are formed in mammals. Recently, we reported the existence of both compounds in rat serum [10]. However, the existence of *m*-tyrosine and *o*-tyrosine in the human body has not been clarified as yet.

This report describes the determination of *m*-tyrosine and *o*-tyrosine in human serum by a highly sensitive and selective method, utilizing high-performance liquid chromatography (HPLC) with fluorimetric detection.

EXPERIMENTAL

Chemicals

L-Phenylalanine, L-*p*-tyrosine, D,L-*m*-tyrosine and D,L-*o*-tyrosine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and purchased from commercial sources. Milli-Q II system (Millipore, Bedford, MA, U.S.A.) ultrapure water was used throughout this study.

Chromatography

A Hitachi 638-50 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) was used with a Hitachi 650-10S fluorimetric detector. The separations

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were achieved on a C_{18} reversed-phase column (Cosmosil ODS, particle size 5 μm, 250 × 4.6 mm I.D.; Nakarai Chemicals, Kyoto, Japan). The eluent was 1% acetic acid containing 1% sodium chloride at a flow-rate of 0.8 ml/min. The fluorescence was monitored with excitation at 258 nm and emission at 288 nm for phenylalanine, and excitation at 275 nm and emission at 305 nm for *p*-tyrosine, *m*-tryrosine and *o*-tyrosine. All separations were carried out at room temperature.

A Hitachi 835 amino acid analyser was used. The eluent was an MCI buffer (Mitsubishi Chemical Industries, Tokyo, Japan) for physiological fluid analysis.

Sample preparation

The sixteen subjects investigated (seven men, nine women) were our university students. The blood samples (venous blood) were drawn between 10.00 and 11.00 a.m. and centrifuged at 1000 *g* for 5 min to obtain serum samples. A 2-ml serum sample was deproteinized by the addition of 0.5 ml of 1.0 *M* trichloroacetic acid. The mixture was centrifuged at 12 000 *g* for 10 min at 4°C. After filtration through a 0.22-μm membrane filter, 10—100 μl of the clear supernatant were directly injected into the liquid chromatograph.

Quantification

The equations for the calibration curves were obtained by linear regression analysis. Peak areas increased linearly with concentrations up to approximately 10—250 ng/ml for *m*-tyrosine and *o*-tyrosine and 5—250 μg/ml for *p*-tyrosine and phenylalanine, and the correlation coefficients (r) were 0.994, 0.991, 0.998 and 0.994, respectively. The lower limit of detection (signal-to-noise ratio of 2.5) was approximately 0.75 ng/ml for *p*-, *m*- and *o*-tyrosine and 250 ng/ml for phenylalanine, and the coefficients of variation (C.V.) were below 7.9%. The concentrations of the compounds in serum were determined from peak areas by comparison with standards which were carried through the whole sample preparation procedure. Reproducibility was assured from repeated injections of standard solutions on the same day. The coefficients of variation for the standards were 1—4%.

Analytical recovery and assay precision

Recoveries were determined over the serum concentration range of 10, 20 and 30 ng/ml for *m*- and *o*-tyrosine, and of 10, 30 and 50 μg/ml for *p*-tyrosine and phenylalanine. They ranged from 95.9 to 107.3%, with a mean value of 101.6 ± 3.5% (S.D.). The intra- and inter-assay coefficients of variation ranged from 2.1 to 6.2% and 4.2 to 7.2%, respectively. To determine the possibility of interference from other compounds which may be present in serum, the assay selectivity was evaluated by assaying serum samples containing 5 ng/ml of the following compounds: norepinephrine, epinephrine, normetanephrine, 3,4-dihydroxyphenylalanine, dopamine and metanephrine. The above compounds did not interfere with the determination of *p*-tyrosine, *m*-tyrosine, *o*-tyrosine and phenylalanine.

RESULTS AND DISCUSSION

As shown in Fig. 1A, *p*-tyrosine, *m*-tyrosine, *o*-tyrosine and phenylalanine are completely separated in 23 min. The peaks represent 2.5 ng of *p*-, *m*- and *o*-tyrosine, and 2.5 μg of phenylalanine.

Fig. 1B shows a typical chromatogram of a serum sample. *m*-Tyrosine and *o*-tyrosine were detected in serum in addition to *p*-tyrosine and phenylalanine. The peaks 1—4 in Fig. 1B were identified by two methods. First, the peaks of the tyrosine isomers and phenylalanine were characterized on the basis of their HPLC retention behaviour and co-injection with the reference compounds. Then, the effluent corresponding to each peak was collected and subjected to ion-exchange chromatography using an amino acid analyser. The retention times of each peak were identical with those of the corresponding authentic samples.

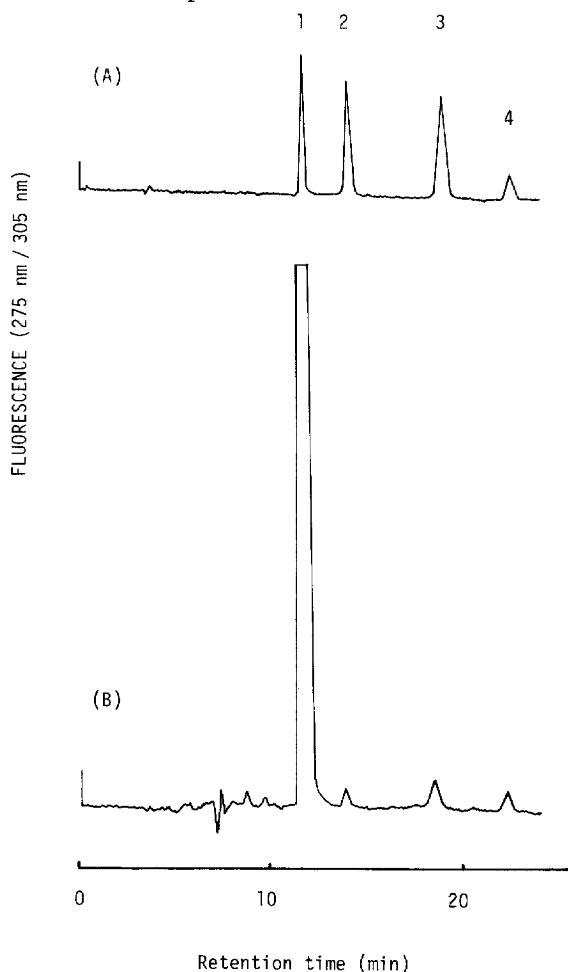

Fig. 1. Chromatograms of (A) a synthetic mixture of reference compounds and (B) a serum sample from a healthy subject. Injection volume was 100 μl. The concentrations in B were 3.1 ng/ml *m*-tyrosine, 3.4 ng/ml *o*-tyrosine and 10.14 μg/ml phenylalanine. Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

Serum concentrations (mean ± S.D.) of *m*-tyrosine and *o*-tyrosine determined from sixteen healthy subjects were 2.6 ± 1.2 and 3.1 ± 0.9 ng/ml, respectively. No significant difference between the concentrations of *m*-tyrosine and *o*-tyrosine was observed between the sexes. The rat serum concentrations of *m*-tyrosine and *o*-tyrosine were 3.3 ± 1.9 and 3.6 ± 1.8 ng/ml, respectively [10]. In addition, the mean values of *p*-tyrosine and phenylalanine in human serum were found to be 9.45 ± 1.05 and 9.64 ± 0.76 μg/ml, respectively.

The above-described HPLC assay with fluorimetric detection provides a fast, reliable and simple method for the determination of serum levels of *m*-tyrosine and *o*-tyrosine.

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