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## Note

# High-performance liquid chromatographic determination of *m*-tyrosine and *o*-tyrosine in rat urine

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Many authors have reported the presence of *m*- and *o*-substituted phenol derivatives of tyramine [1,2], octopamine [3,4] and hydroxymandelic acid [5,6] of endogenous origin in human urine and adrenal gland, and it has been demonstrated that these compounds are derived from *m*-hydroxyphenylalanine (*m*-tyrosine) and *o*-hydroxyphenylalanine (*o*-tyrosine) [7,8]. These findings suggest that *m*-tyrosine and *o*-tyrosine may be formed in mammals. In fact, *m*-tyrosine and *o*-tyrosine have been detected in rat and human serum [9,10]. In addition, we have reported the formation of *m*-tyrosine and *o*-tyrosine from L-phenylalanine and have suggested that the conversion of phenylalanine to *m*-tyrosine and *o*-tyrosine may be catalysed by phenylalanine hydroxylase (EC 1.14.16.1) and tyrosine hydroxylase (EC 1.14.16.2) [11]. However, there have been no reports on the determination of *m*-tyrosine and *o*-tyrosine from the urine.

The purpose of this study is firstly to describe a method that permits rapid, simple and reproducible extraction of urinary *m*-tyrosine and *o*-tyrosine by using Bond Elut SCX and $C_{18}$ minicolumns and, secondly, to report the values for urinary *m*-tyrosine and *o*-tyrosine excretion.

## EXPERIMENTAL

### *Materials*

L-Phenylalanine, L-*p*-tyrosine, DL-*m*-tyrosine, DL-*o*-tyrosine and α-methyl-DL-*p*-tyrosine were obtained from Sigma (St. Louis, MO, U.S.A.), Bond Elut $C_{18}$ and Bond Elut SCX columns from Analytichem International, (Harbor City, CA, U.S.A.), DL-ethionine from Wako (Osaka, Japan) and DL-*p*-chlorophenylalanine from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of

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the highest purity commercially available. Milli-Q II system (Millipore, Bedford, MA, U.S.A.) ultrapure water was used throughout.

*Apparatus*

A Hitachi Model 635 high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F-1000 fluorescence spectromonitor equipped with a 12-$\mu$l flow-cell were used. 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*-, *m*- and *o*-tyrosine. Peak areas were calculated by a Hitachi Model D-2000 chromato-integrator. Other chromatographic conditions were as described previously [10].

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

*Animals and samples*

Adult male Wistar rats weighing 200–250 g were fasted for 16 h prior to the experiment but had free access to water. The rats were administered intraperitoneally (i.p.) *p*-chlorophenylalanine (720 mg/kg) and ethionine (80 mg/kg) and/or $\alpha$-methyltyrosine (250 mg/kg). On the next day, L-phenylalanine (80 mg/kg) in saline was adminstered i.p. to the rats. Urine (24 h) was collected and stored at 4 °C if assayed within a week or at −20 °C if kept longer before analysis.

*Extraction procedure*

A urine sample (2 ml) was adjusted to pH 8.5 with 0.5 *M* sodium hydroxide and applied to a Bond Elut $C_{18}$ minicolumn prewashed with methanol and water. Phenylalanine and *p*-, *m*- and *o*-tyrosine were eluted with water. The eluate was adjusted to pH 2 with 0.5 *M* hydrochloric acid and applied to a Bond Elut SCX cation-exchange column prewashed with methanol and water. Amino acids were eluted from the column with 10 ml of 0.5 *M* hydrochloric acid and evaporated to dryness at 37 °C under reduced pressure. The residue was dissolved in 2 ml of 1% acetic acid, and an aliquot of the solution was injected into the HPLC column.

*Calibration for the quantification in urine*

The calibration graphs were obtained by assaying spiked urine samples and subsequent extraction according to the above procedure. At least four different standard concentrations were used and analysed twice. The concentrations of standards were similar to those reported for the recovery assay. The compounds in urine were determined by external calibration.

## RESULTS AND DISCUSSION

*Chromatography*

As shown in Fig. 1A, *p*-, *m*- and *o*-tyrosine and phenylalanine are completely separated in 20 min. The peaks represent 0.2 $\mu$g of *p*-tyrosine, 0.2 ng of *m*- and *o*-tyrosine and 0.4 $\mu$g of phenylalanine. Reproducibility was estimated from repeated injections of standard solutions on the same day. The coefficients of vari-

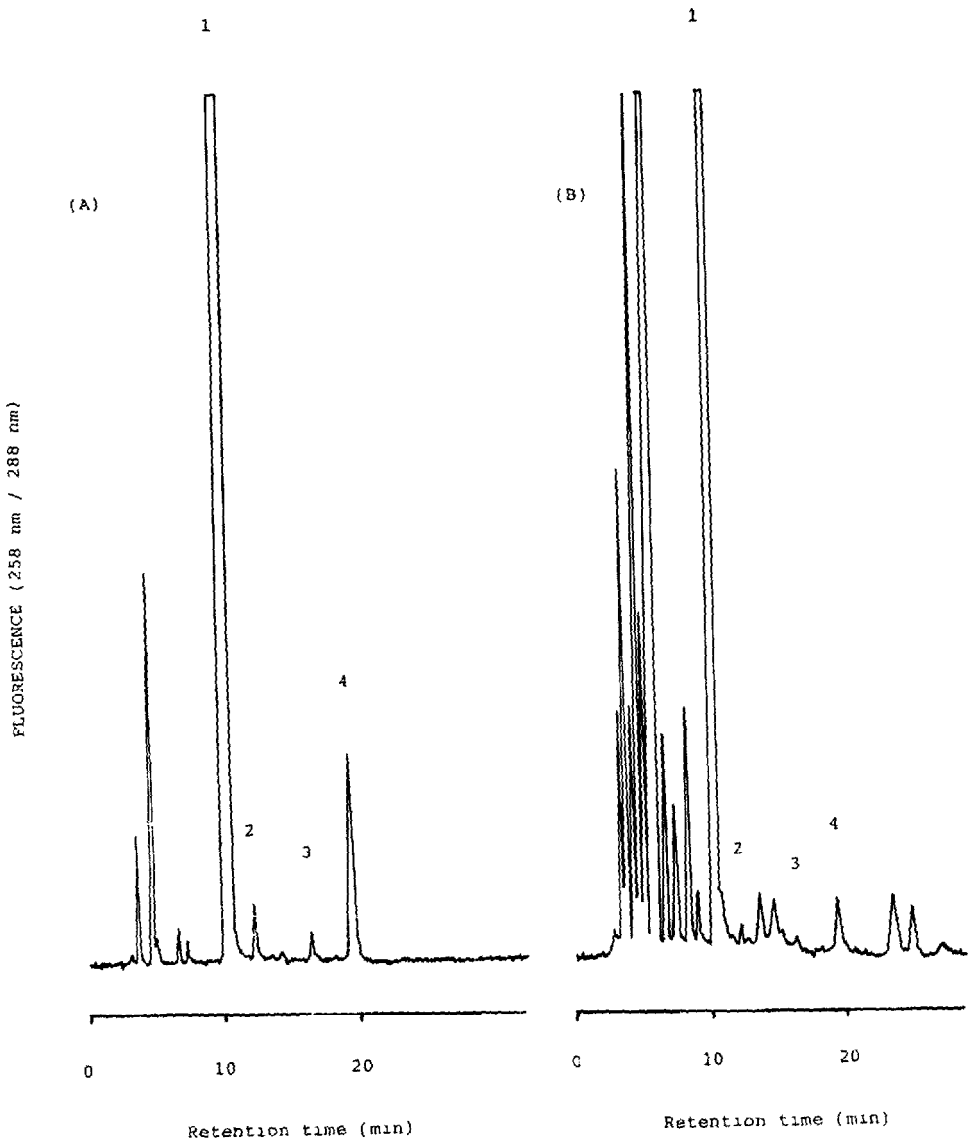


Fig. 1. Chromatograms of (A) a synthetic mixture of reference compounds and (B) a urine sample after administration of *p*-chlorophenylalanine and ethionine. Injection volume was 100 μl. The concentrations in (A) were 2 μg/ml for *p*-tyrosine, 2 ng/ml for *m*-tyrosine, 2 ng/ml for *o*-tyrosine and 4 μg/ml for phenylalanine. Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

ation for the standards were 1–3%. Peak areas increased linearly with concentrations up to ca. 2.5–100 ng/ml for *m*- and *o*-tyrosine, 2.5–80 μg/ml for *p*-tyrosine and 5–120 μg/ml for phenylalanine. All coefficients of correlation were at least 0.990.

Fig. 1B shows a typical chromatogram of a urine sample following the administration of a phenylalanine hydroxylase inhibitor. No internal standards were used because (i) α-methyltyrosine investigated for this purpose was used as in-

hibitor of tyrosine hydroxylase, (ii) good precision was obtained with the external standard method and (iii) the extraction efficiencies determined from spiked urine samples were quantitative and reproducible.

The peaks 1–4 in Fig. 1B were identified by two methods. First, the peaks of *p*-, *m*- and *o*-tyrosine and phenylalanine were identified on the basis of HPLC retention behaviour and co-injection with reference compounds. Then the corresponding peaks were collected and subjected to ion-exchange chromatography using an amino acid analyser. As shown in Fig. 2, *m*-tyrosine, *p*-tyrosine, phenylalanine and *o*-tyrosine were eluted at 114, 119, 127 and 129 min, respectively. The retention time of each peak was identical with that of the corresponding authentic sample. These results imply that the present method is not subject to interferences by coexisting substances and is suitable for the determination of *p*-, *m*- and *o*-tyrosine and phenylalanine.

*Analytical parameters*

To assess the precision of the analytical procedure, both intra-assay and inter-assay variations were examined. Intra-assay variation was determined in quadruplicate at two concentrations: 5 and 20 μg/ml for *p*-tyrosine and phenylalanine and 2.5 and 5 ng/ml for *m*-tyrosine and *o*-tyrosine. Inter-assay variation was determined at the same two concentrations from four replicate runs. The coefficients of variation in the intra-assay study varied between 2.9 and 13.5%, whereas that in the inter-assay ranged from 4.5 to 13.9%.

To check the recovery, urine samples spiked with known amounts of *p*-tyrosine (2.5 μg/ml), *m*-tyrosine (20 ng/ml), *o*-tyrosine (20 ng/ml) and phenylalanine (2.5 μg/ml) were analysed by the assay procedure. Mean recoveries of *p*-, *m*- and *o*-tyrosine and phenylalanine from urine were 83, 87, 81 and 106%, respectively.

*Daily urinary excretion of p-, m- and o-tyrosine and phenylalanine in rats*

Daily urinary excretion of *p*-, *m*- and *o*-tyrosine and phenylalanine was examined. Urinary concentrations (mean ± S.D.) of *m*-tyrosine and *o*-tyrosine determined from the seven samples of urine were 20.1 ± 5.8 and 21.2 ± 3.8 ng per day, respectively. The mean values of *p*-tyrosine and phenylalanine were found to be 16.6 ± 3.1 and 51.9 ± 7.8 μg per day, respectively. These values are similar to those described by many authors [12,13]. These results indicate that the urine levels of *m*-tyrosine and *o*-tyrosine detected in this experiment were reliable.

*Effect of administration of p-chlorophenylalanine, ethionine and α-methyltyrosine*

It was shown previously that phenylalanine hydroxylase activity was markedly inhibited by the administration of *p*-chlorophenylalanine and ethionine [14] and that tyrosine hydroxylase activity was suppressed by the administration of α-methyltyrosine [15]. The effects of *p*-chlorophenylalanine, ethionine and α-methyltyrosine are shown in Table I. The concentrations of *m*- and *o*-tyrosine in the urine were significantly increased by the administration of L-phenylalanine. This indicates that the administration of L-phenylalanine to rats stimulated the urinary excretion of *m*- and *o*-tyrosine, with a concomitant increase in the level of phenylalanine in the urine. The concentrations of *m*- and *o*-tyrosine in the

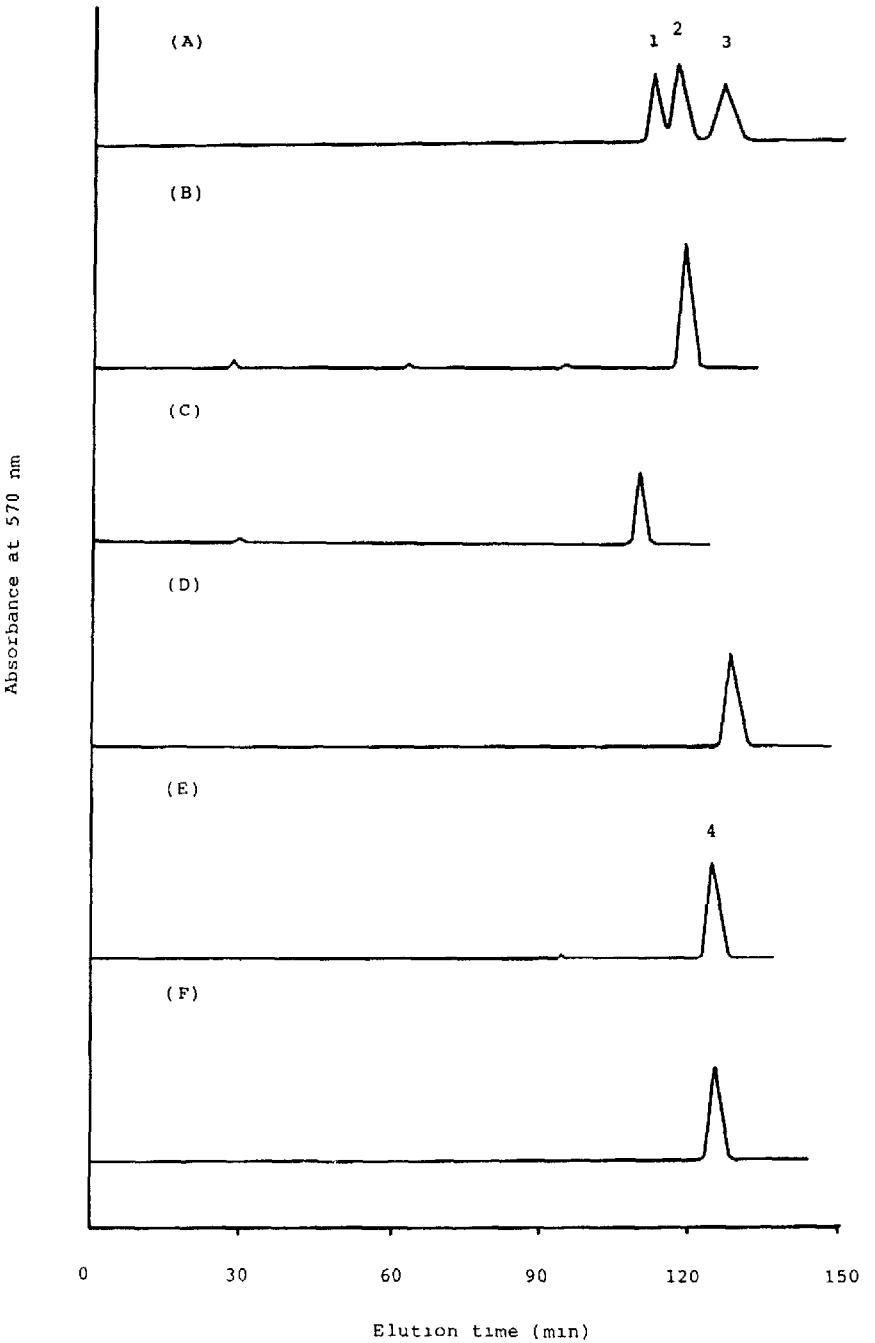


Fig. 2. Ion-exchange chromatograms of phenylalanine and tyrosine isomer fractions. The (B) *p*-, (C) *m*-, (D) *o*-tyrosine and (F) phenylalanine fractions collected, and authentic samples of tyrosine isomers (A) and phenylalanine (E) were subjected to ion-exchange chromatography. The concentrations in (A) and (E) were 12.5 nmol per 50 $\mu$l for *p*-, *m*- and *o*-tyrosine and phenylalanine. Peaks: 1 = *m*-tyrosine; 2 = *p*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

TABLE I

URINARY CONCENTRATIONS OF PHENYLALANINE, *p*-, *m*- AND *o*-TYROSINE IN RATS

The rats were administered intraperitoneally *p*-chlorophenylalanine (720 mg/kg), ethionine (80 mg/kg) and α-methyltyrosine (250 mg/kg), and urine was collected for 24 h after dosing. L-Phenylalanine was administered at the dose of 80 mg/kg.

| | Concentration | | | |
|---|---|---|---|---|
| | *p*-Tyrosine (μg/day) | *m*-Tyrosine (ng/day) | *o*-Tyrosine (ng/day) | Phenylalanine (μg/day) |
| Control | 16.6± 3.1 | 20.1± 5.8 | 21.2± 3.8 | 51.9± 7.8 |
| *p*-Chlorophenylalanine, ethionine | 30.8± 6.7 | 194.2±39.3 | 222.3±43.7 | 241.7± 59.2 |
| α-Methyltyrosine | 54.7±12.4 | 30.4± 6.2 | 37.1± 4.6 | 122.4± 18.7 |
| Phenylalanine | 19.8± 5.2 | 58.6±12.5 | 64.4±12.5 | 214.7± 46.5 |
| *p*-Chlorophenylalanine, ethionine+phenylalanine | 35.3± 7.1 | 599.7±86.6 | 578.2±63.7 | 1890.1±194.1 |
| α-Methyltyrosine +phenylalanine | 86.2±18.0 | 35.8±11.5 | 51.9±12.3 | 140.0± 56.1 |

urine were 1.5 times higher than the control value by the administration of α-methyltyrosine, which is an inhibitor of tyrosine hydroxylase. However, in the tyrosine hydroxylase inhibitor-treated rats, there was no significant increase in the concentration of *m*- and *o*-tyrosine after the administration of L-phenylalanine. On the other hand, the concentrations of urinary *m*- and *o*-tyrosine following administration of *p*-chlorophenylalanine and ethionine were 10 times higher than the control value. In addition, when phenylalanine hydroxylase activity was inhibited, the concentration of urinary phenylalanine showed a marked increase. Furthermore, in the phenylalanine hydroxylase inhibitor-treated rats, the urinary excretion of *m*- and *o*-tyrosine was further increased after the administration of L-phenylalanine. These results are consistent with a report that the in vivo formation of *m*- and *o*-tyrosine is caused mainly by tyrosine hydroxylase, not by phenylalanine hydroxylase [9].

In conclusion, our newly developed method is applicable to the routine assay of urinary *m*- and *o*-tyrosine in clinical studies.

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