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Simultaneous determination of stable isotopically labelled L-histidine and urocanic acid in human plasma by stable isotope dilution mass spectrometry

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ABSTRACT

A capillary gas chromatographic–mass spectrometric method for the simultaneous determination of stable isotopically labelled L-histidine (L-[3,3-$^2H_2$,1′,3′-$^{15}N_2$]histidine, L-His-[M + 4]) and urocanic acid ([3-^{2}H,1′,3′-$^{15}N_2$]urocanic acid, UA-[M + 3]) in human plasma was developed using DL-[2,3,3,5′-$^2H_4$,2′-^{13}C,1′,3′-$^{15}N_2$]histidine (DL-His-[M + 7]) and [2,3,5′-$^2H_3$,2′-^{13}C,1′,3′-$^{15}N_2$]urocanic acid (UA-[M + 6]) as internal standards. L-Histidine and urocanic acid were derivatized to $^{\alpha}$N-(trifluoroacetyl)-^{im}N-(ethoxycarbonyl)-L-histidine *n*-butyl ester and ^{im}N-(ethoxycarbonyl)urocanic acid *n*-butyl ester. Quantification was carried out by selected ion monitoring of the molecular ions of the respective derivatives of L-His-[M + 4], DL-His-[M + 7], UA-[M + 3] and UA-[M + 6]. The sensitivity, specificity, precision and accuracy of the method were demonstrated to be satisfactory for measuring plasma concentrations of L-His-[M + 4] and UA-[M + 3] following administration of trace amounts of L-His-[M + 4] to humans.

INTRODUCTION

Histidinaemia is a hereditary metabolic disorder characterized by mental and/or speech retardations and is caused by a virtual deficiency of the liver enzyme, histidine ammonia-lyase, which catalyses β-elimination of ammonia from L-histidine to produce urocanic acid [1]. The metabolism of L-histidine to urocanic acid *in vivo* can be investigated by treating human subjects with stable isotopically labelled L-histidine. In an attempt to detect the heterozygote state [2–4] of histidinaemia by stable isotope methodology, we have previously developed a gas chromatographic–mass spectrometric (GC–MS) method for the determination of endogenous (unlabelled) and exogenous (labelled) histidine in human plasma following administration of stable isotopically labelled L-histidine (L-[3,3,5′-$^2H_3$,3′-^{15}N]histidine) [5].

This paper describes the development of a capillary GC–MS method for the simultaneous determination of stable isotopically labelled L-histidine (L-[3,3-$^2H_2$,1′,3′-$^{15}N_2$]histidine) and urocanic acid ([3-^{2}H,1′,3′-$^{15}N_2$]urocanic acid) in human plasma.

EXPERIMENTAL

Materials

L-Histidine free base and urocanic acid were obtained from Sigma (St. Louis, MO, USA), trifluoroacetic anhydride from Kanto (Tokyo, Japan) and diethyl pyrocarbonate from Aldrich (Milwaukee, WI, USA). L-[ring-2-^{14}C]Histidine (specific activity 1.92 GBq/mmol) was purchased

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from CEA (Gif-sur-Yvette, France). Stable isotopically labelled compounds, *i.e.*, L-[3,3-$^{2}H_2$,1′,3′-$^{15}N_2$]histidine (L-His-[M + 4]), DL-[2,3,3,5′-$^{2}H_4$,2′-^{13}C,1′,3′-$^{15}N_2$]histidine (DL-His-[M + 7]), [3-^{2}H,1′,3′-$^{15}N_2$]urocanic acid (UA-[M + 3]) and [2,3,5′-$^{2}H_3$,2′-^{13}C,1′,3′-$^{15}N_2$]urocanic acid (UA-[M + 6]), were synthesized in our laboratory [6,7]. [ring-2-^{14}C]Urocanic acid (1.92 MBq/mmol) was synthesized by the enzymatic reaction of L-[ring-2-^{14}C]histidine using histidine ammonia-lyase (EC 4.3.1.3) from *Pseudomonas fluorescens* (Sigma).

The radioactive urocanic acid was purified by anion-exchange column chromatography (Dowex 1-X8, 200–400 mesh; 5 cm × 1.5 cm I.D. column). The radiochemical purity was more than 94.5%, determined by radio thin-layer chromatography (JTC-600; Aloka, Tokyo, Japan).

All other chemicals and reagents were of analytical-reagent grade and were used as received.

Preparation of standards

Stock solutions of L-His-[M + 4] (107.3 μg/ml), DL-His-[M + 7] (1.936 μg/ml), UA-[M + 3] (47.30 μg/ml) and UA-[M + 6] (2.332 μg/ml) were prepared in 2% ethanol in distilled water. All analyses were performed by diluting the stock solutions with 2% ethanol in distilled water.

Gas chromatography–mass spectrometry with selected ion monitoring (GC–MS-SIM)

Capillary GC–MS-SIM analysis was carried out on a QP2000 gas chromatograph–mass spectrometer equipped with a data processing system (Shimadzu, Kyoto, Japan). GC was performed on a Durabond (DB-5) fused-silica capillary column (40 m × 0.32 mm I.D.) with the stationary phase coated at a 0.1-μm film thickness (J & W Scientific, Rancho Cordova, CA, USA). Helium was used as the carrier gas at a column head pressure of 78.4 kPa. A split–splitless injection system (Shimadzu SPL-9) operated in the splitless mode was used with a septum purge flow-rate of 10 ml/min and a split flow-rate of 60 ml/min. The purge activation time was 2 min. The initial column temperature was set at 100°C. After the sample injection, it was maintained for 2 min and then increased at 25°C/min to 200°C and subsequently at 10°C/min to 250°C. The temperature of the injector was 250°C. The mass spectrometer was operated in the electron impact (EI) mode at an energy of 70 eV and the ion source temperature was set at 270°C. The multiple-ion detector was focused on the molecular ions at *m/z* 379 for $^{\alpha}$N-(trifluoroacetyl)-^{im}N-(ethoxycarbonyl)-L-histidine *n*-butyl ester (L-His-TEB) and at *m/z* 266 for ^{im}N-(ethoxycarbonyl)urocanic acid *n*-butyl ester (UA-EB), and at *m/z* 383, 386, 269 and 272 for the corresponding derivatives of labelled histidines and urocanic acids, L-His-[M + 4], DL-His-[M + 7], UA-[M + 3] and UA-[M + 6], respectively.

Sample preparation for GC–MS-SIM

Extraction. To 0.5 ml of human plasma in a conical centrifuge tube (100 × 13 mm I.D.) were added 193.6 ng of DL-His-[M + 7] and 233.2 ng of UA-[M + 6] as the internal standards dissolved in 100 μl of 2% ethanol in distilled water. The plasma sample was deproteinized and extracted with ethanol (2 × 2 ml) on a vortex mixer for *ca.* 0.5 min. After centrifugation at 1500 *g* for 5 min, the ethanol solution was transferred into another conical centrifuge tube and evaporated at 50–60°C under a stream of nitrogen. The residue was dissolved in 0.5 ml of ethanol and then applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 0.5 ml of ethanol and then eluted with 1 ml of distilled water. The eluate was transferred into a 2-ml micro product V-vial (Wheaton, Millville, NJ, USA) and then evaporated to dryness at 50°C under a stream of nitrogen.

Derivatization. To the residue containing L-histidine and urocanic acid were added 200 μl of 3 *M* hydrogen chloride in *n*-butanol. The reaction mixture was sealed under a nitrogen atmosphere and heated at 100°C for 15 min. After removal of the solvent at 70°C under a stream of nitrogen, 200 μl of dichloromethane were added and the solution was evaporated to dryness. The residue was reconstituted in 250 μl of 20% trifluoroacetic anhydride (TFAA) in dichloromethane and heated at 150°C for 5 min under a nitrogen atmo-

sphere. After the reaction, the excess of reagent and solvent were evaporated at room temperature under a gentle stream of nitrogen. The residue was dissolved in 200 μl of 1.5% diethyl pyrocarbonate (DEPC) in dichloromethane, heated at 150°C for 20 min and then evaporated to dryness at room temperature under a gentle stream of nitrogen.

Purification. The derivatized sample was dissolved in a small volume (50–100 μl) of dichloromethane and subjected to thin-layer chromatographic (TLC) purification (Kieselgel $60F_{254}$; Merck, Darmstadt, Germany). The TLC plate was developed with dichloromethane–methanol (10:1, v/v) and the UV–visual zones corresponding to R_F values of 0.30 for L-histidine and of 0.37 for urocanic acid were scraped off. The derivatives were extracted with 2 ml of acetone twice and the solvent was evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was reconstituted with 50 μl of dichloromethane for GC–MS-SIM analysis.

Recovery

To 0.5 ml of human plasma were added 57.5 Bq of L-[ring-2-^{14}C]histidine or 87.3 Bq of [ring-2-^{14}C]urocanic acid. The sample was then carried through the sample preparation procedure described above. The recoveries of L-histidine and urocanic acid were calculated by comparing the radioactivities before and after the extraction procedures. The radioactivity was measured with a liquid scintilation counter (LSC1000; Aloka).

Calibration

To each of seven standards containing a known ratio of L-His-[M + 4] to UA-[M + 3] (1.07:0.95, 5.37:4.73, 21.46:23.65, 107.30:47.30, 214.60:94.60, 536.50:236.50 and 2146.0:473.00) were added 193.6 ng of DL-His-[M + 7] and 233.2 ng of UA-[M + 6] as internal standards and 11.60 μg of unlabelled L-histidine and 51.80 ng of unlabelled urocanic acid as carriers. Each sample was prepared in triplicate. After evaporation of the solvent, the samples were derivatized according to the procedure described above. A 0.5–1.0-μl portion of the dichloromethane solution (50 μl) was analysed by GC–MS-SIM. The peak-height ratios (*m/z* 383 to 386 for L-histidine and *m/z* 269 to 272 for urocanic acid) were determined in triplicate. The calibration graph was obtained as the peak-height ratios *versus* the concentrations (ng/ml) of L-His-[M + 4] or of UA-[M + 3] on each analysis of the standard mixtures.

Accuracy

Accuracy was examined by assaying five preparations of human plasma spiked with 53.65 or 536.50 ng/ml of L-His-[M + 4] and 193.6 ng of DL-His-[M + 7] as the internal standard and with 47.30 or 237.00 ng of UA-[M + 3] and 233.2 ng of UA-[M + 6] as the internal standard. After preparation of the sample for GC–MS-SIM as described above, the peak-height ratios (*m/z* 383 to 386 and *m/z* 269 to 272) were measured.

RESULTS AND DISCUSSION

Recently we reported evidence for a stepwise mechanism via a carbanion intermediate in the elimination of ammonia from L-histidine to urocanic acid *in vitro,* catalysed by histidine ammonia-lyase (EC 4.3.1.3) from *Pseudomonas fluorescens* [8–10]. The reaction mechanism was rationalized based on the observation that the enzyme-catalysed hydrogen exchange occurred at C-5′ of the imidazole ring in the reaction of L-[3,3,5′-$^{2}H_3$,3′-^{15}N]histidine with the enzyme. This indicates that L-histidine labelled with deuterium at C-5′ in the imidazole ring is inappropriate as a biological internal standard because of its instability caused by the enzymatic reaction. Successful application of stable isotope methodology to the biochemical and clinical investigations is always dependent on the availability of compounds labelled at predesigned positions that are chemically and biologically inert. We have synthesized multi-labelled L-histidine and urocanic acid containing at least three non-exchangeable stable isotopes with high isotopic purity, *i.e.,* L-[3,3-$^{2}H_2$,1′,3′-$^{15}N_2$]histidine (L-His-[M + 4]) [6] for a biological internal standard, DL-

[2,3,3,5′-$^{2}H_4$,2′-^{13}C,1′,3′-$^{15}N_2$]histidine (DL-His-[M + 7]) and [2,3,5′-$^{2}H_3$,2′-^{13}C,1′,3′-$^{15}N_2$]urocanic acid (UA-[M + 6]) for analytical internal standards. [3-^{2}H,1′,3′-$^{15}N_2$]Urocanic acid (UA-[M + 3]), which would be formed metabolically from L-His-[M + 4], was also synthesized for development of the GC–MS assay. Detailed discussion concerning the synthesis of DL-His-[M + 7], UA-[M + 6] and UA-[M + 3] will be described elsewhere [7].

For the simultaneous assay of L-histidine and urocanic acid, solvent extraction and purification from the plasma sample followed by GC derivatization should preferably be the same for the two compounds. The simultaneous derivatization of L-histidine and urocanic acid was carried out by the following three-step reaction to give $^{\alpha}$N-(trifluoroacetyl)-^{im}N-(ethoxycarbonyl)-L-histidine *n*-butyl ester (L-His-TEB) [5,11,12] and ^{im}N-(ethoxycarbonyl)urocanic acid *n*-butyl ester (UA-EB): initial esterification with 3 *M* HCl in *n*-butanol at 100°C for 15 min, followed by trifluoroacetylation with TFAA at 150°C for 5 min and then ethoxycarbonylation with diethyl pyrocarbonate (DEPC) at 150°C for 20 min. The optimum conditions for each step were determined by following the reaction by radio-TLC, using human blank plasma spiked with trace amounts of radioactive L-histidine and urocanic acid. The trifluoroacetylation step is not required for the derivatization of urocanic acid but is necessary for the simultaneous derivatization of both L-histidine and urocanic acid. The reaction conditions employed for trifluoroacetylation left urocanic acid *n*-butyl ester unaffected and subsequent ethoxycarbonylation gave the desired UA-EB derivative.

Fig. 1 shows the EI mass spectra of TEB derivatives of unlabelled L-histidine and stable isotopically labelled histidines (L-His-[M + 4] and DL-His-[M + 7]). The isotopic purities were calculated to be 97.3 % for M + 4 and 95.2% for M + 7, based on the ion intensities in the region of the molecular ion of each compound. Fig. 2 shows the EI mass spectra of EB derivatives of unlabelled urocanic acid and labelled urocanic acids (UA-[M + 3] and UA-[M + 6]). The isotopic

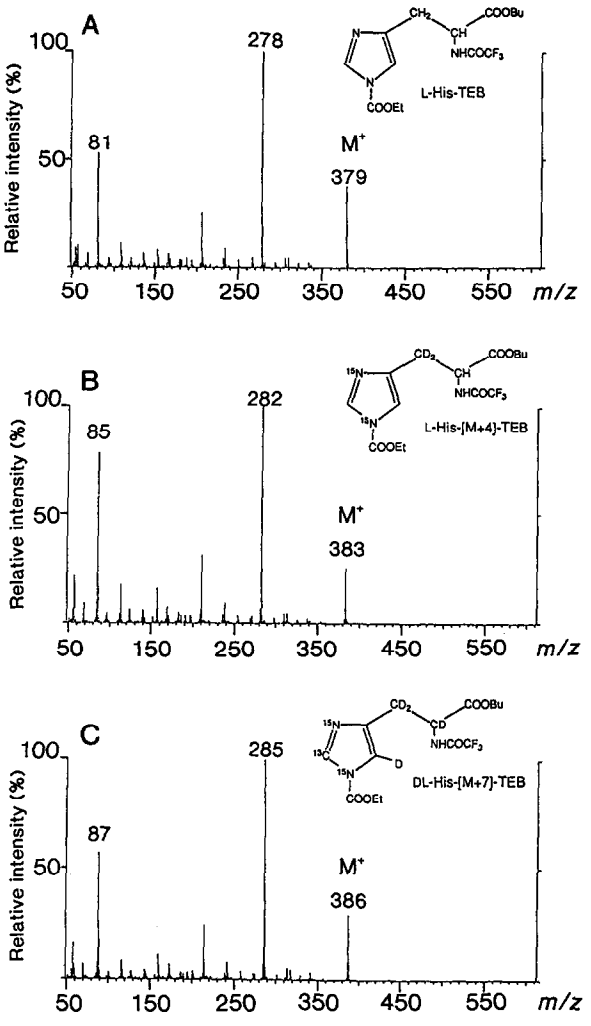

Fig. 1. Electron impact mass spectra of TEB derivatives of unlabelled L-histidine (A) and stable isotopically labelled histidines, *i.e.*, L-His-[M + 4] (B) and DL-His-[M + 7] (C). Bu = Butyl; Et = ethyl.

purities were 93.9% for M + 3 and 93.8% for M + 6. When the molecular ions were monitored, the sensitivity of the capillary GC–MS-SIM assays was *ca.* 10 pg per injection for the pure reference L-histidine (*m/z* 379) and *ca.* 50 pg per injection for urocanic acid (*m/z* 266), with a signal-to-noise ratio of more than 5 (Fig. 3).

The efficiency of extraction of L-histidine and urocanic acid from plasma was examined on the basis of recovery using radioactive L-histidine and urocanic acid. The plasma sample was de-

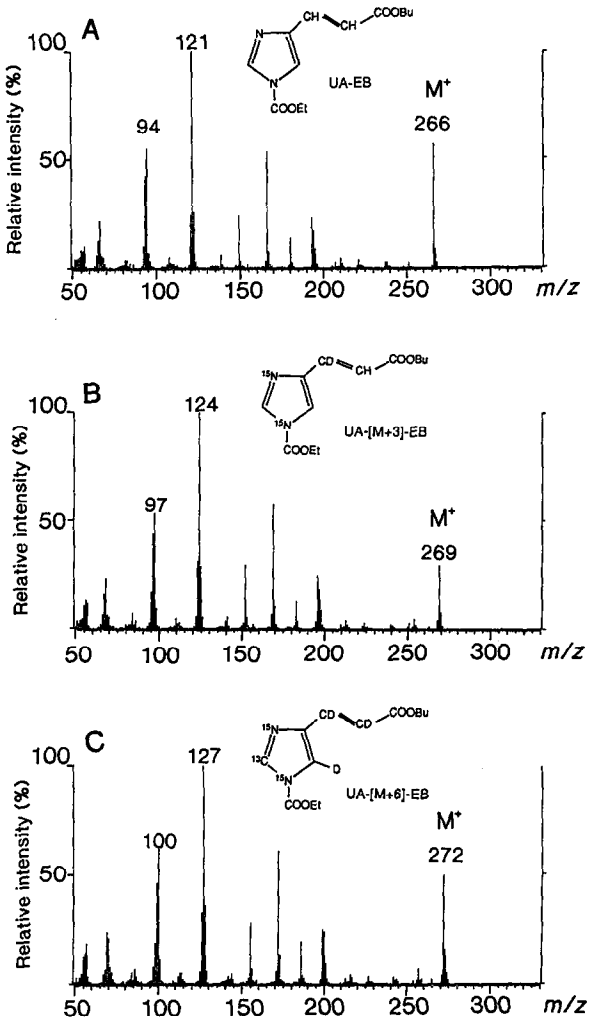

Fig. 2. Electron impact mass spectra of EB derivatives of unlabelled urocanic acid (A) and stable isotopically labelled urocanic acids, *i.e.*, UA-[M + 3] (B) and UA-[M + 6] (C).

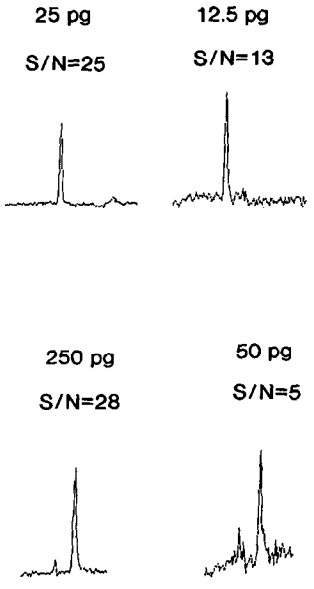

Fig. 3. Sensitivity for L-histidine (top) and urocanic acid (bottom).

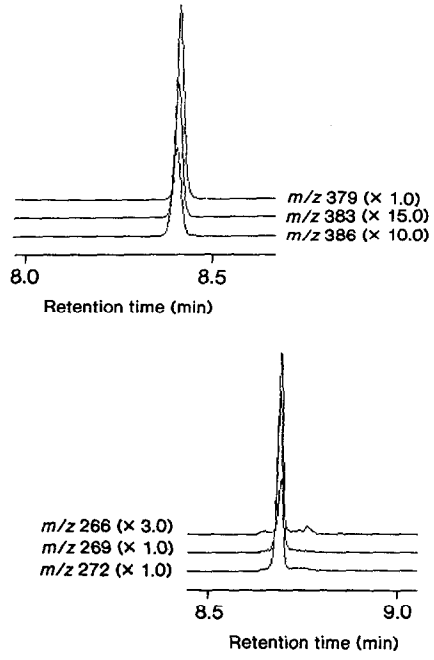

Fig. 4. Selected ion recordings of TEB derivatives of unlabelled L-histidine (*m/z* 379), L-His-[M + 4] (*m/z* 383) and DL-His-[M + 7] (*m/z* 386) and of EB derivatives of unlabelled urocanic acid (*m/z* 266), UA-[M + 3] (*m/z* 269) and UA-[M + 6] (*m/z* 272) after processing a plasma sample.

proteinized and extracted with 2 ml of ethanol twice. After removal of the solvent, the extract was dissolved in ethanol (0.5 ml) and subjected to a Sep-Pak C_{18} cartridge. The cartridge was washed with 0.5 ml of ethanol to remove endogenous compounds present in plasma and eluted with 1 ml of water. The recoveries were 78.5–80.4% (n = 3) for L-histidine and 71.1–74.7% (n = 3) for urocanic acid. After the aqueous solution had been dried, the residue was derivatized according to the procedure described above and then purified by TLC. Fig. 4 shows selected ion

TABLE I

ACCURACY OF GC–MS-SIM ANALYSIS OF L-HIS-[M + 4] AND UA-[M + 3] IN HUMAN PLASMA

Compound	Added (ng/ml)	Found (ng/ml)						Relative error (%)	C.V. (%)
		Individual values[a]					Mean ± S.D.		
L-His-[M + 4]	53.65	52.19	52.19	53.79	49.79	53.47	52.29 ± 1.57	−2.53	3.01
	536.50	550.09	506.44	545.14	543.86	538.91	536.89 ± 17.48	−0.07	3.26
UA-[M + 3]	47.30	50.39	47.31	47.79	45.42	45.89	47.36 ± 1.96	+0.13	4.13
	237.00	244.29	241.84	253.42	248.98	253.89	248.48 ± 5.38	+4.84	2.16

[a] Each individual value is the mean of triplicate measurements.

recordings of TEB derivatives of unlabelled L-histidine (*m/z* 379), L-His-[M + 4] (*m/z* 383) and DL-His-[M + 7] (*m/z* 386), and of EB derivatives of unlabelled urocanic acid (*m/z* 266), UA-[M + 3] (*m/z* 269) and UA-[M + 6] (*m/z* 272) after processing from spiked plasma. Blank plasma samples were found to contain no interfering substances derived from plasma.

Calibration graphs were prepared in the ranges 1–2000 ng of labelled L-histidine (L-His-[M + 4]) and 1–500 ng of labelled urocanic acid (UA-[M + 3]) with DL-His-[M + 7] (193.6 ng) and UA-[M + 6] (233.2 ng) as the internal standards for the GC–MS assays. The mixture was assayed as L-His-TEB and UA-EB derivatives by monitoring the molecular ion intensities at *m/z* 383 (L-His-[M + 4]), *m/z* 386 (DL-His-[M + 7]), *m/z* 269 (UA-[M + 3]) and *m/z* 272 (UA-[M + 6]). The peak-height ratios were plotted against the concentrations (ng/ml) of L-His-[M + 4] and UA-[M + 3]. The graphs were linear over the ranges 1–2000 ng [$y = (6.2521 \cdot 10^{-3})x - 0.0063$, $r = 0.9999$] for L-His-[M + 4] and 1–500 ng [$y = (4.2308 \cdot 10^{-3})x - 0.0068$, $r = 0.9998$] for UA-[M + 3].

The accuracy of measurements was determined for L-His-[M + 4] and UA-[M + 3] added to 0.5-ml aliquots of pooled plasma. The plasma samples contained fixed amounts of DL-His-[M + 7] (193.6 ng) and UA-[M + 6] (233.2 ng) as the internal standards and different amounts of L-His-[M + 4] (53.65 and 536.50 ng) and UA-[M + 3] (47.30 and 237.00 ng). Table I shows that the amounts of L-His-[M + 4] and UA-[M + 3] determined were in good agreement with the actual amounts added, the relative error being less than 2.53% of for L-His-[M + 4] and 4.84% for UA-[M + 3]. The inter-assay coefficients of variation (C.V.) ($n = 5$) were 3.01% for 53.65 ng/ml and 3.26% for 536.50 ng/ml of L-His-[M + 4] and 4.13% for 47.30 ng/ml and 2.16% for 237.00 ng/ml of UA-[M + 3]. The intra-assay C.V. values ($n = 3$) were less than 3% for both L-His-[M + 4] and UA-[M + 3].

The present method provides a sensitive and reliable technique for the simultaneous determination of plasma concentrations of stable isotopically labelled L-histidine and urocanic acid with good accuracy and precision. The method can be applied to pharmacokinetic and metabolic studies of L-histidine following administration of stable isotopically labelled L-histidine to patients with histidinaemia.

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REFERENCES

1 B. N. La Du, in J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson (Editors), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1978, p. 317.

2 Y. Kuroda, M. Ito, T. Ogawa, E. Takeda, K. Toshima and M. Miyao, *Clin. Chim. Acta,* 96 (1979) 139.
3 I. Matsuda, K. Matsuo, F. Endo, I. Uehara, N. Nagata, Y. Jinno, S. Chikazawa, T. Miyakita and H. Miura, *Clin. Chim. Acta,* 119 (1982) 319.
4 I. Matsuda, F. Endo and N. Nagata, *Lancet,* i (1982) 162.
5 T. Furuta, Y. Kasuya, H. Shibasaki and S. Baba, *J. Chromatogr.,* 413 (1987) 1.
6 T. Furuta, Y. Kasuya, H. Takahashi and S. Baba, *J. Chem. Res. (S),* (1987) 86.
7 T. Furuta, H. Takahashi, H. Shibasaki and Y. Kasuya, *J. Chem. Soc. Perkin Trans. 1,* submitted for publication.
8 T. Furuta and Y. Kasuya, in T. A. Baillie and J. R. Jones (Editors), *Synthesis and Applications of Isotopically Labelled Compounds, Proceedings of the 3rd International Symposium, Innsbruck, July 17–21, 1988,* Elsevier, Amsterdam, 1989, p. 747.
9 T. Furuta, H. Takahashi and Y. Kasuya, *J. Am. Chem. Soc.,* 112 (1990) 3633.
10 T. Furuta, H. Takahashi and Y. Kasuya, *J. Chromatogr.,* 562 (1991) 363.
11 D. Roach and C. W. Gehrke, *J. Chromatogr.,* 44 (1969) 269.
12 I. M. Moodie, *J. Chromatogr.,* 99 (1974) 495.