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DETERMINATION OF STABLE ISOTOPICALLY SUBSTITUTED HISTIDINE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A gas chromatographic–electron-impact mass spectrometric method for the determination of stable isotopically substituted histidine in human plasma has been developed. Histidine was derivatized to $^{\alpha}$N-trifluoroacetyl-^{im}N-carbethoxyhistidine *n*-butyl ester (TCB derivative) by a three-step reaction: an initial esterification by 3 *M* *n*-butanolic hydrochloric acid, followed by trifluoroacetylation with trifluoroacetic anhydride and then ethoxycarbonylation with diethyl pyrocarbonate. Quantitation was carried out by selected-ion monitoring on the molecular ions (*m/z* 379, 383 and 385) of the respective TCB derivatives of histidine, [1-^{15}N,5,β,β-$^{2}H_3$]histidine (histidine-[M+4]) and [1,3-$^{15}N_2$,5,α,β,β-$^{2}H_4$]histidine (histidine-[M+6]). The sensitivity, specificity, precision and accuracy of the method were demonstrated to be satisfactory for application to a pharmacokinetic study of histidine after administration of a trace amount of stable isotopically substituted histidine (histidine-[M+4]) in humans.

INTRODUCTION

Histidinaemia is a hereditary metabolic disorder characterized by mental and/or speech retardations and caused by a virtual deficiency of the liver enzyme, histidine ammonia-lyase, which catalyses the non-oxidative deamination of histidine to urocanic acid.

Since the development of newborn screening programmes to identify patients with histidinaemia, a number of newborns with atypical histidinaemia have been found. A histidine loading test has been employed as one of the most common methods of evaluating the liver enzymatic activity to differentiate between patients with typical histidinaemia requiring strict dietary management and those with the atypical type. An inherent problem is that this method does not permit a distinction between endogenous and exogenous histidine in biological fluids after dosing. This means that a large dose of histidine is required to obtain high con-

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centrations beyond the endogenous level of histidine in the patients.

This paper describes the development of a gas chromatographic–mass spectrometric–selected-ion monitoring (GC–MS–SIM) method for the determination of stable isotopically substituted histidine after administration of [1-^{15}N,5,β,β-$^{2}H_3$]histidine (histidine-[M+4]) to human subjects. The method should make it possible to reveal the precise pharmacokinetics of histidine, even after dosing small amounts of the labelled histidine, because of its high sensitivity, precision and specificity to measure low nanogram levels of histidine in human plasma. The useful application of the method will be found in clinical studies, especially in a diagnosis to detect the heterozygote state of the disorder.

EXPERIMENTAL

Materials

L-Histidine, free base (Wako, Osaka, Japan), trifluoroacetic anhydride (Nakarai, Kyoto, Japan) and diethyl pyrocarbonate (Aldrich, Milwaukee, WI, U.S.A.) were commercially obtained. L-[ring-2-^{14}C]Histidine (specific activity 59 mCi/mmol) was purchased from Amersham (Buckinghamshire, U.K.). Stable isotopically substituted histidines, DL-[1-^{15}N,5,β,β-$^{2}H_3$]histidine (histidine-[M+4]) and DL-[1,3-$^{15}N_2$,5,α,β,β-$^{2}H_4$]histidine (histidine-[M+6]) were synthesized in our laboratory [1]. All other chemicals and reagents were of analytical-reagent grade and used without further purification.

Gas chromatography–mass spectrometry–selected-ion monitoring

GC–MS–SIM measurements were made with a Shimadzu QP1000 gas chromatographic–mass spectrometric system equipped with a data processing system. Gas chromatography was performed on a glass column (1 m×3.0 mm I.D.) packed with 1.5% OV-17 on Shimalite W (80–100 mesh). The column temperature was 240°C, the injector temperature 260°C and the ion source temperature 280°C. Helium was used as the carrier gas at a flow-rate of ca. 20 ml/min. The multiple-ion detector was focused on the molecular ions of $^{\alpha}$N-trifluoroacetyl-^{im}N-carbethoxyhistidine *n*-butyl ester (*m/z* 379) and the corresponding derivatives of histidine, histidine-[M+4] (*m/z* 383) and histidine-[M+6] (*m/z* 385).

Sample preparation for GC–MS–SIM

Extraction. Unless otherwise stated, the following procedure was used. To 0.5 ml of human plasma in a conical centrifuge tube (100×13 mm) were added 200 ng of the internal standard, histidine-[M+6], dissolved in 2% ethanol–distilled water. The plasma sample was deproteinized and extracted with 2.0 ml of ethanol on a vortex mixer for ca. 1 min. After centrifugation at 1500 *g* for 10 min, the ethanol phase was transferred into another conical centrifuge tube and evaporated at 50°C under a stream of nitrogen. The residue was dissolved in 0.5 ml of distilled water and the solution was washed with 0.5 ml of chloroform on a vortex mixer (1 min). After centrifugation at 1500 *g* for 5 min, the aqueous phase was transferred into a 2.0-ml micro product vial with PTFE-lined screw cap (Whea-

ton, Millville, NJ, U.S.A.) and then evaporated to dryness at 50°C under a stream of nitrogen.

Derivatization. To the residue containing histidine were added 200 μl of 3 *M* *n*-butanolic hydrochloric acid. 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 again. The residue was reconstituted in a mixture of dichloromethane–trifluoroacetic anhydride (4:1, v/v, 250 μl) and then heated at 150°C for 5 min. After the reaction, the excess 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 in dichloromethane, heated at 150°C for 20 min, and then evaporated to dryness.

Purification. The derivatized sample was dissolved in a small volume of dichloromethane (50–100 μl) and subjected to thin-layer chromatographic (TLC) purification (Kieselgel $60F_{254}$, E. Merck, Darmstadt, F.R.G.). The TLC plate was developed with ethyl acetate–benzene (1:1, v/v) and the zone corresponding to the R_F value of 0.39 was scraped off. The derivative was extracted with 2 ml of acetone twice and the solvent was evaporated. The residue was reconstituted with 40 μl of dichloromethane for the GC–MS–SIM analysis. The derivative was stable at −20°C for at least two months.

Calibration curve

To each of ten standards containing 1.00, 5.01, 10.02, 20.05, 50.12, 100.24, 200.48, 501.20, 1002.4 and 2004.8 ng of histidine-[M+4], 214.52 ng of histidine-[M+6] as the internal standard and 15.0 μg of non-substituted histidine as a carrier were added. After evaporation of the solvent (2% ethanol) to dryness, the samples were derivatized according to the procedure described above. A 1–5 μl volume of the dichloromethane solution (40 μl) was analysed by GC–MS–SIM. The calibration curve was obtained from the ln–ln plot of the peak-height ratios versus the concentrations (ng/ml) of histidine-[M+4].

Recovery

To 0.5 ml of human blank plasma containing 16.9 μg/ml endogenous histidine, ca. 10 nCi of [ring-2-^{14}C]histidine (26.3 ng) were added. The sample was then carried through the entire sample preparation procedure described above. The absolute recovery was calculated by comparing the radioactivities before and after the extraction and derivatization procedures.

Accuracy

Accuracy was examined by assaying five preparations of human blank plasma spiked with 50.12 or 501.20 ng/ml histidine-[M+4] and 214.52 ng/ml histidine-[M+6] as the internal standard. After preparation of the sample for GC–MS–SIM as described above, the peak-height ratio (*m/z* 383 to *m/z* 385) was measured.

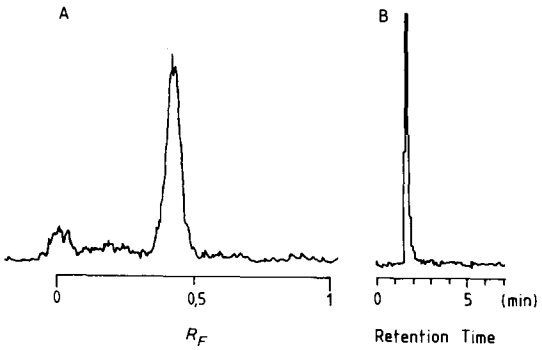

Fig. 1. Radio-TLC (A) and radio-GC (B) profiles of TCB derivative of histidine after processing from plasma spiked with radioactive histidine. (A) Solvent system for TLC, ethyl acetate–benzene (1:1, v/v); (B) GC system, 1.5% OV-17 column (1 m, 230°C), nitrogen gas was used as a carrier at a flow-rate of 40 ml/min.

RESULTS AND DISCUSSION

The stable isotope dilution technique in combination with GC–MS has provided a powerful tool for clinical and biochemical investigations into the metabolism of amino acids in humans, as has been demonstrated for phenylalanine, tyrosine [2–6] and tryptophan [7–9]. Application of this technique to the pharmacokinetic and metabolic studies of histidine requires two types of stable isotopically substituted histidine for use as biological and analytical internal standards. We have synthesized histidine-[M+4] and histidine-[M+6]. Detailed discussion concerning the synthesis will be soon described elsewhere [1].

In addition, this amino acid is one of the most difficult and elusive substances that can be chromatographed in the vapour phase [10]. The derivatization of histidine to $^{\alpha}$N-trifluoroacetyl-^{im}N-carbethoxyhistidine *n*-butyl ester (TCB derivative) [10,11] was carried out by a three-step reaction with an initial esterification by 3 *M* hydrogen chloride in *n*-butyl alcohol at 100°C for 15 min, followed by trifluoroacetylation with trifluoroacetic anhydride at 150°C for 5 min and ethoxycarbonylation with diethyl pyrocarbonate at 150°C for 20 min. The optimal conditions at each step were determined by following the reaction with radio-TLC and radio-GC, using human blank plasma spiked with a trace amount of radioactive histidine. As shown in Fig. 1A and B, the GC and TLC behaviour demonstrated that the derivatization proceeded almost quantitatively. The stability of the derivative was examined by repeated radio-GC and radio-TLC analyses of the same set of standard samples over a one- or two-month period. The derivatized sample in dichloromethane was found to be stable at −20°C for at least two months. During the past decade, several other derivatives of histidine, such as trimethylsilylhistidine [12,13], pentafluoropropionylhistidine [14] have been reported for the separation and quantitation of protein amino acids by GC. However, these derivatives lack stability and give only low-intensity molecular ions.

Fig. 2 shows the electron-impact mass spectra of TCB derivatives of non-sub-

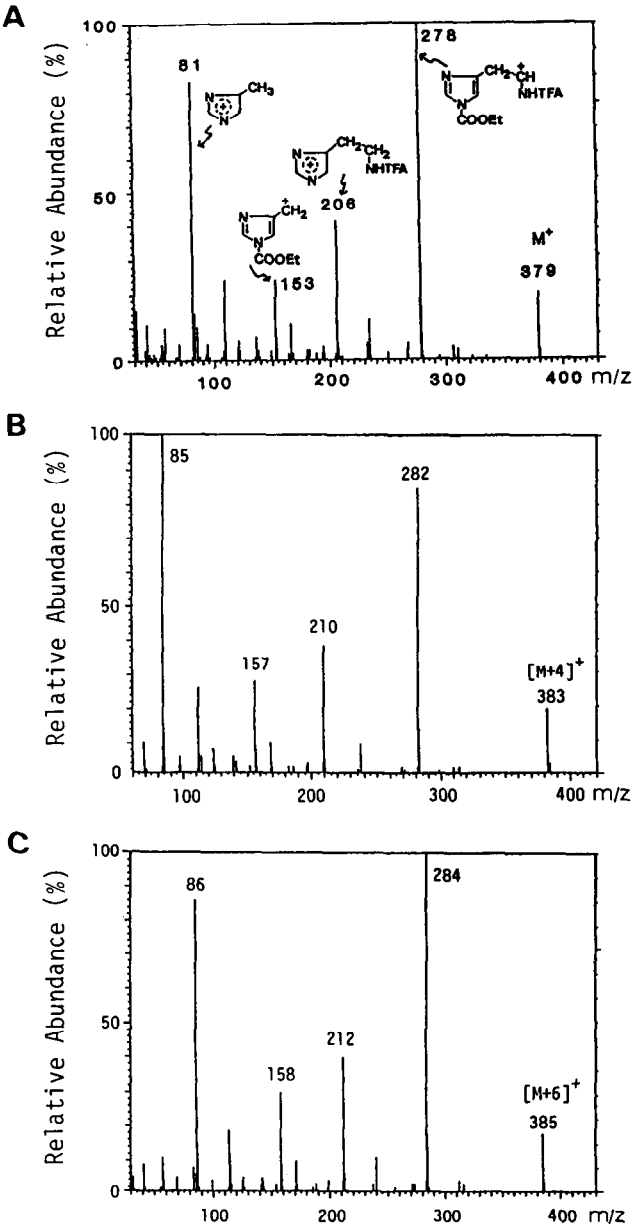

Fig. 2. Electron-impact mass spectra of TCB derivatives of non-substituted (A) and stable isotopically substituted histidines, i.e. histidine-[M+4] (B) and histidine-[M+6] (C).

stituted and two types of stable isotopically substituted histidine (histidine-[M+4] and histidine-[M+6]). The isotopic purities were calculated as 96.5% for M+4 and 93.0% for M+6, based on the ion intensities in the region of the molecular ion of each compound. When the molecular ions were monitored, the lower limit of detection determined on the basis of the signal-to-noise ratio was 200 pg per injection. An intensive fragment ion (m/z 278) formed by the loss of

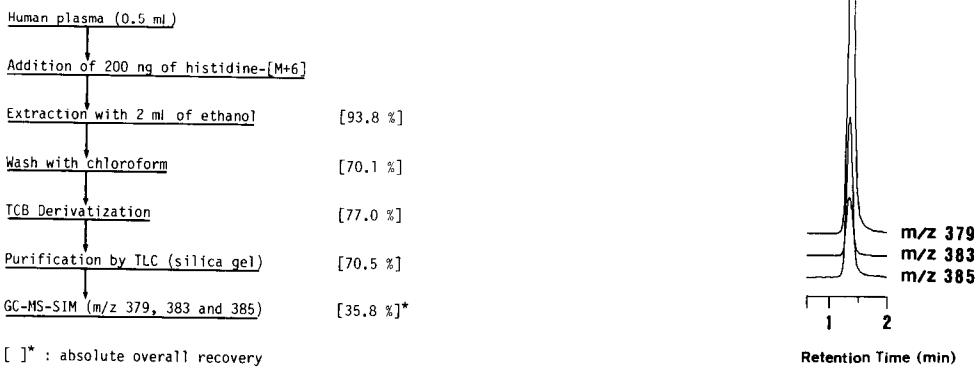

Fig. 3. Extraction procedure of histidine from human plasma.

Fig. 4. Mass fragmentogram of TCB derivatives of histidine (m/z 379), histidine-[M+4] (m/z 383) and histidine-[M+6] (m/z 385) after processing from a plasma sample.

n-butyloxycarbonyl ion from the molecular ion was not appropriate for monitoring, because an interfering peak derived from blank plasma was observed when monitoring the ion at m/z 282, corresponding to the ion $[M+4-COOBu]^{+\cdot}$ of histidine-[M+4] in Fig. 2B.

The condition for extraction of histidine from plasma was examined on the basis of recovery using radioactive histidine. The employed procedures and the recovery at each step are shown in Fig. 3. An examination of the effect of the extraction volume of ethanol (0.5, 1.0, 1.5, 2.0 and 2.5 ml) on the absolute recovery indicated an increasing recovery of histidine with increasing volume of ethanol up to 1.0 ml, above which no further increase was obtained. The volume of 2.0 ml of ethanol was chosen to give effective deproteinization. After evaporation of the solvent, the extract was dissolved in water (0.5 ml) and washed with chloroform (0.5 ml) to remove endogenous compounds present in plasma. After the aqueous phase had been dried, the residue was derivatized according to the procedure described above and then purified by TLC. The absolute overall recovery was 35.8%. Fig. 4 shows the mass fragmentogram of TCB derivatives of histidine (m/z 379), histidine-[M+4] (m/z 383) and histidine-[M+6] (m/z 385) after processing from spiked plasma. Blank plasma samples were found to contain no interfering substances derived from plasma.

A calibration curve was prepared in the range 1.00–2004.8 ng of histidine-[M+4] with 214.52 ng of histidine-[M+6] as the internal standard for the GC–MS assay. The mixture was assayed as the TCB derivative by monitoring the molecular ions at m/z 383 for histidine-[M+4] and at m/z 385 for histidine-[M+6]. The peak-height ratio was plotted against the concentration (ng/ml) of histidine-[M+4]. The curve was linear over the range 5–2000 ng/ml [$y=(0.7347\cdot10^{-3})x-0.0093$]. A least-squares analysis gave a correlation coefficient of 0.9999.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF HISTIDINE-[M+4] BY GC-MS-SIM

Added (ng/ml)	Found (ng/ml)						Coefficient of variation (%)	Relative error (%)
	Individual values★					Mean ± S.D.		
50.12	52.08	53.33	52.85	51.78	53.09	52.63 ± 0.60	1.13	5.0
501.20	500.27	492.67	496.31	483.03	483.19	491.09 ± 6.95	1.41	2.0

★Each individual value is the mean of triplicate measurements.

The accuracy of measurement was determined for histidine-[M+4] added to 0.5-ml aliquots of pooled plasma containing 16.9 μg/ml endogenous histidine. The plasma samples contained 214.52 ng of the internal standard and different amounts (50.12 and 501.20 ng) of histidine-[M+4]. The amounts of histidine-[M+4] were measured by the present GC–MS–SIM method. Table I shows that the amounts of histidine determined were in good agreement with the actual amounts added, the relative error being less than 5.0%. The intra-assay coefficients of variation were 3.89% for 50.12 ng/ml and 2.53% for 501.20 ng/ml and the inter-assay values were 1.13 and 1.41%, respectively.

The present method provides a sensitive and reliable technique for determining plasma levels of histidine with good accuracy and precision. The method should be applied to pharmacokinetic and metabolic studies of histidine after administration of a small amount of stable isotopically substituted histidine to patients with histidinaemia.

REFERENCES

1 T. Furuta, Y. Kasuya, H. Takahashi and S. Baba, J. Chem. Res., submitted for publication.
2 F.K. Trefz, D.J. Byrd, M.E. Blaskovics, W. Kochen and P. Lutz, Clin. Chim. Acta, 73 (1976) 431.
3 M.-J. Zagalak, H.-Ch. Curtius, W. Leimbacher and U. Redweik, J. Chromatogr., 142 (1977) 523.
4 H.-Ch. Curtius, M.J. Zagalak, K. Baerlocher, J. Schaub, W. Leimbacher and U. Redweik, Helv. Paediatr. Acta, 32 (1977) 461.
5 S. Tokuhisa, K. Saisu, H. Yoshikawa, T. Tsuda, T. Morishita and S. Baba, Chem. Pharm. Bull., 26 (1978) 3647.
6 F.K. Trefz, T. Erlenmaier, D.H. Hunneman, K. Bartholome and P. Lutz, Clin. Chim. Acta, 99 (1979) 211.
7 H. Wegmann, H.-Ch. Curtius and U. Redweik, J. Chromatogr., 158 (1978) 305.
8 H. Wegmann, H.-Ch. Curtius, R. Gitzelmann and A. Otten, Helv. Paediatr. Acta, 34 (1979) 497.
9 H.-Ch. Curtius, H. Farner and F. Rey, J. Chromatogr., 199 (1980) 171.
10 I.M. Moodie, J. Chromatogr., 99 (1974) 495.
11 D. Roach and C.W. Gehrke, J. Chromatogr., 44 (1969) 269.
12 D. Roach, C.W. Gehrke and R.W. Zumwalt, J. Chromatogr., 43 (1969) 311.
13 C.W. Gehrke and K. Leimer, J. Chromatogr., 57 (1971) 219.
14 N. Mahy and E. Gelpi, Chromatographia, 11 (1978) 573.