Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 6

PREPARATION OF MULTI-LABELLED UROCANIC ACIDS WITH ^{2}H, ^{13}C AND ^{15}N

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SUMMARY

Two types of multi-labelled urocanic acids with stable isotopes, *i.e.*, [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid and [2,3,5'-$^{2}H_3$,2'-^{13}C,1',3'-$^{15}N_2$]urocanic acid were synthesized by the enzymatic reaction of DL-[3,3-$^{2}H_2$,1',3'-$^{15}N_2$]histidine or DL-[2,3,3,5'-$^{2}H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine with histidine ammonia-lyase (*Pseudomonas fluorescens*) at pH 9.0. The reaction of DL-[2,3,3,5'-$^{2}H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine was achieved in D_2O buffer system (pD 9.0) to avoid the enzyme-catalyzed hydrogen exchange at C-5' of the imidazole ring. The isotopic purities were demonstrated to be 94 atom%, based upon the ion intensities in the region of the molecular ions of respective gas chromatographic derivatives measured by GC-MS.

Key Words: [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid, [2,3,5'-$^{2}H_3$,2'-^{13}C,1',3'-$^{15}N_2$]urocanic acid, stable isotope, labelled urocanic acid, labelled histidine

INTRODUCTION

Histidinemia is a hereditary metabolic disorder characterized by mental and/or speech retardations[1] and caused by a virtual deficiency of the liver enzyme, histidine ammonia-lyase, which catalyzes β-elimination of ammonia from L-histidine to produce urocanic acid.[2] 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[3-5] of histidinemia by stable isotope

0362–4803/92/060437–07$05.00

Received 17 December, 1992
Revised 14 February, 1992

methodology, we have previously synthesized stable isotopically labelled histidines;[6,7] L-[3,3-$^2H_2$,1',3'-$^{15}N_2$]histidine as biological internal standard and DL-[2,3,3,5'-$^2H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine as analytical internal standard for the GC-MS assay.[8-10]

In the present study, two types of multi-labelled urocanic acids containing at least three non-exchangeable stable isotopes, *i.e.*, [3-^2H,1',3'-$^{15}N_2$]urocanic acid and [2,3,5'-$^2H_3$,2'-^{13}C,1',3'-$^{15}N_2$]urocanic acid were synthesized to investigate the pharmacokinetics of L-histidine in humans by stable isotope methodology.

EXPERIMENTAL

Materials and Methods.– ^1H NMR spectra were recorded on a Bruker AM-400 400 MHz spectrometer for solutions in D_2O using sodium 3-trimethylsilyl-propionate-d_4 as internal standard. Capillary gas chromatographic-mass spectrometric (GC-MS) analysis was done on a Shimadzu QP2000 GC-MS equipped with a data-processing system. GC-MS employed a Durabond (DB-5) fused-silica capillary column (30 m x 0.32 mm i.d., film thickness 0.1-μm; J & W Scientific Inc.). L-Histidine free base, urocanic acid, and histidine ammonia-lyase (*Pseudomonas fluorescens*) were purchased from Sigma (St. Louis, MO, USA). Stable isotopically labelled histidines, *i.e.*, DL-[3,3-$^2H_2$,1',3'-$^{15}N_2$]histidine and DL-[2,3,3,5'-$^2H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine were synthesized in our laboratory.[6,7] All other chemicals and reagents were of analytical-reagent grade and were used without further purification.

[3-^2H,1',3'-$^{15}N_2$]Urocanic Acid.– Histidine ammonia-lyase (1000 units, *Pseudomonas fluorescens*) was incubated in 21 mL of 70 mM carbonate buffer at pH 9.0 for 30 min at 25 °C in the presence of 10 mM $MgCl_2$ and 100 mM glutathione (reduced form). To the incubation mixture was added DL-[3,3-$^2H_2$,1',3'-$^{15}N_2$]histidine (40 mg, 0.25 mmol; 95.3 atom%) and then incubated at 25 °C for 24 h. The enzymatic reaction was terminated by adding 150 mL of EtOH. The precipitated protein was filtered with the aid of charcoal and the solvent was evaporated at 50 °C under reduced pressure. The reaction mixture was purified by anion-exchange column chromatography (Dowex I-X8 200-400 mesh, 5 X 1.5 cm i.d.; 0.5 M CH_3COOH as the eluting solvent). Recrystallization from aqueous EtOH gave pure [3-^2H,1',3'-$^{15}N_2$]urocanic acid (15 mg, 85% from the L-isomer of labelled histidine) as colorless crystals: ^1H NMR (400 MHz; D_2O) δ_H 8.67 (1H, t, J = 5.7 Hz, 2'-H), 7.68 (1H, s, 5'-H), and 6.55 (1H, s, 2-H). A proton signal at δ_H 7.32 (unlabelled compound; 1H, d, J = 16.2 Hz, 3-H) had

completely disappeared in the labelled compound. GC-MS [^{im}N-(ethoxycarbonyl)-urocanic acid *n*-butyl ester][10] *m/z* 269 (M^+, relative intensity; 30%), 196 (M^+ - COOEt, 25%), 169 (M^+ - *n*-C_4H_9OCO + H, 59%), and 124 (M^+ - COOEt - *n*-C_4H_9O + H, 100%). Isotopic purity of the [^2H,$^{15}N_2$]-form was 93.9 atom%.

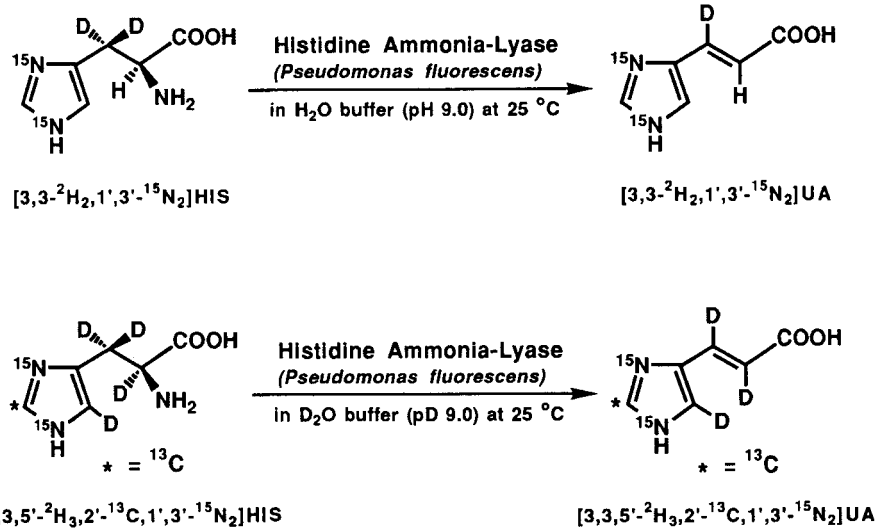
[2,3,5'-$^2H_3$,2'-^{13}C,1',3'-$^{15}N_2$]Urocanic Acid.- According to the procedure for preparation of [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid, DL-[2,3,3,5'-$^2H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine (120 mg, 0.74 mmol; 95.2 atom%) was allowed to react with histidine ammonia-lyase (2000 units) to give [2,3,5'-$^2H_3$,2'-^{13}C,1',3'-$^{15}N_2$]urocanic acid (40 mg, 75% from the L-isomer of labelled histidine) as colorless crystals. The carbonate buffer solution was prepared in D_2O (99.75 atom%, Merck) as a solvent. ^{1}H NMR (400 MHz; D_2O) δ_H 8.67 (1H, dt, J = 5.7, 220.7 Hz, 2'-H). Proton signals at δ_H 7.32 (unlabelled compound; 1H, d, J = 16.2 Hz, 3-H), 7.68 (1H, s, 5'-H), and 6.55 (1H, d, J = 16.2 Hz, 2-H) had completely disappeared in the labelled compound. GC-MS [^{im}N-(ethoxycarbonyl)-urocanic acid *n*-butyl ester][10] *m/z* 272 (M^+, relative intensity; 49%), 199 (M^+ - COOEt, 26%), 172 (M^+ - *n*-C_4H_9OCO + H, 61%), and 127 (M^+ - COOEt - *n*-C_4H_9O + H, 100%). Isotopic purity of the [$^2H_3$,^{13}C,$^{15}N_2$]-form was 93.8 atom%.

RESULTS AND DISCUSSION

Successful application of stable isotope dilution mass spectrometry to the pharmacokinetic and clinical investigations is always dependent upon the availability of multi-labelled compounds at predesignated positions that are chemically inert to the conditions employed. The most appropriate sites for the introduction of deuterium in the urocanic acid molecule are the positions of C-2, C-3 and C-5'. Deuterium substitution at C-2' of the imidazole ring is inappropriate because of its chemical instability. Gerlinger *et al.* synthesized [2,5'-$^2H_2$]- and [2,3-$^2H_2$]urocanic acids by using a combination of chemical and enzymatic method.[11] The method, however, does not provide suitable compounds that satisfy with the requirement for pharmacokinetic studies. In previous papers,[6,7] we have described an efficient and concise synthesis of multi-labelled DL-histidine involving the selective deuteriation at C-2, C-3, or C-5'. The synthetic sequence has also offered a convenient method of introducing stable isotopes (^{15}N and/or ^{13}C) during the construction of imidazole ring of histidine.

Two types of multi-labelled urocanic acids were synthesized by the enzymatic reaction of DL-[3,3-$^2H_2$,1',3'-$^{15}N_2$]histidine or DL-[2,3,3,5'-$^2H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine[6,7] with histidine ammonia-lyase from *Pseudomonas*

fluorescens (Scheme I). DL-[3,3-$^{2}H_2$,1',3'-$^{15}N_2$]Histidine (95.3 atom%) was incubated in a carbonate buffer (pH 9.0) with histidine ammonia-lyase (1000 units) at 25 °C for 24 h in the presence of $MgCl_2$ and glutathione (reduced form). The reaction mixture was purified by anion-exchange column chromatography, followed by recrystallization from aqueous EtOH to give pure [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid (85% yield from the L-isomer in labelled racemic histidine). The mass spectrum of gas chromatographic



Scheme I. Synthesis of Stable Isotopically Labelled Urocanic Acids.

derivative of the labelled urocanic acid, ^{im}N-(ethoxycarbonyl)-urocanic acid *n*-butyl ester,[10] showed that the molecular ion at *m/z* 269 was three mass units higher than that of the unlabelled compound. As shown in Figure 1, the ^{1}H NMR spectrum of [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid showed no proton signal at the C-3 methine (δ_H 7.32). The proton at C-2' (δ_H 8.67) showed a triplet signal (J = 5.7 Hz) due to nitrogen-15 labelled at N-1' and N-3'. The isotopic purity of the [^{2}H,$^{15}N_2$]-form was estimated to be 93.9 atom%, based upon the ion intensities in the region of the molecular ion measured by GC-MS.

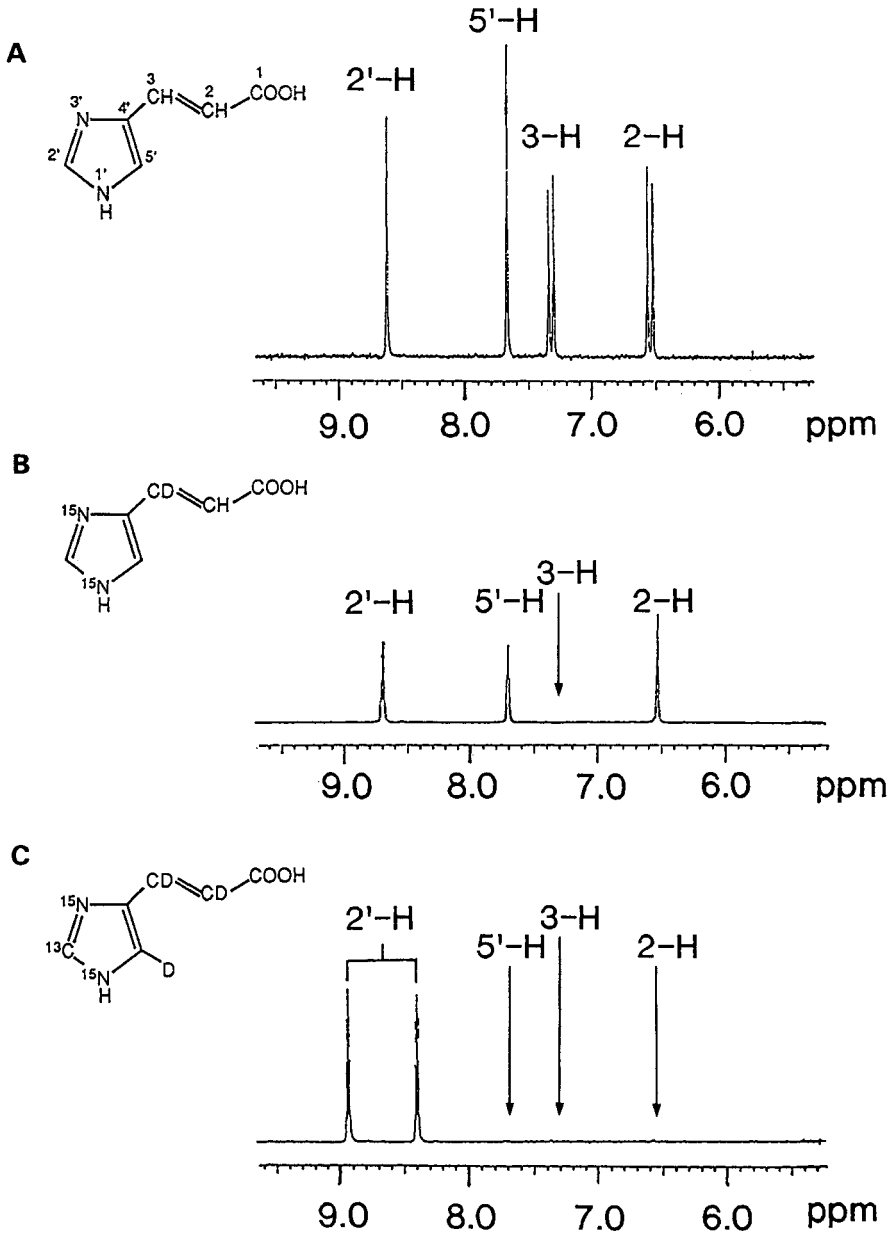


Figure 1. ^{1}H NMR Spectra of Unlabelled Urocanic Acid (A), [3-^{2}H,1',3'-$^{15}N_2$]Urocanic Acid (B), and [2,3,5'-$^{2}H_3$,2'-^{13}C,1',3'-$^{15}N_2$]Urocanic Acid (C).

We have previously demonstrated the enzyme-catalyzed hydrogen exchange at C-5' of the imidazole ring in the reaction of L-[5'-^{2}H]histidine with histidine ammonia-lyase.[12-14] The hydrogen exchange with solvent (H_2O) was rationally explained by a stepwise reaction mechanism via a carbanion intermediate in the elimination of ammonia catalyzed by the enzyme. The enzymatic reaction of DL-[2,3,3,5'-$^{2}H_4$,2'-^{13}C,1',3'-$^{15}N_2$]histidine (95.2 atom%) was therefore carried out using D_2O buffer (pD 9.0) as a solvent to give [2,3,5'-$^{2}H_3$,2'-^{13}C,1',3'-$^{15}N_2$]urocanic acid (75% yield from the L-isomer in labelled racemic histidine). The mass spectrum showed that the molecular ion at *m/z* 272 was six mass units higher than that of the unlabelled compound (isotopic purity as [$^{2}H_4$,$^{15}N_2$,^{13}C]-form, 93.8 atom%). The ^{1}H NMR spectrum of [2,3,5'-$^{2}H_3$,2-^{13}C,1',3'-$^{15}N_2$]urocanic acid (Figure 1) showed no proton signal at C-5' (δ_H 7.68), indicating the retention of deuterium at C-5' of the imidazole ring. In addition, there were no proton signals at C-2 (δ_H 6.55) and C-3 (δ_H 7.32). The H-^{13}C resonance at C-2' (δ_H 8.67, $J_{H\text{-}^{15}N}$ = 5.7 Hz) was observed as doublet ($J_{H\text{-}^{13}C}$ = 220.8 Hz).

The present study provides two types of multi-labelled urocanic acids containing at least three non-exchangeable stable isotopes with high isotopic purity. DL-[2,3,5'-$^{2}H_3$,2'-^{13}C,1',3'-$^{15}N_2$]Urocanic acid shows the mass difference of 3 a.m.u. from [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid and is appropriate for use as an analytical internal standard for the GC-MS assay to determine simultaneously endogenous (unlabelled) urocanic acid and [3-^{2}H,1',3'-$^{15}N_2$]urocanic acid following administration of L-[3,3-$^{2}H_2$,1',3'-$^{15}N_2$]histidine to humans.

ACKNOWLEDGMENTS

This work was supported, in part, by Takeda Science Research and by a Grant-in-Aid for Scientific Research (No. 03807143) from the Ministry of Education, Science and Culture, Japan.

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