Preparation of Deuterium-Labeled Isoniazid for Isotope Dilution Analysis

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A method was established for preparation of deuterated isoniazid(^{2}H-INAH). In this method isonicotinic acid *N*-oxide was deuterated by base-catalyzed exchange reaction and converted to ^{2}H-INAH. The deuterium content of the ^{2}H-INAH obtained by this method was 95.2 atom %D. This ^{2}H-INAH is suitable for use as an internal standard in gas chromatography-mass spectrometry.

Keywords isoniazid; deuterium label; isotope dilution; GC-MS; base-catalyzed deuteration

Isoniazid (INAH) is a drug commonly used in the treatment of tuberculosis. We have reported the detection of free hydrazine as a metabolite of INAH,[1] and have also described the effects of ascorbic acid on the metabolism and excretion of INAH.[2] In these studies, INAH was determined as benzaldehyde isonicotinylhydrazone by gas chromatography (GC)[3] or by gas chromatography-mass spectrometry (GC-MS).[4] This assay method required complex procedures, such as condensation, extraction and derivatization. Thus, the use of a suitable internal standard is of importance for obtaining reliable and reproducible results in this assay.

Isotope dilution analysis is a good method for correcting for losses of a compound during sample preparation. Accordingly, we developed a procedure for preparation of deuterated INAH (^{2}H-INAH) and then we used this isotope-labeled material in the determination of INAH in plasma.

Experimental

Materials Isonicotinic acid *N*-oxide was purchased from Aldrich Chemical Co. Deuterium oxide (99.75 atom%D) and sodium deuteroxide (40% in D_2O, 99 atom%D) were obained from E. Merck.

Preparation of ^{2}H-INAH ^{2}H-INAH was prepared from isonicotinic acid *N*-oxide as follows (Chart 1).

Isonicotinic acid *N*-oxide (3 g) was dissolved in a mixture of 30 ml of D_2O and 3 ml of 40% NaOD in D_2O. The solution was refluxed for 3 h and then cooled in an ice bath. On acidification of the solution to pH 1 with concentrated HCl, deuterated isonicotinic acid *N*-oxide was precipitated and separated in a yield of 2.7 g.

This *N*-oxide (2.7 g) was then refluxed with 100 ml of HCl–EtOH for 4 h. The EtOH was evaporated off and the residue was dissolved in 120 ml of $CHCl_3$ and washed with Na_2CO_3 solution. The $CHCl_3$ layer was separated and the solvent was evaporated off to give deuterated isonicotinic acid ethylester *N*-oxide (2.1 g). According to the method of Hamana,[5] the ester was dissolved in 60 ml of $CHCl_3$ and the solution was added dropwise to 1.2 ml of PCl_3 at 0 °C. The solution was refluxed for 1 h, and then mixed with 30 g of ice water. The mixture was made basic with 10 N NaOH and extracted with $CHCl_3$. The organic layer was dried over anhydrous sodium sulfate and evaporated, giving deuterated isonicotinic acid ethylester as an oily residue (1.0 g).

According to the method of Rohlich,[6] 1.0 g of the deuterated isonicotinic acid ethylester was refluxed with 2 g of hydrazine hydrate for 30 min. The reaction mixture was cooled in an ice bath and ^{2}H-INAH precipitated. The precipitate was separated and recrystallized twice from EtOH to give 0.7 g of ^{2}H-INAH: mp 170 °C.

Extraction and Derivatization Procedures INAH (5—50 μg) was added to 1 ml of control plasma with a fixed amount (20 μg) of ^{2}H-INAH. Then 20 ml of CH_3CN was added and the plasma samples were shaken for 30 min and centrifuged. The upper CH_3CN layer was removed and evaporated. The residue was dissolved in 5 ml of 0.2 N acetate buffer solution (pH 5.0), and this solution was mixed with 0.5 ml of ethanolic benzaldehyde solution (0.05 ml/ml) and shaken for 30 min. The solution was extracted with 20 ml of dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated. The residue was dissolved in 30 μl of *N*,*N*-dimethylformamide and derivatized to *tert*-butyldimethylsilylether (TBDMS) by treatment with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Tokyo Kasei).

GC-MS Conditions GC-MS was carried out in a JEOL D-300 GC-MS computer system. A glass column (1 m × 2 mm i.d.) peaked with 1.5% OV-1 on Chromosorb W (80—100 mesh) was used. Helium gas flow rate, 20 ml/min; column temperature, 230 °C; accelerating voltage, 3 kV; ionizing energy, 20 eV. The selected monitoring ions were those at *m/z* 282 and 284.

Results and Discussion

There are two possible stable isotopes for labeling INAH, ^{15}N and ^{2}H. ^{15}N-labeling is easily achieved by using ^{15}N-hydrazine and isonicotinic acid ester. This method should be very simple and give a high yield, but a label with a high ^{15}N content, such as $^{15}N_2$-hydrazine sulfate (95 atom%), is very expensive.

The other possible method is deuterium-labeling. Kawazoe *et al.*[7] reported hydrogen–deuterium exchange of α-hydrogen in pyridine, quinoline, isoquinoline, and their *N*-oxide on heating them in D_2O solution containing sodium hydroxide or sodium carbonate. Accordingly, we examined the base-catalyzed hydrogen exchange of isonicotinic acid *N*-oxide in D_2O solution and obtained the deuterium-labeled compound in a good yield.

The incorporation of deuterium into INAH was assessed by proton nuclear magnetic resonance (^{1}H-NMR) and MS analyses. As shown in Fig. 1, ^{1}H-NMR data of the product showed complete loss of the doublet signal centered at 8.6 ppm due to the α-hydrogen in the pyridine ring. The MS spectrum of the product indicated that the molecular ion at *m/z* 139 and the base peak at *m/z* 108 were shifted by 2 mass units from those of unlabeled INAH.

The isotopic purity of ^{2}H-INAH was calculated from the

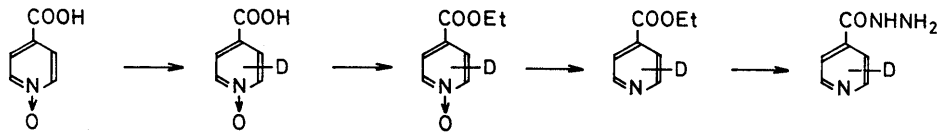

Chart 1. Scheme for Synthesis of Deuterated Isoniazid

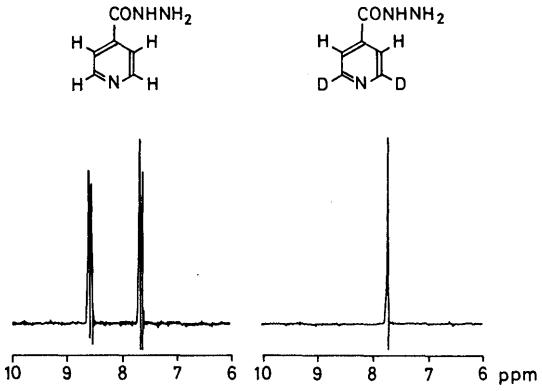

Fig. 1. ^{1}H-NMR Spectra of Isoniazid and Deuterium-Labeled Isoniazid (100 MHz, in CD_3OD)

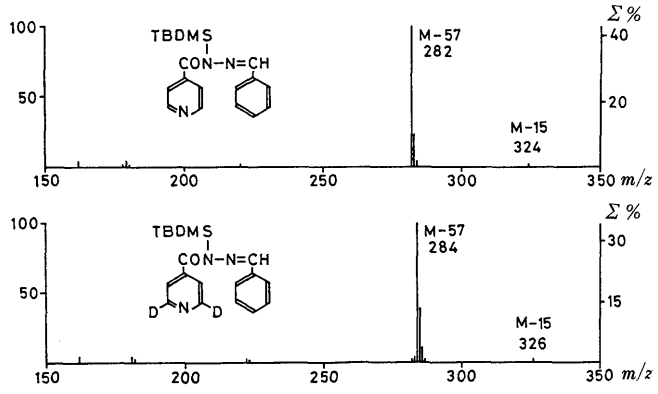

Fig. 2. MS Spectra of Isoniazid and Deuterium-Labeled Isoniazid as TBDMS Derivatives of Their Hydrazones

ion intensity at the molecular ion region. It was found that the ^{2}H-INAH was composed of the $^2H_2$-species (92.4%), the $^2H_1$-species (5.5%) and unlabeled INAH (2.1%), and its deuterium content was evaluated as 95.2 atom%D. This value did not change upon increase of the time (15 h) or temperature (120 °C) of the hydrogen–deuterium exchange reaction.

The MS spectra of INAH and ^{2}H-INAH as the TBDMS derivatives of their hydrazones are shown in Fig. 2. No molecular ion was observed, but base peak ions were seen at *m/z* 282 for INAH and at *m/z* 284 for ^{2}H-INAH. These MS spectra indicate that the deuterium label is stable during the derivatization procedures.

For application of ^{2}H-INAH to isotope dilution analysis,

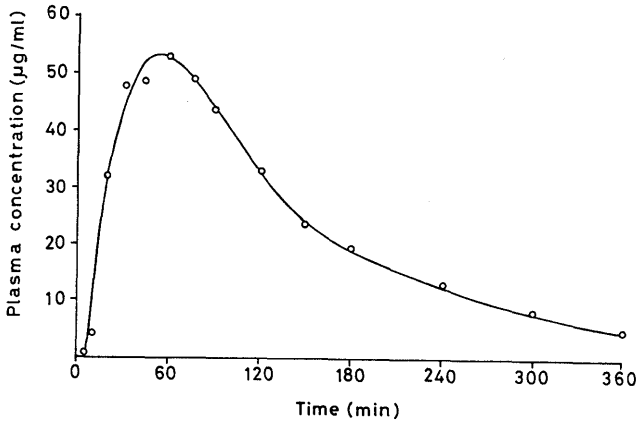

Fig. 3. Plasma Concentration of Isoniazid after Oral Administration to a Rabbit

a working curve was prepared with an extract of rabbit plasma containing known amounts of INAH and ^{2}H-INAH. Selected ion monitoring (SIM) was carried out at *m/z* 282 and 284. Both SIM peaks of INAH and ^{2}H-INAH were observed at 3 min after sample injection. The SIM peak area was corrected for the contributions at *m/z* 282 for ^{2}H-INAH and at *m/z* 284 for INAH. The peak area ratio was correlated with the amount ratio, the correlation coefficient obtained by regression analysis being 0.9996. The average coefficient of variation was $\pm 4.7\%$ in the concentration range of 7.5—75 μg/ml in plasma ($n=6$).

The plasma concentration of INAH in rabbits after oral administration of INAH (50 mg/kg) was measured with ^{2}H-INAH as an internal standard. An example of the change in plasma concentration of INAH with time after oral administration of INAH to a rabbit is shown in Fig. 3.

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