Use of Nuclear Magnetic Resonance Spectroscopy and Selective ^{13}C -Labeling for Pharmacokinetic Research in Man: Detection of Benzoic Acid Conversion to Hippuric Acid

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This paper demonstrates that the stable isotope tracer technique using NMR spectroscopy and the selective ^{13}C labeling of protonated carbons can provide a relatively sensitive method to investigate pharmacokinetic problems in man. The urinary excreted $[1,3,5\text{-}^{13}\text{C}_3]\text{hippuric acid}$ ($[^{13}\text{C}]\text{HA}$) formed from orally administered $[1,3,5\text{-}^{13}\text{C}_3]\text{benzoic acid}$ ($[^{13}\text{C}]\text{BA}$) as a model substrate was successfully quantitated without any separation procedures by proton-decoupled ^{13}C -NMR spectroscopy of 10-fold concentrated urine in a 10 min accumulation time. In spite of the low dosage (10 mg BA), the C3,5 resonances of $[^{13}\text{C}]\text{HA}$ were detected with favorable signal-to-noise ratios to quantitate $[^{13}\text{C}]\text{HA}$ concentration. The administered $[^{13}\text{C}]\text{BA}$ was found to be quantitatively biotransformed to HA and excreted in urine within 4 h. The lower limit of detection was estimated to be 50 nmol in an NMR tube, which was improved about one order of magnitude over that of BA labeled in the quaternary carbon (C7). The potential of an inverse detection experiment using heteronuclear multiple quantum coherence was also investigated in order to detect $[^{13}\text{C}]\text{HA}$ in urine with a higher sensitivity. The inverse experiment improved the sensitivity by a factor of 2–3 over $^{13}\text{C}\{^1\text{H}\}$ -NMR, although the specificity of detection was relatively poor.

Key words nuclear magnetic resonance spectroscopy; ^{13}C label; pharmacokinetics; benzoic acid; hippuric acid

The usefulness of the stable isotope tracer technique using ^{13}C -labeling of substrates followed by nuclear magnetic resonance (NMR) spectroscopy of biofluids has become accepted in metabolic investigations.¹⁾ The greatest single disadvantage associated with this tracer technique has been the lack of sensitivity compared with the stable isotope tracer techniques coupled with gas chromatography–mass spectrometry. This is a serious disadvantage particularly in pharmacokinetic research where many samples must be analyzed in a limited machine time. However, NMR spectroscopy has recently undergone many hardware and software developments which have resulted in ever increasing sensitivity. Thus, it is of particular interest to investigate the efficiency and sensitivity of the ^{13}C -labeling and NMR approach in pharmacokinetic research.

This approach was found useful in the previous paper,²⁾ where the biotransformation of benzoic acid (BA) to hippuric acid (HA) in a human subject was detected by $^{13}\text{C}\{^1\text{H}\}$ -NMR of urine (9.4T, 10 min accumulation) following ingestion of $[7\text{-}^{13}\text{C}]\text{BA}$ (2 mg/kg). Subsequently, the same biotransformation in the rat was followed under the similar NMR conditions with an approximately ten-fold higher sensitivity using BA selectively labeled in the protonated carbons, i.e., $[2,4,6,7\text{-}^{13}\text{C}_4]\text{BA}$ (2 mg/kg).³⁾ The sensitivity improvement was due to the large nuclear Overhauser enhancements and short spin-lattice relaxation times (T_1) of the protonated carbons (C2,6) as well as the double labeling in the same chemical circumstance. The NMR sensitivity for protonated carbons was thought to be further improved by an inverse detection experiment using heteronuclear multiple quantum coherence (HMQC),⁴⁾ by which protons connected to ^{13}C can be selectively detected with a sensitivity comparable to that of ^1H -NMR. Thus, in this paper, the potential of the selective ^{13}C -labeling and NMR approach for pharma-

cokinetic research in man was investigated by urinalysis using both $^{13}\text{C}\{^1\text{H}\}$ -NMR and the HMQC experiment after dosing with a much smaller amount of $[1,3,5\text{-}^{13}\text{C}_3]\text{BA}$ (0.2 mg/kg) as a model substrate.

MATERIALS AND METHODS

Materials $[2\text{-}^{13}\text{C}]\text{Sodium acetate}$ (99.1 atom% ^{13}C) was purchased from Nippon Sanso Co. (Tokyo Japan). $[1,3,5\text{-}^{13}\text{C}_3]\text{BA}$ ($[^{13}\text{C}]\text{BA}$, 99.3 atom% ^{13}C) was synthesized according to the previously described method.⁵⁾ Deuterium oxide (99.95 atom% ^2H), ca. 40% sodium deuterioxide $^2\text{H}_2\text{O}$ solution and sodium 3-trimethylsilyl-[2,2,3,3- $^2\text{H}_4$]-propionate (TSP) were purchased from Merck (Darmstadt, Germany). Other reagents were purchased from Kanto Kagaku Co. (Tokyo, Japan).

Administration A healthy male subject (64 kg weight) received a single oral dose of $[^{13}\text{C}]\text{BA}$ (10 mg) with 200 ml of water after an overnight fast. Half an hour after the administration of the BA, the subject was allowed to drink 100 ml of water. Three hours after the administration, the subject was allowed to drink freely. Urine samples were collected immediately before administration, and then 0–0.5, 0.5–1, 1–2, 2–4, 4–6 h postdose. The volume of urine collected for each period was in the range of 100–200 ml. The urine samples were stored at -20°C until analyzed.

Sample Preparation Ten ml of urine collected for each period was freeze-dried. The residue was reconstituted in approximately 1 ml of water containing sodium hydroxide (0.5 mmol) and $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ (7.35 μmol) for

Chart 1

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$^{13}\text{C}\{^1\text{H}\}$ -NMR, or in approximately 1 ml of $^2\text{H}_2\text{O}$ containing sodium deuterioxide (0.5 mmol) and $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ (1.46 μmol) for the HMQC experiment. The mixture was subjected to sonication to completely dissolve HA. The resulting suspension was centrifuged (2500 rpm, 10 min), and about 0.4 ml of the supernatant was transferred to a 5 mm NMR tube.

NMR Measurements $^{13}\text{C}\{^1\text{H}\}$ -NMR spectra were measured on a Bruker AM-400 spectrometer (9.4 T) at 300 K using a 5 mm NMR tube. The following $^{13}\text{C}\{^1\text{H}\}$ -NMR conditions were selected, estimating T_1 of C3,5 of HA to be similar to that (3.8 s) of BA.⁶⁾ Parameters were spectral width, 25000 Hz; time domain points, 32768; 75° pulse; recycle time, 2.7 s; 216 scans; and line broadening, 2.0 Hz. Proton NMR and HMQC spectra were measured on a Bruker ARX-400 spectrometer (9.4 T) at 300 K using an inverse probe. The inverse experiment was performed using a one-dimensional version of the usual HMQC pulse sequence⁴⁾ with or without ^{13}C GARP⁷⁾ decoupling during ^1H data acquisition. Parameters for ^1H -NMR and HMQC measurements were spectral width, 5208 Hz; time domain points, 8192; recycle time, 2.8 s; 206 scans; and line broadening, 1.0 Hz. The total accumulation time was 10 min for all acquisitions. Chemical shifts were referenced to that of TSP ($\delta_{1\text{H}}$ and $\delta_{13\text{C}}$ 0).

Quantitation Method The residue obtained by freeze-drying of the control urine (10 ml) was reconstituted in 1 ml of a standard aqueous solution of non-labeled sodium hippurate (460 mM) and sodium acetate (506 mM). The resulting suspension was treated and analyzed six times by ^{13}C -NMR spectroscopy as described above. The relative sensitivity for equal moles of sodium hippurate and sodium acetate was calculated from the integral intensities of the resonances due to C3,5 ($\delta_{13\text{C}}$ 131.5) of the former and C2 ($\delta_{13\text{C}}$ 26.2) of the latter. The relative sensitivity (C3,5/C2) was 6.85 (cv. 4.2%). The amount of $[1,3,5\text{-}^{13}\text{C}_3]\text{HA}$ ($[^{13}\text{C}]\text{HA}$) in urine was quantitated based on the relative sensitivity, the ratio of the integral intensities of resonances [C3,5 of $[^{13}\text{C}]\text{HA}$ /C2 of $[2\text{-}^{13}\text{C}]\text{sodium acetate}$], and the amount of $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ added.

RESULTS AND DISCUSSION

In the previous study,³⁾ the C3,5 resonances of BA ($\delta_{13\text{C}}$ 131.0) and HA ($\delta_{13\text{C}}$ 131.5) were assigned, and found to be satisfactorily separated in water despite the positions being three bonds away from the metabolic site (C7). Thus, $[1,3,5\text{-}^{13}\text{C}_3]\text{BA}$ ($[^{13}\text{C}]\text{BA}$) was used as a precursor in the present research. $[^{13}\text{C}]\text{BA}$ (10 mg) was administered to a human subject and the excreted urine was analyzed by $^{13}\text{C}\{^1\text{H}\}$ -NMR after concentration by a factor of 10. There was no appreciable resonance due to endogenous BA or HA in the spectrum of control urine under the NMR conditions used, although endogenous resonances probably due to urea, creatinine, and citric acid were observed.⁸⁾ In the $^{13}\text{C}\{^1\text{H}\}$ -NMR spectra of urine following ingestion of BA, additional resonances at $\delta_{13\text{C}}$ 131.5 and 136.1 were clearly observed, and they were due to C3,5 and C1 of $[^{13}\text{C}]\text{HA}$ formed from administered $[^{13}\text{C}]\text{BA}$, as illustrated in Fig. 1. The C3,5 resonances of BA and HA in the concentrated urine were confirmed to be well separated with the line broadening of 2 Hz as shown in Fig. 2A. Figure 3 shows the time-course of partial $^{13}\text{C}\{^1\text{H}\}$ -NMR spectra of urine. The resonance intensities rapidly decreased with time after dosing. In spite of the low dosage, the spectra showed favorable signal-to-noise (S/N) ratios using only a 10-min accumulation time, which was a significant contrast to the resonances of the quaternary carbon (C1) in the spectra.

The amounts of urinary excreted $[^{13}\text{C}]\text{HA}$ were determined as expressed in Table 1. $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ was used as an internal standard for the quantitation. This can be regarded as valid because there was no appreciable resonance due to endogenous acetate in the $^{13}\text{C}\{^1\text{H}\}$ spectra of control urine under the NMR conditions used. The calibration of NMR sensitivity for $[^{13}\text{C}]\text{HA}$ (C3,5) and $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ (C2) was performed using those non-labeled analogues. The advantage of this calibration is that no preparation of the authentic labeled metabolite is required.

The C3,5 resonance in 1–2 h urine shown in Fig. 3 corresponded to approximately 500 nmol in the NMR

Fig. 1. $^{13}\text{C}\{^1\text{H}\}$ -NMR Spectrum of 0–0.5 h Urine Obtained from a Human Subject Orally Administered with $[^{13}\text{C}]\text{BA}$ (10 mg)

The resonance at $\delta_{13\text{C}}$ 165 was obviously due to urea, whereas those at $\delta_{13\text{C}}$ 33, 49, 59 and 62 were unidentified, although they were probably due to creatinine and citric acid.⁸⁾

tube. One-tenth of this value, *i.e.*, 50 nmol is probably detected like the C1 resonance in the same spectrum. This value seems to be the lower limit of detection for $[^{13}\text{C}]\text{HA}$, which is almost identical to that of $[2,4,6,7-^{13}\text{C}_4]\text{HA}$ in rat urine,³⁾ and ten times higher than that of BA labeled in the quaternary carbon (C7) in concentrated human urine.²⁾ Thus, $[^{13}\text{C}]\text{HA}$ can be measured to 10 μM in urine by the present ^{13}C -NMR approach. Also, the $[^{13}\text{C}]\text{HA}$ excretion can be followed every few minutes using ureteral catheterization since the urine is excreted at the rate of 2–6 ml/min. These results imply that the selective labeling of protonated carbons can afford a much higher sensitivity than the labeling of quaternary carbons.

The urinary excretion of $[^{13}\text{C}]\text{HA}$ over 4 h amounted

Fig. 2. Spectral Resolution of $[^{13}\text{C}]\text{BA}$ and $[^{13}\text{C}]\text{HA}$ in $^{13}\text{C}\{^1\text{H}\}$ -NMR (A) and ^{13}C -Decoupled HMQC Spectra (B)

The 0.5–1 h urine was treated and placed in an NMR tube as described in Materials and Methods. The urine sample in the tube was spiked with $[^{13}\text{C}]\text{BA}$ (*ca.* 1 mg) and analyzed by $^{13}\text{C}\{^1\text{H}\}$ NMR or by the HMQC experiment.

to virtually 100% of the injected dose. It was found that the $[^{13}\text{C}]\text{HA}$ excretion could be almost completely followed under the present experimental conditions. The time-course was similar to that of the previous experiment using 100 mg of $[7-^{13}\text{C}]\text{BA}$ as a precursor.²⁾ These experiments have demonstrated the feasibility of detecting the biotransformation of BA to HA in a human subject by the selective ^{13}C -labeling and ^{13}C -NMR approach even in such a small dose.

The urinary excretion rate of HA formed from administered BA had been used as an indicator of liver function because the biotransformation occurs primarily in liver and reflects liver reserve.⁹⁾ In the test, a large amount of BA (1–5 g) had to be administered so that the contribution of endogenous HA to the quantitation of exogenous HA was negligible. Also, the quantitation procedures were extremely tedious. In the ^{13}C -NMR approach, a much smaller amount (10 mg) of BA was orally administered, and the urinary exogenous HA was quantitated by very simple procedures. Therefore, the present method could form the basis for a safer and more convenient HA test.

HMQC, which is an inverse detection experiment, has

Table 1. Amounts of $[^{13}\text{C}]\text{HA}$ in 10 ml of Urine (A) and Total Volume of Urine (B) Excreted in Each Period

Time (h)	$[^{13}\text{C}]\text{HA}$ amounts (mg)	
	A ^{a)}	B
0–0.5	0.327	2.776
0.5–1	0.436	8.068
1–2	0.229	3.158
2–4	0.117 ^{b)}	1.432 ^{b)}

a) Ten ml of urine collected for each period was freeze-dried and reconstituted in 1 ml of H_2O . Approximately 0.4 ml of the supernatant was transferred to the NMR tube followed by ^{13}C -NMR analysis. b) The values were not corrected for the contribution by endogenous HA.

Fig. 3. Partial $^{13}\text{C}\{^1\text{H}\}$ -NMR Spectra of Urine Obtained from a Human Subject Orally Administered with $[^{13}\text{C}]\text{BA}$ (10 mg)

Collection periods of urine are indicated at the top. All spectra were plotted at the fixed C2 resonance height of $[2-^{13}\text{C}]\text{sodium acetate}$ spiked. The resonance with asterisk ($\delta_{13\text{C}}$ 129.9) in the spectrum for 2–4 h urine seemed to be due to C2, 6 of endogenous HA.

Fig. 4. ^1H -NMR and HMQC Spectra of 0.5–1 h Urine Obtained from a Human Subject Orally Administered with $[^{13}\text{C}]\text{BA}$ (10 mg)

A, ^1H -NMR; B, HMQC; C, ^{13}C -decoupled HMQC. The region of the water resonance (δ_{H} 4.8) was omitted. The $[2\text{-}^{13}\text{C}]\text{sodium acetate}$ was spiked into the urine sample as described in Materials and Methods in order to examine the selectivity of HMQC detection in the region of higher field.

become routinely available quite recently. In principle, protons bound to ^{13}C can be selectively detected by the experiment with a sensitivity comparable to that of ^1H -NMR spectroscopy. Therefore, ^{13}C -labeled drug metabolites in biological fluids can be followed by the experiment with a much higher sensitivity than $^{13}\text{C}\{^1\text{H}\}$ -NMR detection. However, the application to biological fluids has not been reported in the literature. Thus, an attempt was made to detect $[^{13}\text{C}]\text{HA}$ in concentrated urine using a one-dimensional version of HMQC pulse sequence.

The major technical difficulty with an inverse experiment is suppression of the signals from protons not coupled to ^{13}C . Thus, HMQC spectra were first obtained under the ^{13}C non-decoupled conditions to investigate the problem. As illustrated in Fig. 4A and 4B, the H3,5 resonance (δ_{H} 7.6) of $[^{13}\text{C}]\text{HA}$ was selectively detected as a doublet of quintets in the aromatic region by the HMQC experiment, which was a significant contrast to the case of ^1H -NMR spectroscopy. The monitoring of urinary excreted $[^{13}\text{C}]\text{HA}$ by the ^{13}C -decoupled HMQC experiment was thought possible because no H3,5 resonance due to endogenous HA appeared in the non-decoupled HMQC spectrum. On the other hand, many endogenous resonances in the region of higher field remained. However, $[2\text{-}^{13}\text{C}]\text{acetate}$ spiked was observed as an intense doublet resonance at δ_{H} 1.9, which showed how selective the HMQC detection is. The H2 resonance of endogenous acetate was found difficult to suppress because of the longer T_1 of the nucleus. The HMQC spectrum was subsequently obtained under the ^{13}C -decoupled conditions as shown in Fig. 4C, where the

H3,5 resonance of $[^{13}\text{C}]\text{HA}$ appeared as a triplet. The resonance broadened to a small extent due to the decoupling. The spectral resolution of the H3,5 resonances of BA and HA was confirmed as shown in Fig. 2B. The S/N ratio of the H3,5 resonance was found to be improved by a factor of 2–3, compared with that of the C3,5 resonance in the corresponding $^{13}\text{C}\{^1\text{H}\}$ -NMR spectrum. Needless to say, the gain in sensitivity is expected to be increased to 4–6, assuming the resonance appears as a singlet.¹⁰⁾

These preliminary results suggest that the biotransformation of BA to HA can be followed with a higher sensitivity by the ^{13}C -decoupled HMQC experiment of urine following ingestion of $[^{13}\text{C}]\text{BA}$. This also means a great reduction in the accumulation time with the same S/N ratios as those of $^{13}\text{C}\{^1\text{H}\}$ spectra. However, the direct ^{13}C -NMR affords a more simple approach with higher specificity of detection at the present stage. It is noteworthy that the biotransformation of BA to HA in a human subject could be followed by the ^{13}C -NMR approach using only 0.1 mmol of $[^{13}\text{C}]\text{BA}$ as a substrate. The sensitivities of the NMR approaches using the selective ^{13}C -labeling are further improved by the use of a larger NMR tube as well as a higher magnetic field. Thus, the NMR approaches promise to be of great value in detecting and quantitating ^{13}C -labeled drug metabolites in biofluids with minimal sample pretreatments in pharmacokinetic research of man.

REFERENCES AND NOTES

- 1) Simpson T. J., "Isotopes in the Physical and Biomedical Sciences,"

- Vol. 2, ed. by Buncel E., Jones J. R., Elsevier, Amsterdam, 1991, pp. 431—474; Bain A. D., Lao L., *ibid.*, pp. 411—429; Malet-Martino M. C., Martino R., *Biochimie*, **74**, 785 (1992); London R. E., *Prog. Nucl. Magn. Res. Spectrosc.*, **20**, 337 (1988).
- 2) Baba S., Akira K., Sakuma C., *Yakugaku Zasshi*, **110**, 586 (1990).
  - 3) Akira K., Takagi N., Takeo S., Shindo H., Baba S., *Anal. Biochem.*, **210**, 86 (1993).
  - 4) Bax A., Sparks S. W., Torchia A., *Methods Enzymol.*, **176**, 134 (1989).
  - 5) Akira K., Baba S., *J. Labelled Compd. and Radiopharm.*, **31**, 109 (1991).
  - 6) Rabenstein D. L., Keire D. A., *Pract. Spectrosc.*, **11**, 323 (1991).
  - 7) Shaka A. J., Barker P. B., Freeman R., *J. Magn. Res.*, **64**, 547 (1985).
  - 8) Yamamoto H., Arai T., Khoda N., Yamaguchi S., *Igaku No Ayumi*, **144**, 929 (1988); Nicholson J. K., Wilson I. D., *Prog. Drug Res.*, **31**, 427 (1987).
  - 9) Quick A. J., *Am. J. Clin. Pathol.*, **10**, 222 (1940); Aoyama H., Kamiyama Y., Ukikusa M., Ozawa K., *J. Lab. Clin. Med.*, **108**, 456 (1986).
  - 10) The H3,5 resonance cannot be obtained as a singlet at the present stage because the simultaneous decoupling of H2,6 and H4 is impossible under the ^{13}C GARP decoupling conditions.