ABSTRACT:

Antipyrine is a useful probe to evaluate variation of in vivo activities of oxidative hepatic drug-metabolizing enzymes. Here we describe an approach using $^{13}$C labeling and NMR spectroscopy for the direct and simultaneous analysis of major metabolites of antipyrine in human urine. [C-Methyl-$^{13}$C]antipyrine (500 mg) was dosed orally to human volunteers, and the post-dose urine was analyzed by 100-MHz $^{13}$C NMR spectroscopy under the conditions of distortionless enhancement by polarization transfer (DEPT) without any pretreatments such as deconjugation, chromatographic separation, or solvent extraction. Consequently, all the major metabolites in urine were successfully detected with favorable signal-to-noise ratios in the limited acquisition time (30 min). The reproducibility of the NMR detection was sufficient for the quantitative evaluation of the metabolic profile. A quantitative method is proposed using a combination of inverse gated decoupling and DEPT experiments with [2-$^{13}$C]sodium acetate as an internal standard. The present approach is useful and practical to evaluate variation of in vivo activities of the conjugation enzymes as well as oxidative enzymes responsible for the formation of antipyrine metabolites in humans. This direct approach would enhance the value of the antipyrine test because of its simplicity and convenience.

Antipyrine, an antipyretic and analgesic, has been extensively used as a probe to study the influence of age, diseases, drugs, heredity, and environmental factors on oxidative hepatic drug-metabolizing capacity (Hartleb, 1991; St. Peter and Awni, 1991). In humans, antipyrine is metabolized by several forms of cytochrome P450 (Sharer and Wrighton, 1996), and the resulting oxidative metabolites are extensively conjugated and excreted in urine (Bassmann et al., 1985; Palette et al., 1991; Moreau et al., 1992). The main oxidative metabolites are 3-hydroxymethylantipyrine (HMA), 4-hydroxyantipyrine (OHA), and norantipyrine (NORA). Glucuronide conjugation is the major phase II pathway (Fig. 1) (Bottcher et al., 1982b).

The quantitation of antipyrine and its metabolites excreted in urine has been used to understand variation in oxidative hepatic drug-metabolizing capacity (Ohashi et al., 1991; Robertz Vaupel et al., 1992; Ali et al., 1995; Yang et al., 1996). Several methods using high-performance liquid chromatography (HPLC) have been reported for the determination of antipyrine and its metabolites (Danhof et al., 1979; Teunissen et al., 1983; Bassmann et al., 1985; Mikati et al., 1988; Palette et al., 1991; Velic et al., 1995). However, the direct and simultaneous determination of all phase I and phase II metabolites in biological materials has not been reported using HPLC, except for a radio-HPLC method (Velic et al., 1995). However, the method is troublesome to use and unsuitable for application to humans because of the radiation hazard. Although enzymic or chemical deconjugation followed by HPLC analysis is still the standard approach, analytical problems arise from the diversity of metabolites and the instability of NORA and OHA liberated after the deconjugation (Danhof et al., 1979; Bottcher et al., 1982a, 1984; Teunissen et al., 1983; Palette et al., 1991), which varies according to the conditions of hydrolysis and the nature of the conjugates (Bottcher et al., 1982a, 1984; Teunissen et al., 1983; Moreau et al., 1992).

A stable isotope tracer technique using $^{13}$C labeling of substrates (approximately 100% enrichment) followed by NMR spectroscopy of biofluids has been demonstrated to be useful for pharmacokinetic research (Baba et al., 1990, 1995; Malet-Martino and Martino, 1992; Akira et al., 1993, 1997, 1998; Akira and Shinohara, 1996). Because of the high specificity of detection, the application of the tracer technique enables analysis of biological fluids without resorting to extraction and chromatographic separations. Therefore, the technique saves much time and analytical effort, and the decomposition and loss of compounds can be minimized. In a previous study, antipyrine metabolites in rat urine were successfully detected using the NMR approach with $^{13}$C labeling (Akira et al., 1999). In the present study, the NMR approach with $^{13}$C labeling has been used for the direct analysis of major metabolites that occur in human urine after oral dosing with $^{13}$C-labeled antipyrine.

Materials and Methods

Chemicals and Reagents. [C-Methyl-$^{13}$C]antipyrine (>99 atom% $^{13}$C), [C-methyl-$^{13}$C]OHA (>99 atom% $^{13}$C), HMA, and OHA sulfate (OHA-S) were synthesized as previously described (Akira et al., 1999). [2-$^{13}$C]Sodium acetate was purchased from Sigma-Aldrich (St. Louis, MO). The deuterated standard, sodium acetate-d$_3$ (99 atom% $^{13}$C), was purchased from Cambridge Isotope Laboratories (Andover, MA). Other reagents were of analytical grade or better.
3-trimethylsilyl[2,2,3,3-2H₄]propionate (1–2 mg) and deuterium oxide (100 ml) of aqueous solution of [2-13 C]sodium acetate (448 mCi/ml) as an internal standard (IS). The mixture was centrifuged (3000 rpm, 10 min), and about 0.4 ml of the supernatant was transferred to a 5-mm NMR tube containing sodium 3-trimethylsilyl[2,2,3,3-2H₄]propionate (1–2 mg) and deuterium oxide (100 μl) for field-frequency lock.

Subject and Administration. Two healthy male subjects [24 years, 50 kg (1); 39 years, 59 kg (2)] received a single oral dose of [13 C]antipyrine (500 mg) dissolved in 100 ml of water after an overnight fast. Informed consent was obtained from the subjects. Urine samples were collected immediately before administration, and then at 0 to 12, 12 to 24, 24 to 36, and 36 to 48 h postdose. The volume of urine collected for each period was in the range of 212 to 328 ml for subject 1 and 365 to 585 ml for subject 2. The pH value was in the range of 5.8 to 6.7. The urine samples were stored at −20°C until analyzed.

NMR Measurements. Ten (subject 1) or 20 ml (subject 2) of urine collected for each period was freeze-dried. The residue was reconstituted in 1 ml of aqueous solution of [2-13 C]sodium acetate (448 μCi/ml) as an internal standard (IS). The mixture was centrifuged (3000 rpm, 10 min), and about 0.4 ml of the supernatant was transferred to a 5-mm NMR tube containing sodium 3-trimethylsilyl[2,2,3,3-2H₄]propionate (1–2 mg) and deuterium oxide (100 μl) for field-frequency lock.

13C NMR spectra were measured at 300 K on a Bruker DRX500 spectrometer under the conditions of usual 1H decoupling (13C[1H]), distortionless enhancement by polarization transfer (DEPT) with 1H decoupling (Morris, 1984), or inverse gated decoupling (Shoolery, 1977). The 13C chemical shifts were referenced to that of sodium 3-trimethylsilyl[2,2,3,3-2H₄]propionate (δ13C 0). Parameters for the 13C[1H]-NMR were spectral width, 30581 Hz; time domain points, 65536; 45° pulse; acquisition time, 1.07 s; pulse delay, 2.00 s; accumulation 580 (30 min); zero filling to 131,072; and line broadening, 1.0 Hz. Parameters for the DEPT experiments were spectral width, 30,581 Hz; time domain points, 65536; 90° pulse; acquisition time, 1.07 s; pulse delay, 50 s; accumulation 1224 (17.4 h); zero filling to 131,072; and line broadening, 0.5 Hz. The spin-lattice relaxation times (T₁) of the labeled carbons of the metabolites and the IS in urine were determined using the post-dose urine by inversion recovery experiments as follows: HMA glucuronide (HMA-G), 0.2 s; HMA, 1.1 s; NORA glucuronide (NORA-G), 1.6 s; OHA-S, 2.4 s; OHA gluturonide (OHA-G), 1.6 s; IS (acetate), 9.3 s. Thus the delay time in the inverse gated decoupling experiments was set at 50 s, which is about 5 times the longest T₁ (9.3 s) because the spin system almost completely returns to equilibrium in 5 T₁ when the flip angle is 90°.

Quantitation Method by NMR. The 12- to 24-h post-dose urine of subject 2 was treated as described above and analyzed by 13C NMR under the inverse gated decoupling conditions. The integral intensities of the resonances due to the labeled antipyrine metabolites, creatinine, and the IS were measured, and then the molar ratios of the metabolites and the IS in the urine sample were determined. The same sample was subsequently analyzed by the DEPT experiments, and the relative integral intensities of the metabolites and the IS were measured. From these results, the relative NMR sensitivity (metabolite/IS) for equal moles of the metabolites and the IS was calculated as follows: HMA-G, 1.8; HMA, 1.5; NORA-G, 1.6; OHA-S, 1.8; OHA-G, 1.5; creatinine (CH₃COOH), 1.6. The metabolites in other urine samples were quantitated by the DEPT experiments, based on the relative sensitivity, the amount of the IS added, and the ratio of the resonance integral intensities (metabolite/IS).

HPLC Measurements. HMA in urine was determined using an HPLC system equipped with a Waters M600E multisolvent delivery system, Waters U6K injector, Waters Lambda-Max model 481 LC spectrophotometer set at 254 nm (Waters Corp., Milford, MA), and Inertsil ODS-2 column (250 × 4.6-mm, i.d., 5 μm, GL Sciences, Tokyo, Japan) with a precolumn. The mobile phase was a mixture of acetonitrile/H₂O/acetic acid (5:95:0.5, v/v, solution A) and acetonitrile/H₂O/acetic acid (90:10:0.5, v/v, solution B) with a linear gradient of 100 to 0% solution A from 0 to 30 min. The flow rate was 1 ml/min.

Urine was treated according to the method reported by Teunissen et al. (1983), except that enzymic hydrolysis was omitted. Briefly, urine (0.5 ml) was extracted with 5 ml of a mixture of chloroform and ethanol (9:1, v/v) after addition of phenacitin (14.6 μg) as an IS and sodium chloride (200 mg). A linear calibration curve with a correlation coefficient of 0.999 was obtained using blank urine containing known amounts of HMA (2.0–31.7 μg) and phenacitin (14.6 μg).

Results and Discussion

In our previous study, antipyrine metabolites in rat urine, i.e., HMA-G, HMA, NORA sulfate, NORA-G, OHA, OHA-S, OHA-G, and 4,4′-dihydroxyantipyrine sulfate, were directly and simultaneously detected using [C-methyl-13 C]antipyrine as a substrate and NMR spectroscopy (Akira et al., 1999). Main urinary metabolites of antipyrine in humans have been reported to be HMA-G, HMA, NORA-G, OHA-S, and OHA-G (Bottcher et al., 1984; Bassmann et al., 1985; Moreau et al., 1992) as shown in Fig. 1, although minor metabolites such as 3-carboxyantipyrine (Paletet et al., 1991) and 4,4′-dihydroxyantipyrine glucuronide (Bassmann et al., 1985) and a small amount of antipyrine also occur. Thus, it appeared that the 13C-labeled antipyrine method could be useful for the direct 13C NMR detection of urinary antipyrine metabolites in humans.

Labeled antipyrine was orally administered to humans, and the excreted urine was analyzed by both of the 13C[1H]-NMR and the DEPT experiments as shown in Figs. 2 and 3. Many resonances due to antipyrine metabolites and endogenous metabolites such as urea and creatinine were observed in the 13C[1H]-NMR spectra, as illustrated in Fig. 2B. The major resonances at δ13C 64.5, 57.9, 16.1, 12.1, and 11.8 observed in the 13C[1H]-NMR spectra were also detected in the DEPT spectra as shown in Figs. 2C and 3. These resonances were assigned to HMA-G, HMA, NORA-G, OHA-S, and OHA-G, respectively, comparing the chemical shifts with those of the spectra of urine from rats dosed with the labeled antipyrine and the 13C-labeled NORA (Akira et al., 1999). The assignment of HMA and OHA-S was confirmed by spiking those authentic samples. When the post-dose urine was incubated with β-glucuronidase in a similar manner as previously described (Akira et al., 1999), the resonances of HMA-G, NORA-G, and OHA-G disappeared with a concurrent increase of the resonance of HMA (δ13C 57.9) and appearance of the resonances of

FIG. 1. Structures of antipyrine and metabolites.

* labeled position.
OHA (δ\(^{13}\)C 11.0) and NORA (δ\(^{13}\)C 14.2). In the control experiments without β-glucuronidase, the NMR spectra showed no apparent change, which confirmed the resonances before the deconjugation to be glucuronide conjugates of these phase I metabolites.

All of the major antipyrine metabolites reported to occur in human urine were identified on the \(^{13}\)C NMR spectra by the above experiments. The results showed that the major antipyrine metabolites could be directly detected in one operation by the combined use of \(^{13}\)C NMR and \(^{13}\)C labeling of the C-methyl carbon without any pretreatments such as deconjugation, extraction, and chromatography. The spectra shown in Fig. 2B are the first complete profile of antipyrine metabolism in humans. There have been some reports to suggest unknown metabolites of antipyrine (Uchino et al., 1983; Palette et al., 1991). These NMR data are useful as a clue to identify some new metabolites.

The signal-to-noise ratios of the resonances due to the major metabolites, obtained using the limited acquisition time, were considered sufficient for the quantitative evaluation of the metabolic profile. The signal-to-noise ratios of the DEPT spectra were enhanced by about 2-fold, compared with those of the \(^{13}\)C(\(^{1}\)H)-NMR spectra in the same accumulation time, as illustrated in Fig. 2. When the analysis of

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**Fig. 2.** \(^{13}\)C(\(^{1}\)H)-NMR spectrum of control urine (A), and \(^{13}\)C(\(^{1}\)H)-NMR (B) and DEPT spectra of 12- to 24-h post-dose urine (C), from subject 1 orally administered with \([^{13}\text{C}]\)antipyrine (500 mg).

1, urea; 2, HMA-G; 3, creatinine (CH\(_2\)); 4, HMA; 5, creatinine (CH\(_3\)); 6, IS (acetate); 7, NORA-G; 8, antipyrine; 9, OHA-S; and 10, OHA-G.

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**Fig. 3.** DEPT spectra of urine collected immediately before (A) and at 0 to 12 h (B), 12 to 24 h (C), 24 to 36 h (D) and 36 to 48 h (E) after oral administration of \([^{13}\text{C}]\)antipyrine (500 mg) to subject 2.

Key is identical to Fig. 2.

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**Fig. 4.** Inverse gated decoupling spectrum of the 12- to 24-h post-dose urine analyzed in Fig. 3.

Key is identical to Fig. 2.
the post-dose urine in Fig. 2 was repeated five times under the DEPT conditions, the coefficients of variation of the integral intensity ratios between the metabolites and the IS were as follows: HMA-G, 2.0%; HMA, 1.9%; NORA-G, 2.1%; OHA-S, 2.1%; and OHA-G, 1.7%. The proposed approach is thus suitable for evaluating variation of in vivo activities of conjugation enzymes as well as oxidation enzymes responsible for the formation of antipyrine metabolites in humans.

To quantitate the 13C-labeled metabolites, the calibration of NMR sensitivity for individual metabolites and an IS is generally performed using the authentic compounds (Akira et al., 1993, 1997). This is usually necessary because sensitivity under DEPT conditions differs depending on the number and $T_1$ values of the 1H nuclei attached to the monitored 13C nuclei, and the 13C–1H coupling constants. However, in these experiments, calibration of NMR sensitivity was achieved without the use of authentic labeled glucuronides, which are difficult to prepare (Palette et al., 1994), by a combination of DEPT and inverse gated decoupling experiments.

The post-dose urine analyzed by the DEPT experiments (see Fig. 3C) was subsequently analyzed by the inverse gated decoupling experiments as shown in Fig. 4. Although the NMR sensitivity under the inverse gated decoupling conditions was extremely poor, favorable signal-to-noise ratios were obtained by the long accumulation. The relative NMR sensitivity between the metabolites and the IS obtained from these experiments was used to quantitate the metabolites as described under Materials and Methods. The percentage of excreted amounts of the five major metabolites were obtained as shown in Table 1. The cumulative excretion of individual metabolites and the total cumulative excretion in 48 h were similar to those reported previously (Palette et al., 1991; Moreau et al., 1992). The elimination half-lives of HMA-G, HMA, NORA-G, OHA-S, and OHA-G were 35, 25, 27, 23, and 31 h for subject 1, and 38, 45, 38, 23, and 31 h for subject 2, respectively.

Palette et al. (1991) reported an HPLC method to determine the three main oxidative antipyrine metabolites, i.e., HMA, NORA, and OHA, in human urine before and after deconjugation, by which the amounts of conjugated metabolites were indirectly estimated. However, the procedures were considerably troublesome, and the amount of OHA-S was not obtained because the enzymic deconjugation of OHA-S was disturbed by sodium metabisulphite added to stabilize OHA and NORA (Moreau et al., 1992). The present NMR approach directly and simultaneously detects all the major metabolites including OHA-S in stable chemical forms so that more accurate and reliable results are obtained.

The resonances due to creatinine were detected with favorable signal-to-noise ratios, as shown in Figs. 2 and 3. Thus, the urinary output of labeled metabolites can be conveniently estimated based on the creatinine level using spot urine samples, which may make the antipyrine test (Hartleb, 1991) more useful and practical. The excreted amounts of creatinine for each 12-h period, which were calculated based on its methyl resonance in the same way as in antipyrine metabolites considering the natural abundance of 13C (1.1%), were in the normal range: subject 1, 0.70, 0.62, 0.64, and 0.77 g; subject 2, 0.71, 0.59, 0.63, and 0.77 g.

To investigate the validity of the quantitation method, the concentration of HMA in the post-dose urine was measured by the reverse-phase HPLC (see Fig. 5). Consequently, the amounts determined by HPLC were roughly identical to those obtained by NMR as shown in Table 1. Some factors possibly affect the NMR quantitation. The viscosity of urine and the presence of small amounts of paramagnetic substances in urine can shorten $T_1$ values (Nicholson and Wilson, 1987). Another possible factor is the error of probe tuning between the samples. Although these points need further investigation, the experimental results shown here seem to suggest the validity of the quantitation method by NMR.

In conclusion, the direct NMR approach with 13C labeling has been demonstrated to be useful for the analysis of metabolic profile of antipyrine in human urine. We hope that the simplicity and conve-

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**TABLE 1**  
*Amounts of antipyrine metabolites excreted in urine following administration of [13C]antipyrine to human volunteers*

<table>
<thead>
<tr>
<th>Excretion (% of Dose)</th>
<th>Urine</th>
<th>HMA-G</th>
<th>HMA</th>
<th>NORA-G</th>
<th>OHA-S</th>
<th>OHA-G</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subject 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–12</td>
<td>1.3</td>
<td>1.8 (1.6)</td>
<td>6.8</td>
<td>2.9</td>
<td>9.4</td>
<td>22.1</td>
<td></td>
</tr>
<tr>
<td>12–24</td>
<td>1.6</td>
<td>2.0 (1.9)</td>
<td>5.4</td>
<td>2.2</td>
<td>7.3</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>24–36</td>
<td>1.2</td>
<td>1.5 (1.6)</td>
<td>3.3</td>
<td>1.3</td>
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<td>12.0</td>
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<tr>
<td>36–48</td>
<td>1.0</td>
<td>1.0 (0.8)</td>
<td>2.9</td>
<td>1.0</td>
<td>3.7</td>
<td>9.6</td>
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<tr>
<td>0–48</td>
<td>5.0</td>
<td>6.4 (5.8)</td>
<td>18.4</td>
<td>7.4</td>
<td>25.1</td>
<td>62.2</td>
<td></td>
</tr>
<tr>
<td><strong>Subject 2</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0–12</td>
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<td>1.6 (1.5)</td>
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<td>1.6</td>
<td>9.7</td>
<td>20.8</td>
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<tr>
<td>12–24</td>
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<td>5.1</td>
<td>1.1</td>
<td>6.5</td>
<td>16.2</td>
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<tr>
<td>24–36</td>
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<tr>
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<td>4.2</td>
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<tr>
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<td>19.1</td>
<td>4.0</td>
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<td>60.5</td>
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</table>

**FIG. 5.** HPLC chromatogram of the 12- to 24-h post-dose urine from subject 2.  
1, HMA; 2, IS (phenacetin).
nience of this NMR approach will serve to enhance the value of the antipyrine test in biochemical and clinical pharmacological research.

References


