0090-9556/99/2711-1248–1253$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 27, No. 11
Printed in U.S.A.

DIRECT DETECTION OF ANTIPYRINE METABOLITES IN RAT URINE BY ^{13}C LABELING AND NMR SPECTROSCOPY

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(Received January 25, 1999; accepted July 15, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

Antipyrine is a useful probe to evaluate variation of in vivo activities of oxidative hepatic drug-metabolizing enzymes. Here we describe a new approach using ^{13}C labeling and NMR spectroscopy for the direct and simultaneous detection of all phase I and phase II metabolites of antipyrine in rat urine. [*C*-methyl-^{13}C]Antipyrine was synthesized and administered orally to rats (100 mg/kg), and the 0- to 24-h postdose urine was analyzed by 100-MHz ^{13}C NMR spectroscopy under the conditions of distortionless enhancement by polarization transfer without any pretreatments such as deconjugation, chromatographic separation, and 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 assignments of the resonances were performed by enzymic modification and spiking authentic samples. The reproducibility of the NMR detection was sufficient for the quantitative evaluation of the metabolic profile. Effects of 3-methylcholanthrene on antipyrine metabolism were examined by this approach to evaluate variation of in vivo phase I and phase II metabolism of antipyrine in rats. The present approach is useful and practical to evaluate variation of in vivo activities of conjugation enzymes as well as oxidation enzymes responsible for the formation of antipyrine metabolites in rats. This direct approach would enhance the value of the antipyrine test because of the simplicity and convenience.

Antipyrine, one of the antipyretic and analgesic drugs, 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 (St Peter and Awni, 1991; Hartleb, 1991). Antipyrine is metabolized by several forms of cytochrome P-450 (Sharer and Wrighton, 1996), and the resulting oxidative metabolites are extensively conjugated and excreted in urine of both humans (Bassmann et al., 1985; Palette et al., 1991; Moreau et al., 1992) and rats (Velic et al., 1995). The main oxidative metabolites in humans are 3-hydroxymethylantipyrine (HMA)[1], 4-hydroxyantipyrine (OHA), and norantipyrine (NORA) (Fig. 1). The main oxidative metabolites in rats are these three metabolites and 4,4′-dihydroxyantipyrine (DOHA). Whereas glucuronide conjugation is the major phase II pathway in humans, sulfoconjugation is prominent in rats (Bottcher et al., 1982b).

The quantitation of antipyrine and its metabolites excreted in urine has been used to understand the variation of the oxidative hepatic drug-metabolizing capacity (Buters and Reichen, 1990; Groen et al., 1992; Anadon et al., 1995; Ali et al., 1995; Yang et al., 1996). A number of methods using HPLC have been reported for the determination of antipyrine and its metabolites (Danhof et al., 1979a; Eichelbaum et al., 1981; Teunissen et al., 1983a; 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, except a

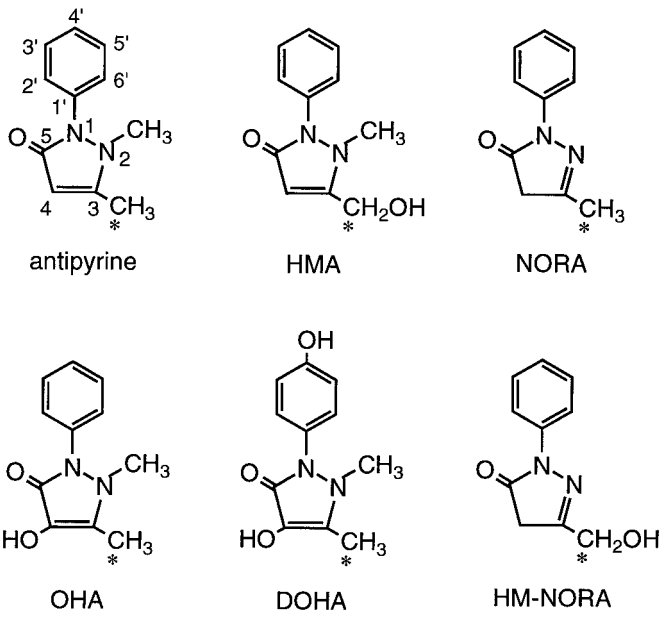

FIG. 1. *Structures of antipyrine and oxidative metabolites.*
∗, labeled position.

[1] Abbreviations used are: HMA, 3-hydroxymethylantipyrine; DEPT, distortionless enhancement by polarization transfer; MS, mass spectra; 3-MC, 3-methylcholanthrene; HMA-G, 3-hydroxymethylantipyrine glucuronide; NORA, norantipyrine; NORA-S, norantipyrine sulfate; NORA-G, norantipyrine glucuronide; OHA, 4-hydroxyantipyrine; OHA-S, 4-hydroxyantipyrine sulfate; OHA-G, 4-hydroxyantipyrine glucuronide; DOHA, 4,4′-dihydroxyantipyrine; DOHA-S, 4,4′-dihydroxyantipyrine sulfate; HM-NORA, 3-hydroxymethyl-norantipyrine; I.S., internal standard.

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radio-HPLC method (Velic et al., 1995) that is troublesome to use and unsuitable for the application to humans because of the radiation hazard. Enzymic or chemical deconjugation followed by HPLC analysis is still the standard approach; this causes analytical problems due to the lability of NORA, OHA, and DOHA liberated after the deconjugation (Danhof et al., 1979a; Bottcher et al., 1982a, 1984; Teunissen et al., 1983a; Palette et al., 1991), variable susceptibility of individual metabolites to deconjugation (Bottcher et al., 1982a,b, 1984; Teunissen et al., 1983a; Moreau et al., 1992), and the volatility of NORA during desiccation (Teunissen et al., 1983a). Thus studies of antipyrine metabolism are still hampered by the lack of a convenient determination method.

The usefulness of the stable isotope tracer technique using ^{13}C labeling of substrates followed by NMR spectroscopy of biofluids has become accepted in metabolic investigations (London 1988; Simpson 1991; Malet-Martino and Martino, 1992). Owing to 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. We have demonstrated the usefulness of the NMR approach with ^{13}C labeling (approximately 100% enrichment) for pharmacokinetic research in terms of sensitivity and specificity (Baba et al., 1990, 1995; Akira et al., 1993, 1997a, 1998; Akira and Shinohara, 1996). In the present study, the NMR approach with ^{13}C labeling has been presented for the direct detection of all phase I and phase II metabolites that occur in rat urine after oral dosing with [^{13}C]antipyrine.

Materials and Methods

Chemicals and Reagents. [4-^{13}C]Ethyl acetoacetate (99 atom % ^{13}C) was purchased from Nippon Sanso (Tokyo, Japan). Ethyl acetoacetate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). OHA was purchased from Aldrich (Tokyo, Japan). Chlorosulfonic acid, *N*-bromosuccinimide, and silica gel (Wakogel C-300) were purchased from Wako Pure Chemical Industries (Osaka, Japan). HMA was synthesized according to the method reported by Buijs et al. (1986). β-Glucuronidase (bovine liver, 114 U/g) was purchased from Funakoshi (Tokyo, Japan). Antipyrine, NORA, and other reagents were purchased from Kanto Chemical (Tokyo, Japan).

Instrumentation. ^1H NMR and ^1H-decoupled ^{13}C ($^{13}C\{^1H\}$) NMR spectra of compounds synthesized or isolated from urine were measured in chloroform-d_1 or methanol-d_4 on Varian (Tokyo, Japan) GEMINI300 or Bruker (Tsukuba, Japan) DPX400 spectrometers, and chemical shifts were referenced to those of chloroform (δ^1H 7.26 and $\delta^{13}C$ 77.0) and methanol (δ^1H 3.35 and $\delta^{13}C$ 49.0). Mass spectra (MS) were recorded on a ThermoQuest (San Jose, CA) TSQ7000 spectrometer. Melting points were determined on a Yanako (Kyoto, Japan) MP-S3 apparatus and were uncorrected. The isotopic purity of ^{13}C-labeled compounds was estimated on the basis of the ion intensities in the region of the molecular ion on MS.

Isolation of DOHA sulfate (DOHA-S) from urine was performed using an HPLC system equipped with Inertsil PREP-ODS column (250 × 30 mm i.d., 10 μm; GL Sciences, Tokyo, Japan) with a precolumn, as described previously (Akira et al., 1997b), except for UV detection at 243 nm and the mobile phase made up from solvent A, methanol/0.5% acetic acid in H_2O (1:1, v/v), and solvent B, methanol. Metabolites were completely eluted within 30 min using the following linear gradient: 0 min: 20% B; 30 min: 80% B.

^{13}C NMR spectra of urine samples were measured at 300 K on a Bruker DPX400 spectrometer using a 5-mm i.d. NMR tube, under the usual ^1H-decoupling conditions ($^{13}C\{^1H\}$) or the conditions of distortionless enhancement by polarization transfer (DEPT) with ^1H decoupling (Morris, 1984). Parameters were spectral width, 26178 Hz; time domain points, 65536; pulse width 6.3 μs (80° pulse); acquisition time, 1.25 s; recycle time, 3.25 s; zero filling; and line broadening, 1.0 Hz. The flip angle of the θ pulse was set at 45° in the DEPT experiments unless otherwise stated. The total accumulation time was 30 min in all experiments. The ^{13}C chemical shifts were referenced to that of sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate ($\delta^{13}C$ 0).

Synthesis of ^{13}C-Labeled Compounds. The following synthetic steps were investigated using unlabeled compounds, and the structures were confirmed by the ^1H NMR spectroscopy before the syntheses of labeled compounds. [*C*-methyl-^{13}C]NORA was prepared by refluxing a solution of [4-^{13}C]ethyl acetoacetate (2.0 g) and phenylhydrazine (1.65 ml) in 20 ml of H_2O/ethanol (1:1, v/v). The compound was obtained as a yellow solid after purification by flash column chromatography over 60 g of silica gel with chloroform/methanol (400:1, v/v) as the eluent (2.59 g; 97% based on ethyl acetoacetate; >99 atom % ^{13}C); m.p. 126.4–127.2°C; ^1H NMR (chloroform-d_1) δ^1H 2.19 (3H, d, J = 129.0 Hz, C-methyl), 3.43 (2H, s, methylene), 7.16 to 7.86 (5H, aromatic protons); $^{13}C\{^1H\}$NMR (chloroform-d_1) $\delta^{13}C$ 17.0 (C-methyl); MS (EI): *m*/*z* 175 (M^+, 100%), 105 (23%), 91 (47%), 77 (51%). Anal Calcd. for $C_9{}^{13}C_1H_{10}N_2O$: C, 68.56; H, 5.75; N, 15.99. Found: C, 68.46; H, 5.84; N, 15.94.

[*C*-methyl-^{13}C]Antipyrine was synthesized by methylation of [*C*-methyl-^{13}C]NORA (2.53 g) with iodomethane according to the method described by Huetter et al. (1987). The compound was obtained as a white powder (1.78 g) after purification by flash column chromatography over 150 g of silica gel with chloroform/methanol (100:1, v/v) as the eluent. The subsequent recrystallization gave crystalline white powder (1.37 g; 50% based on NORA; >99 atom % ^{13}C); m.p. 109.9–111.4°C (from hexane/ethanol (20:1); ^1H NMR (chloroform-d_1) δ^1H 2.23 (3H, dd, J = 129.2 and 0.85 Hz, C-methyl), 3.06 (3H, s, *N*-methyl), 5.40 (1H, m, methine), 7.26 to 7.45 (5H, aromatic protons); $^{13}C\{^1H\}$NMR (chloroform-d_1) $\delta^{13}C$ 13.2 (C-methyl); MS (EI): *m*/*z* 189 (M^+, 60%), 97 (88%), 77 (100%), 57 (83%). Anal Calcd. for $C_{10}{}^{13}C_1H_{12}N_2O$: C, 69.82; H, 6.39; N, 14.80. Found: C, 70.03; H, 6.54; N, 14.81.

[*C*-methyl-^{13}C]OHA was synthesized according to the method reported by Pschorr (1896). Briefly, [*C*-methyl-^{13}C]antipyrine (102 mg) was converted to 4-bromo-[*C*-methyl-^{13}C]antipyrine by the reaction with equimolar amount of *N*-bromosuccinimide, and then the brominated compound was hydrolyzed by potassium hydroxide. [*C*-methyl-^{13}C]OHA was obtained as a white solid after purification by silica gel column chromatography with chloroform/methanol (200:1, v/v) as the eluent (14.5 mg; 14% based on antipyrine; >99 atom % ^{13}C); m.p. 179.6–181.2°C; ^1H NMR (methanol-d_4) δ^1H 2.25 (3H, d, J = 129.3 Hz, C-methyl), 2.95 (3H, s, *N*-methyl), 4.61 (1H, s, OH), 7.37 to 7.56 (5H, aromatic protons); $^{13}C\{^1H\}$NMR (methanol-d_4) $\delta^{13}C$ 9.20 (C-methyl); MS (EI): *m*/*z* 205 (M^+, 8%), 77(12%), 57 (100%). Anal Calcd. for $C_{10}{}^{13}C_1H_{12}N_2O_2 \cdot 1/5H_2O$: C, 63.27; H, 5.98; N, 13.41. Found: C, 63.16 H, 6.19 N, 13.16.

Synthesis of OHA Sulfate (OHA-S). To a solution of OHA (0.31 g) in 21 ml of dehydrated pyridine was added dropwise chlorosulfonic acid (200 μl), keeping the temperature below 10°C by an ice-water bath followed by stirring for 50 h at room temperature. The reaction mixture was neutralized with 3.2% sodium hydroxide in methanol (about 9 ml), and evaporated to dryness. The oily residue was washed with 100 ml of diethyl ether, dissolved in 20 ml of ethanol, and the undissolved material was filtered off. The filtrate was mixed with 120 ml of diethyl ether, and the resulting precipitate was collected by filtration and dried in vacuo to give sodium salt of the title compound as a pale yellow crystalline powder (90 mg; 17%); m.p. 138.3–139.3°C; MS (FAB$^-$): *m*/*z* 283 ([M-Na]$^-$, 100%), 203 (28%). Anal Calcd. for $C_{11}H_{11}N_2NaO_5S \cdot 2H_2O$: C, 38.60; H, 4.42; N, 8.18. Found: C, 38.87; H, 4.39; N, 7.97. ^1H NMR (methanol-d_4) δ^1H 2.39 (3H, s, C-methyl), 3.15 (3H, s, *N*-methyl), 7.41 to 7.57 (5H, aromatic protons).

Animal Experiments and Sample Treatments. Male Wistar rats (185–220 g) were used. Five rats were orally administered with [^{13}C]antipyrine (100 mg/kg) dissolved in saline (10 mg/ml), and two rats were administered with [^{13}C]NORA (100 mg/kg) suspended in olive oil (10 mg/ml) after an overnight fast (for 18 h). In addition, four rats were treated for three consecutive days with 3-methylcholanthrene (3-MC) suspended in olive oil, 30 mg/kg i.p., and then administered with [^{13}C]antipyrine in the same manner as shown above. Rats were placed in individual metabolic cages. Urine that was collected before administration of antipyrine and at 0- to 24- and 24- to 48-h postdose was held below 5°C by an ice-water bath. The metabolic cage was washed with distilled water to completely recover the antipyrine metabolites. Urine (5–15 ml) and the cage wash collected from each rat were combined, and the solution was diluted to 15 to 30 ml with distilled water after filtration, and then stored at

−20°C until analyzed. During the experiments the animals had free access to tap water. They were allowed free access to food at 3 h after the administration. The urine sample was subjected to the following analysis without concentration or after concentrated by a factor of 3 using freeze-drying. An aliquot (0.5 ml) of the urine sample was mixed with 50 μl of a solution of [2-^{13}C]sodium acetate internal standard (I.S.) in deuterium oxide (4.036 mg/ml). Deuterium oxide was used for field-frequency lock. The mixture was centrifuged (2500 rpm, 5 min), and the supernatant was analyzed by ^{13}C NMR spectroscopy. The ratios of resonance integral intensities of the metabolites to that of the I.S. were calculated, and then corrected by the degree of concentration (1 or 3) and the total volume of the urine sample: integral intensity ratio × 1/degree of concentration × urine volume/0.5.

Isolation of [*C*-methyl-^{13}C]DOHA-S from Urine. Male Wistar rats were orally administered with [^{13}C]antipyrine as described above. Urine samples collected over a 0- to 24-h interval from four rats (~40 ml) were incubated with β-glucuronidase (30 mg protein) at 37°C for 3 h to eliminate glucuronidated metabolites of antipyrine. The treated urine was washed with chloroform (100 ml × 2) to eliminate less polar phase I metabolites, and the aqueous fraction was applied as 8-ml aliquots to five pretreated Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). The cartridges were washed with 5 ml of 0.5% acetic acid in water followed by elution with 20 ml of methanol/0.5% acetic acid in H_2O (20:80, v/v). All the eluates were combined and concentrated by a rotary evaporator below 30°C, and then freeze dried. The residue was reconstituted in methanol (0.5 ml), and 20- to 50-μl portions of the solution were injected onto the HPLC column. Three major peaks due to sulfated metabolites of antipyrine were observed at 13, 16 (OHA-S), and 20 min after injection. The peak at 13 min was presumed to be due to DOHA-S, judging from the elution order of antipyrine metabolites reported by Velic et al. (1995). The eluates corresponding to the peak at 13 min were collected, evaporated by a rotary evaporator, and then subjected to freeze drying to give DOHA-S as a yellow oil (3 mg); ^{1}H NMR (methanol-d_4) δ^1H 2.27 (3H, d, J = 129.7 Hz, C-methyl), 3.05 (3H, s, *N*-methyl), 6.96 (2H, d, J = 8.75 Hz, aromatic protons), 7.23 (2H, d, J = 8.75 Hz, aromatic protons); ^{13}C{^{1}H}NMR (methanol-d_4) δ^{13}C 9.91 (C-methyl); MS (FAB$^-$): *m/z* 300 (M^-, 100%), 220 (26%).

Enzymic Modification of Urine Samples. To identify conjugated metabolites of antipyrine, 0.5 ml of the 0- to 24-h postdose urine was transferred to an NMR tube after being mixed with 50 μl of deuterium oxide, incubated at 37°C with or without β-glucuronidase (5 mg-protein), and then analyzed by ^{13}C NMR spectroscopy without additional treatment.

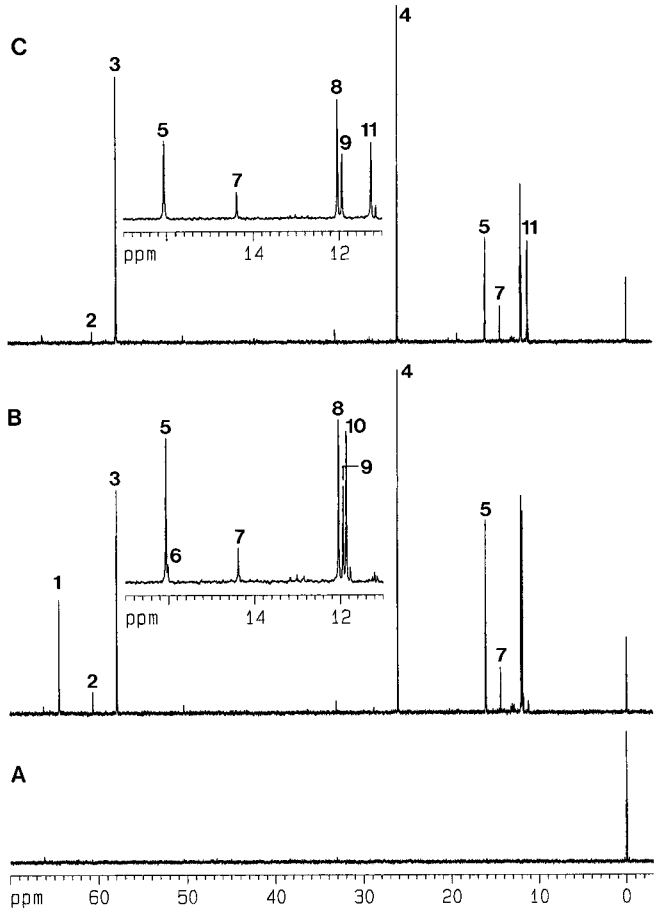

FIG. 2. *^{13}C NMR spectra of control urine (A), 0- to 24-h urine from a rat dosed with [^{13}C]antipyrine (100 mg/kg p.o.) (B), and 0–24 h postdose urine incubated with β-glucuronidase at 37°C for 3 h (C).*

Key: 1 = HMA-G; 2 = HM-NORA (tentative); 3 = HMA; 4 = I.S.; 5 = NORA-S; 6 = NORA-G; 7 = antipyrine; 8 = OHA-S; 9 = DOHA-S; 10 = OHA-G; 11 = OHA.

Results and Discussion

When the NMR approach with ^{13}C labeling is applied to drug metabolism studies, the labeled position has to be selected to get sufficient spectral resolution and sensitivity. As antipyrine is metabolized as shown in Fig. 1, the C3, C4, and C-methyl positions are favorable for labeling in terms of spectral resolution. In the proton-decoupling conditions, the intensities of ^{13}C resonances from protonated carbons are significantly increased due to the nuclear Overhauser enhancement (NOE, maximum 1.99). Also the resonances of protonated carbons are usually more intense than those of nonprotonated carbons due to the short spin-lattice relaxation time (T_1), which allows the accumulation of more scans within a limited acquisition time. Therefore, protonated carbons (C-methyl and C4) are generally preferable for labeling (Akira et al., 1993; Baba et al., 1995). The C-methyl of the carbon of the parent drug retains at least two protons in all metabolite species, whereas the C4 is a quaternary carbon in OHA and DOHA. Thus the C-methyl was considered most suitable for labeling in terms of both spectral resolution and sensitivity. Thus, [*C*-methyl-^{13}C]antipyrine with high incorporation level (>99%) was synthesized from [4-^{13}C]ethyl acetoacetate in two steps.

The labeled antipyrine was orally administered to rats and the excreted urine was analyzed by ^{13}C NMR. The signal-to-noise ratios of the DEPT spectra were enhanced by a factor of 2- to 3-fold, compared with those of the ^{13}C{^{1}H}-NMR spectra in the same accumulation time (Morris, 1984). All of the resonances observed in the ^{13}C{^{1}H}-NMR spectra, except that of ~1% natural abundance of urea, were also observed in the DEPT spectra. Thus, the following NMR spectra were all obtained by the DEPT experiments. In the 0- to 24-h postdose urine, six resonances due to the major metabolites of antipyrine were observed at δ^{13}C 64.4, 57.8, 16.1, 12.0, 11.9, and 11.8 with an antipyrine resonance at δ^{13}C 14.3, as shown in Fig. 2B. Three resonances due to the minor metabolites of antipyrine were also observed at δ^{13}C 60.6, 16.0, and 11.7. The 24- to 48-h postdose urine gave no significant ^{13}C resonance due to antipyrine and its metabolites, which showed the 0- to 24-h urine collection was sufficient to obtain the total metabolic pattern of antipyrine. In rats, antipyrine is mainly biotransformed to HMA, HMA glucuronide (HMA-G), NORA sulfate (NORA-S), OHA-S, DOHA-S, and OHA glucuronide (OHA-G), and these metabolites are excreted in urine with a small amount of antipyrine (Velic et al., 1995). Thus, the major resonances in the methylene region (δ^{13}C 64.4, and 57.8) were considered to be due to HMA and its glucuronide, whereas the major resonances in the methyl region (δ^{13}C 16.1, 12.0, 11.9, and 11.8) due to NORA-S, OHA-S, DOHA-S, and OHA-G. This presumption was found to be correct by the DEPT experiments (θ pulse, 135°) of the urine sample. The resonances at δ^{13}C 57.8, 12.0, and 11.9 were assigned to HMA, OHA-S, and DOHA-S, respectively, by spiking those authentic sam-

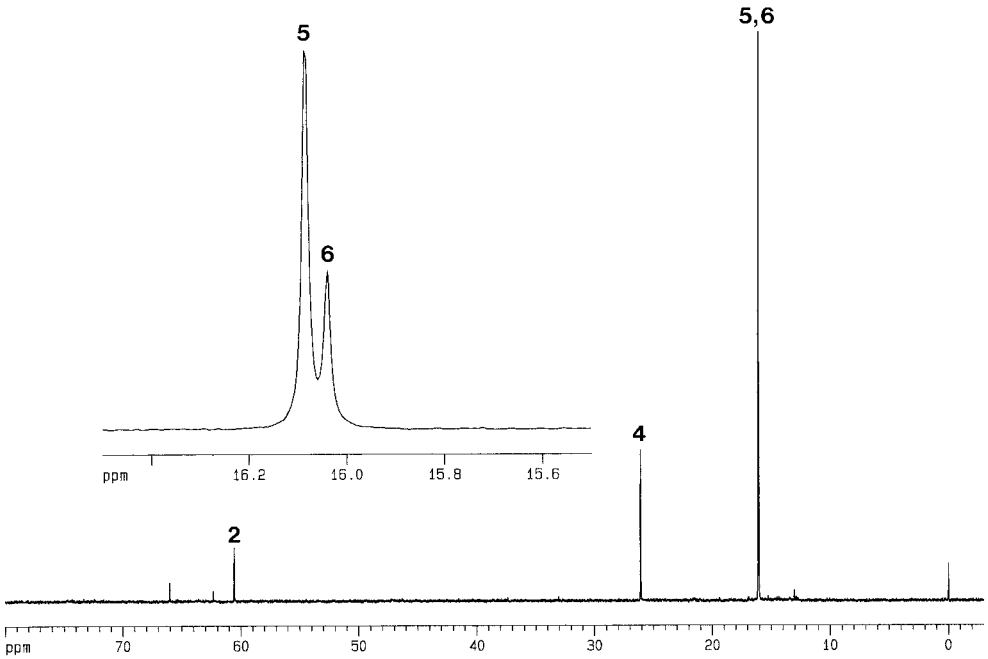

FIG. 3. *^{13}C NMR spectrum of 0- to 24-h urine from a rat dosed with [^{13}C]NORA (100 mg/kg p.o.).*

Key: as in Fig. 2.

ples. Thus, the other major resonance (δ^{13}C 64.4) in the methylene region was assigned to HMA-G.

The postdose urine was incubated with β-glucuronidase to assign the remaining major resonances at δ^{13}C 16.1 and 11.8. Consequently, the resonances at 64.4 (HMA-G), 16.0, 11.8, and 11.7 disappeared with concurrent increase of the resonances at δ^{13}C 57.8 (HMA), 11.2, and 11.1, as shown in Fig. 2C. In the control experiments, the NMR spectrum showed no apparent change, showing the resonances at δ^{13}C 64.4, 16.0, 11.8, and 11.7 to be all glucuronide conjugates. The resonance at δ^{13}C 11.2 was assigned to OHA by spiking the labeled authentic compound. Thus the major resonance at δ^{13}C 11.8 was assigned to OHA-G, and the remaining major resonance at δ^{13}C 16.1 in the methyl region, that was unaffected by β-glucuronidase, was assigned to NORA-S from the metabolic pattern of antipyrine described above.

To confirm the assignment of NORA-S, [^{13}C]NORA was orally administered to rats, and the postdose urine was analyzed by ^{13}C NMR. As shown in Fig. 3, two major resonances due to metabolites of NORA were observed at δ^{13}C 16.1 and 16.0. Bottcher et al. (1985) have reported that NORA is completely conjugated to form NORA-S and NORA glucuronide (NORA-G) in rats. The resonances at δ^{13}C 16.1 and 16.0 were assigned to NORA-S and NORA-G, respectively, because only the resonance at δ^{13}C 16.0 disappeared by the β-glucuronidase treatment. These resonances were greatly shifted downfield compared with those due to C-methyl groups of other antipyrine metabolites, probably due to the characteristic ring structure (Palette et al., 1994). From these experimental results, the above-mentioned assignment of NORA-S was confirmed. Also, the minor glucuronidated metabolite (δ^{13}C 16.0) in Fig. 2B was identified to be NORA-G. The NMR spectrum showed a resonance at δ^{13}C 60.6 due to a minor metabolite of NORA, which was also observed in Fig. 2B. The resonance was found to be due to a methylene group by the DEPT experiments (θ pulse, 135°). Thus, this resonance was tentatively assigned to 3-hydroxymethyl-norantipyrine (HM-NORA, see Fig. 1), recently reported as a minor urinary metabolite of antipyrine in rats (Velic et al., 1995).

All the antipyrine metabolites reported to occur in rat urine were identified on the ^{13}C NMR spectra by the above experiments. The minor metabolite, NORA-G, was also shown to occur in rats, which has been previously unreported. The results showed that all of the major antipyrine metabolites in rats could be directly detected in one operation without any pretreatments such as extraction, chromatography, and deconjugation by the combined use of ^{13}C NMR and ^{13}C labeling of the C-methyl carbon.

The signal-to-noise ratios of the major metabolites, obtained using the limited acquisition time (30 min), were considered sufficient for the quantitative evaluation of the metabolic profile. The analysis of the postdose urine in Fig. 2B was repeated 6 times to examine the reproducibility of the NMR detection. Consequently, the coefficients of variation (%) of the integral intensity ratios between the metabolites and the I.S. were as follows: HMA-G, 6.9; HMA, 1.8; NORA-S, 1.1; AP, 7.3; OHA-S, 2.0; DOHA-S, 3.0; OHA-G, 2.0. In the HPLC analyses of antipyrine metabolites, attention has to be paid to the lability of OHA, DOHA, and NORA liberated after the deconjugation (Danhof et al., 1979a; Bottcher et al., 1982a, 1984; Teunissen et al., 1983a; Palette et al., 1991). However, the NMR approach directly detects their chemically stable conjugates (Teunissen et al., 1983a; Bottcher et al., 1984; Palette et al., 1993) in urine, so that accurate and reliable results are obtained. To compare the urinary excreted amounts in the individual rats, the ratios of resonance integral intensities between the metabolites and the I.S. were calculated and corrected as shown in the experimental section (Table 1). These integral ratios reflect the metabolic profile of antipyrine. It should be noted that relative integrations do not represent molar ratios between the metabolites because the NMR sensitivity for the individual metabolites, which is influenced by the different NOE and T_1 of detected nucleus, differs from one to another (Akira et al., 1993).

The application of ^{13}C NMR to investigating alterations in the metabolism of antipyrine was examined in the case of 3-MC induction. The spectra of the 0- to 24-h postdose urine from 3-MC-treated rats were markedly different from those from normal rats, as shown in Fig. 4. The excretion of NORA-G, OHA-G, and OHA-S increased by

TABLE 1

Urinary excretion pattern of antipyrine metabolites in rats

	[a]Ratio of Integral Intensity							
	HMA	HMA-G	NORA-S	NORA-G	OHA-G	OHA-S	DOHA-S	Antipyrine
Normal rats (five rats)	12.2 ± 0.69	5.2 ± 0.73	11.4 ± 0.91	1.5 ± 0.09	11.2 ± 1.00	10.6 ± 0.91	6.0 ± 0.62	2.5 ± 0.38
3-MC treated rats (four rats)	1.1 ± 0.46**	1.0 ± 0.33**	12.5 ± 1.50	6.1 ± 0.66**	19.1 ± 2.61*	17.4 ± 1.77*	5.1 ± 0.49	

[a] Values listed are means ± S.E. Statistically significant differences (by *t* test) are identified by $*P < .05$ and $**P < .01$. The integral intensity ratios of the metabolites to the I.S. were calculated based on the ^{13}C NMR spectra, and then corrected for the degree of concentration and the total volume of the urine sample.

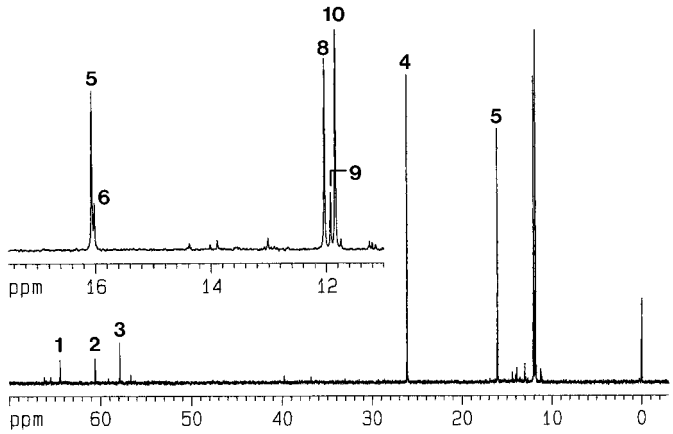

FIG. 4. *^{13}C NMR spectrum of 0–24 h urine from a 3-MC treated rat dosed with [^{13}C]antipyrine (100 mg/kg p.o.).*

Key: as in Fig. 2.

the induction, whereas that of HMA and HMA-G decreased, as shown in Table 1. The excretion of NORA-S and DOHA-S did not change. The increase in OHA (sulfated + glucuronidated) excretion and the decrease in HMA (free + conjugated) excretion have been reported in 3-MC-treated rats (Danhof et al., 1979b; Nakagawa et al., 1983; Teunissen et al., 1983b), which are consistent with our results.

The effects of 3-MC pretreatment on the metabolic formation of NORA is inconsistent across the literature. Teunissen et al. (1983b) have reported a decrease in NORA (sulfated) excretion by the induction, whereas Danhof et al. (1979b) and Nakagawa et al. (1983) reported no change in the excretion. The lability of NORA, liberated after the deconjugation procedures for the HPLC analysis in these reports, may be responsible for this discrepancy. In addition, Bottcher et al. (1984) have directly measured urinary NORA conjugates by thin-layer chromatography coupled with radioisotope tracer techniques, where urine is treated with urease and extracted with methanol. They reported that NORA-S excretion is significantly enhanced by 3-MC induction, and NORA-G is not excreted in urine of both control and 3-MC-treated rats. On the contrary, our results showed no change in NORA-S excretion, and a significant increase in NORA-G excretion and in the total excretion of NORA-S and NORA-G, which are in conflict with the above-mentioned results by other researchers. It should be noted that the 3-MC induction of glucuronidation in antipyrine metabolism was first shown by the detection of significant NORA-G excretion. This contradiction may be due to the difference of strain, age, and body weight of the rats, administration route, and induction method. Our experimental results are, in any event, considered to be most reliable because the NMR approach involves no pretreatments such as deconjugation and solvent extraction.

In conclusion, the ^{13}C NMR approach using ^{13}C-labeling has been demonstrated to be useful and practical for the direct and simultaneous detection of all phase I and phase II metabolites in rat urine. The approach saves time and effort in data acquisition because of the need for little pretreatment of samples and the ability to accommodate relatively short accumulation times. The present approach is useful to evaluate variation of in vivo activities of conjugation enzymes as well as oxidation enzymes responsible for the formation of antipyrine metabolites in the rat. As the metabolic pattern of antipyrine in humans is similar to that in rats, the NMR approach would be also applicable to humans. It is hoped that the introduction of this direct assay may enhance the value of antipyrine test (Hartleb, 1991) in biochemical and clinical pharmacological research because of the simplicity and convenience.

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