Studies on ^{13}C-Phenacetin Metabolism. II.[1] A Combination of Breath Test and Urine Test of *in Vivo* Metabolites in the Diagnosis of Liver Disease

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We determined the optimum ^{13}C-labeling position in phenacetin for use in a breath test to diagnose liver disease based on infrared spectroscopy detection of $^{13}CO_2$ in exhaled air. ([1-^{13}C]Ethoxy)phenacetin gave the best result. This compound was also employed in a urine test using ^{13}C-NMR spectroscopy. In the urine test, healthy subjects gave a higher signal of phenacetin than of its metabolite, phenetidine, whereas in patients with liver disease the situation was the reverse. The combination of the breath and urine tests may be a valuable new tool for the diagnosis of liver disease.

Key words ^{13}C-phenacetin; ^{13}C-phenetidine; breath test; ^{13}C-NMR; ^{13}C-carbon dioxide; liver disease

The study of drug metabolism in humans is of great importance, and non-invasive methods which do not require blood sampling are preferable. We have developed a breath test involving administration of ([1-^{13}C]ethoxy)-phenacetin[1] and detection of $^{13}CO_2$ in exhaled air for the diagnosis of liver diseases. In the present work, we labeled phenacetin with ^{13}C at three different positions to find out which would give the best result in the breath test. We also collected urine samples post-administration, for determination of ^{13}C-phenacetin and its metabolite, ^{13}C-phenetidine, using ^{13}C-NMR spectroscopy.[2–5] The results obtained by the two methods were compared.

Results and Discussion

([1-^{13}C]Ethoxy)phenacetin (**1a**) was prepared as reported previously,[1] by alkylation of *N*-acetyl-*p*-aminophenol with [1-^{13}C]iodoethane. Two other ^{13}C-labeled phenacetins ([1-^{13}C]acetyl)phenacetin (**1b**) and ([2-^{13}C]acetyl)phenacetin (**1c**) were synthesized by acetylation of phenetidine with [1-^{13}C] or [2-^{13}C]acetyl chloride. Three kinds of ^{13}C-phenacetins, ([1-^{13}C]ethoxy)-phenacetin (**1a**), ([1-^{13}C]acetyl)phenacetin (**1b**), and ([2-^{13}C]acetyl)phenacetin (**1c**), were administered to healthy subjects and $^{13}CO_2$ in expired air was measured ($^{13}CO_2/^{12}CO_2$) as ^{13}C-excess percent using infrared spectroscopy. The results of breath tests are shown in Fig. 1. ([1-^{13}C]Ethoxy)phenacetin (**1a**) gave the best result, producing by far the most $^{13}CO_2$ within the shortest time. We therefore used it in all subsequent tests.

Next, the ([1-^{13}C]ethoxy)phenacetin (**1a**) was applied to breath and urine tests for healthy subjects and patients of liver disease. Results of the breath test (Fig. 2.) indicated that healthy subjects produced $^{13}CO_2$ from the drug more quickly and in larger amounts than did patients with liver cirrhosis. The maximum $^{13}CO_2$% dose in cirrhotic patients was only 0.1% at 120 min, compared with 0.2% at 60 min for the controls. In the urine test of healthy controls, ^{13}C-enriched peaks in the ^{13}C-NMR spectra were seen at 66.9 ppm, ([1-^{13}C]ethoxy)phenacetin (**1a**), and 67.5 ppm, ([1-^{13}C]ethoxy)phenetidine, as shown in Fig. 3 (above). Other signals were observed at 32.7 ppm and 58.4 ppm (creatinine), 61.7 ppm (unknown) and 165.0 ppm (urea). ^{13}C-NMR spectrum of urine from patients with acute hepatitis showed a signal of ([1-^{13}C]ethoxy)phenacetin (**1a**) at 66.9 ppm and a large signal of ([1-^{13}C]ethoxy)-phenetidine at 67.5 ppm (Fig. 3 lower). ([1-^{13}C]ethoxy)-phenacetin (**1a**) was increased approximately three times compared with the controls. The metabolic pathways of phenacetin are summarized in Fig. 4. The data thus obtained by ^{13}C-NMR spectroscopy were corrected to

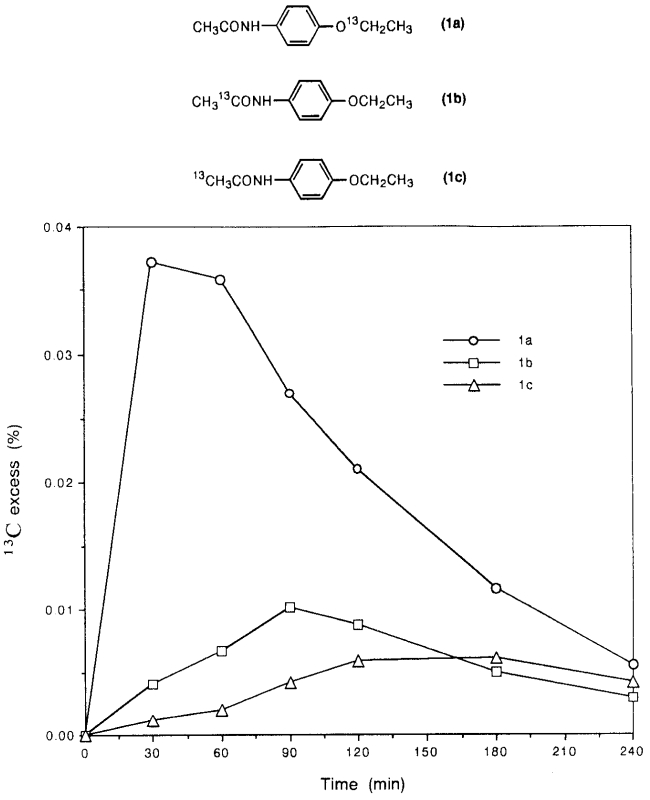

Fig. 1. Time Courses of ^{13}C Excess Percent Measured by Breath Test of ([1-^{13}C]Ethoxy)phenacetin (**1a**), ([1-^{13}C]Acetyl)phenacetin (**1b**), and ([2-^{13}C]Acetyl)phenacetin (**1c**)

Values are expressed as mean (n=2).

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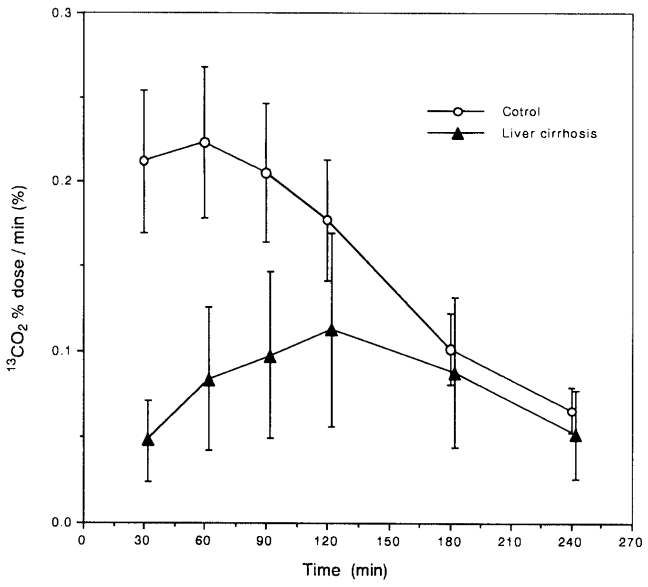

Fig. 2. Time Course of $^{13}CO_2$ Excretion Measured by Breath Test of ([1-^{13}C]Ethoxy)phenacetin (**1a**) for Healthy Subjects ($n=3$) and Patients ($n=7$) with Liver Cirrhosis

Values are expressed as mean ± S.D.

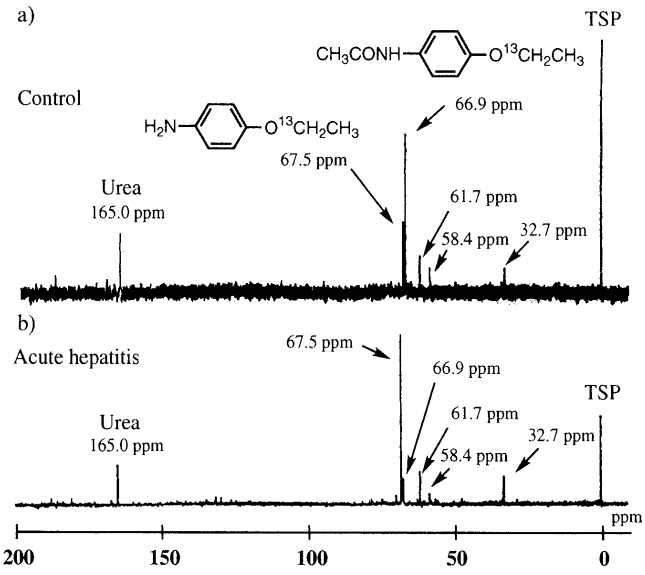

Fig. 3. ^{13}C-NMR Spectra

a) Urine test of healthy subjects (one example) and b) urine test of patients with acute hepatitis after 60 min of ([1-^{13}C]ethoxy)phenacetin (**1a**) oral administration (one example).

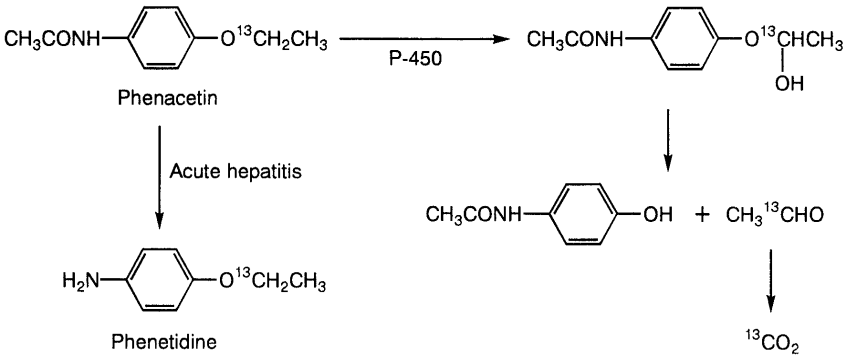

Fig. 4. Metabolic Pathway of Phenacetin

take account of differences in urinary amount and creatinine concentration, using the signal intensity of the internal standard sodium[2,2,3,3-$^2H_4$]-3-(trimethylsilyl)-propionate (TSP), in order to obtain values of ^{13}C-compound % dose (Fig. 5.) Acute hepatitis patients at maximum showed much higher levels of phenetidine. The results of acute hepatitis patients during convalescence are also illustrated, it is noteworthy that the results of those latter patients were similar to those of healthy controls.

The results of the breath test and urine test were in excellent agreement, reflecting the reduced content of cytochrome P-450 in liver disease, which results in the formation of less $^{13}CO_2$ from ([1-^{13}C]ethoxy) phenacetin (**1a**) with a consequent increase in ([1-^{13}C]ethoxy) phenetidine by the alternative metabolic pathway. The combination of the two tests is expected to be useful and convenient for the diagnosis of liver disease, and further studies are planned on patients with other diseases.

Experimental

Materials [1-^{13}C]Acetylchloride (99.7% atom ^{13}C) and [2-^{13}C]acetylchloride (99.8% atom ^{13}C) were supplied by MSD isotopes.

Instruments Melting point (mp) determinations were carried out on a Yazawa micro melting point apparatus, Type BY-1; values are uncorrected. Mass spectra (MS) were recorded on a JEOL DX-302 spectrometer equipped with a JMA DA-5000 data system. Infrared (IR) spectra were measured with a $^{13}CO_2$ analyzer (JASCO EX-130) for the $^{12}CO_2/^{13}CO_2$ breath test. The ^{13}C excess percent was calculated from the IR absorption intensities of $^{13}C=O$ ($2280 \pm 10\,cm^{-1}$) and $^{12}C=O$ ($2380 \pm 10\,cm^{-1}$). ^{1}H-NMR spectra were recorded on a JEOL GSX-400 spectrometer in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard. ^{13}C-NMR spectra were taken on a JEOL GSX-400 (100 MHz) spectrometer in $CDCl_3$ solution referenced to the solvent peak, or in D_2O solution with TSP. ^{13}C-NMR spectral conditions were: number of scans, 1000; acquisition time, 0.678 s; data points, 32 K; pulse width, 7.0 μs.

([1-^{13}C]Acetyl)phenacetin (1b) and ([2-^{13}C]Acetyl)phenacetin (1c) [1-^{13}C]Acetylchloride (1.0 g, 12.7 mmol) was added dropwise to a solution of *p*-phenetidine (1.74 g, 12.7 mmol) in dry benzene (20 ml) at 5 °C under argon. The reaction mixture was stirred for 10 min at this temperature. The crystals were collected by centrifugation. Recrystallization from ethanol–haxane gave ([1-^{13}C]acetyl)phenacetin (**1b**) (2.2 g, 99.9%), mp 134.2—136.1 °C. ^{1}H-NMR ($CDCl_3$) 1.39 (t, 3H, $J=6.9$ Hz, CH_3CH_2), 2.13 (d, 3H, $J_{^{13}CCH}=5.9$ Hz, $CH_3{}^{13}CO$), 4.00 (q, 2H, $J=6.9$ Hz, CH_3CH_2), 6.83 (d, 2H, $J=9.0$ Hz, Ph), 7.37 (d, 2H, $J=9.0$ Hz, Ph). ^{13}C-NMR ($CDCl_3$) 168.1 ($CH_3{}^{13}CO$). EI-MS *m/z* 180 (M^+, 100%), 137 (50%), 109 (69%), 108 (76%).

([2-^{13}C]Acetyl)phenacetin (**1c**) (2.27 g, 100%) was synthesized similarly with [2-^{13}C]acetylchloride (1.0 g, 12.7 mmol) and *p*-phenetidine

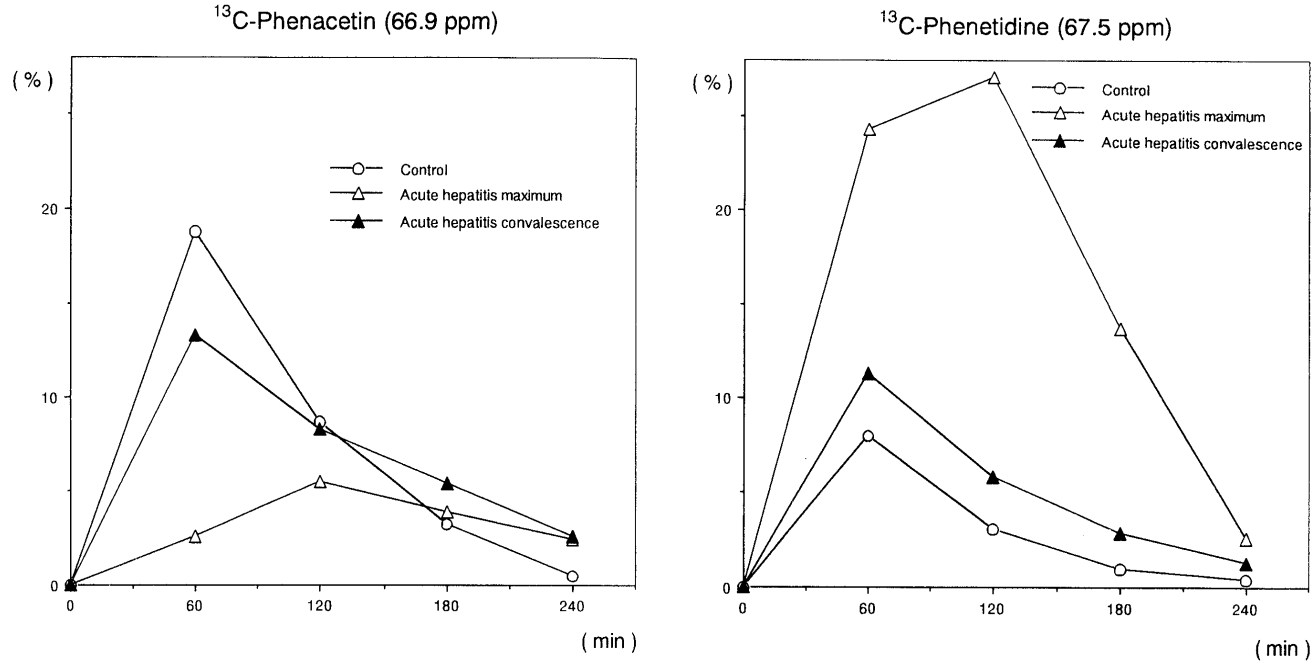

Fig. 5. Time Course of Excretion Contents of ([1-^{13}C]Ethoxy)phenacetin (**1a**) and ([1-^{13}C]Ethoxy)phenetidine Contents in Urine from Healthy Subjects and Acute Hepatitis Patients at Maximum and during Convalescence

Values are expressed as mean ($n=2$).

(1.74 g, 12.7 mmol), mp 135.2—136.7 °C. ^{1}H-NMR ($CDCl_3$) 1.39 (t, 3H, $J=7.0$ Hz, $C\underline{H}_3CH_2$), 2.13 (d, 3H, $J_{^{13}CH}=128.1$ Hz, $^{13}C\underline{H}_3CO$), 4.00 (q, 2H, $J=7.0$ Hz, $CH_3C\underline{H}_2$), 6.83 (d, 2H, $J=9.0$ Hz, Ph), 7.37 (d, 2H, $J=9.0$ Hz, Ph); ^{13}C-NMR ($CDCl_3$) 24.4 ($^{13}\underline{C}H_3CO$). EI-MS m/z 180 (M^+, 96%), 137 (57%), 109 (90%), 108 (100%).

Breath Test by IR Spectroscopy A dose of 3.5 mg/kg or 100 mg/body of a ^{13}C-labeled phenacetin was administered to healthy controls and patients with liver disease. The content of $^{13}CO_2$ in exhaled air was determined by infrared spectroscopy as described previously.[1)]

Urine Test by ^{13}C-NMR Spectroscopy A five ml urine sample was taken and lyophilized. The residue was dissolved in 500 μl of D_2O in a 5 mm i.d. NMR tube. The ^{13}C-NMR spectrum was measured. The signals obtained, ([1-^{13}C]ethoxy)phenacetin (**1a**) at 66.9 ppm and ([1-^{13}C]-ethoxy)phenetidine at 67.5 ppm, were corrected for urinary amount, creatinine concentrations, and intensity of the internal standard (TSP) and values of ^{13}C-compound % dose are shown in Fig. 5.

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