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Evaluation of Liver Function by Co-administration Methodology Using ^{13}C-Labelled Benzoic Acid and Hippuric Acid Coupled with Nuclear Magnetic Resonance Spectroscopy

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Abstract

The amount of hippuric acid synthesized and excreted in the urine after benzoic acid loading (hippuric acid test) is a useful index of liver function. However, the hippuric acid test gives erroneous results in the event of failure of renal excretory function. A new stable isotope co-administration methodology using nuclear magnetic resonance (NMR) spectroscopy has been developed to overcome this defect.

[7-^{13}C]Benzoic acid and [glycine carbonyl-^{13}C]hippuric acid ([gly-^{13}C]hippuric acid), each 0·4–0·6 mmol kg^{-1} were simultaneously administered intravenously as probes to normal or liver-injured rats and the urine was analysed by 100 MHz ^{13}C NMR spectroscopy. Consequently, urinary excretion of [7-^{13}C]hippuric acid formed from [7-^{13}C]benzoic acid and [gly-^{13}C]hippuric acid was successfully traced with very simple and convenient procedures. The urinary excretion of [7-^{13}C]hippuric acid indicated the combined functions of hippuric acid synthesis and renal excretion, whereas that of [gly-^{13}C]hippuric acid was indicative of renal excretion of hippuric acid only. The heights of resonances for C7 of [7-^{13}C]hippuric acid and the glycine carbonyl carbon of [gly-^{13}C]hippuric acid were used to calculate the concentrations of labelled hippuric acids. [7-^{13}C]Hippuric acid was excreted more slowly than [gly-^{13}C]hippuric acid by both normal and liver-injured rats. The liver-injured rats excreted the labelled hippuric acids more slowly than the normal rats. The kinetic parameters were computed for the individual rats on the basis of Michaelis–Menten elimination for benzoic acid and first-order elimination for hippuric acid. The maximum rates of metabolism (V_{max}) (4·8–5·8 μmol min^{-1} kg^{-1}) and the renal elimination rate constants of hippuric acid (K_{re}) (0·010–0·021 min^{-1}) in the liver-injured rats were lower than those (V_{max} 6·7–11·8 μmol min^{-1} kg^{-1}; K_{re} 0·026–0·045 min^{-1}) in the normal rats.

These results have demonstrated that liver function can be evaluated from the V_{max} value even though the renal function of hippuric acid excretion (K_{re}) is impaired. Thus the co-administration methodology is feasible and can remove the defect of the previous hippuric acid test. These results could form the basis for a more convenient and reliable hippuric acid test in man.

Various types of loading test using endogenous and exogenous compounds as probes have been designed for evaluation of the functional reserve of the liver and to diagnose liver diseases in surgical and internal medicine (Branch 1982; Wilkinson & Branch 1984; Goldberg & Brown 1987; St Peter & Awni 1991). Loading tests seem also to be useful for the investigation of the liver toxicity of xenobiotics and the effects of therapeutic agents for liver diseases using experimental animals. A loading test directly related to energy metabolism of hepatocytes is considered advisable for evaluating liver function because energy metabolism, which is based on the supply of adenosine triphosphate (ATP) through mitochondrial oxidative phosphorylation, is of primary importance in the maintenance of functional and structural integrity of the cells. The hippuric acid test introduced by Quick (1933) meets the requirement better than other loading tests reported to date (Aoyama et al 1986). The test utilizes the biotransformation of benzoic acid to hippuric acid by ATP-dependent acyl-CoA synthetase and acyl-CoA:glycine *N*-acyltransferase, a process that occurs mainly in liver mitochondria in man and rats. The capacity to synthesize hippuric acid is associated with ATP supply (Aoyama et al 1986) and with glycine availability in the mitochondria (Quick 1931). In the actual test the hippuric acid excreted in the urine in a specified time after dosing with benzoic acid, by which the liver function is evaluated, is quantitated gravimetrically or spectrophotometrically. Clinically, the decrease in the urinary output of hippuric acid was demonstrated to be reasonably proportional to the extent of hepatic impairment (Quick 1940; Van Sumere et al 1969; Aoyama et al 1986). However, this non-invasive test has found little popularity because of the tedious analytical procedures.

The usefulness of stable isotope tracer technique using ^{13}C-labelling of substrates then nuclear magnetic resonance (NMR) spectroscopy of biofluids has become accepted in metabolic investigations (London 1988; Simpson 1991; Malet-Martino & 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, and so the technique saves much time and analytical effort. We have already established ^{13}C-labelling and NMR approaches for following the urinary excretion of hippuric acid formed from benzoic acid administered to rats and man (Baba et al 1990, 1995; Akira et al 1993, 1994, 1995). The approach substantially simplifies the analytical procedures of the hippuric acid test. Also, the contribution of endogenous hippuric acid to the quantitation of exogenous hippuric acid is negligible even for lower dosages.

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The urinary excretion of hippuric acid formed from the administered benzoic acid is influenced both by the capacity to synthesize hippuric acid and the renal excretory function, because the benzoic acid administered is almost exclusively biotransformed to hippuric acid and excreted in the urine in rats and man (Schachter 1956; Bridges et al 1970; Kubota et al 1988; Baba et al 1990; Akira et al 1993). Thus the hippuric acid test gives misleading results when renal excretory function is impaired. Thus, we have designed a simple stable isotope co-administration methodology for the hippuric acid test using NMR spectroscopy to remove this defect. The methodology involves simultaneous administration, to the same individual, of ^{13}C-labelled benzoic acid and ^{13}C-labelled hippuric acid whose labelled positions differ from each other. The urinary excretion of [^{13}C]hippuric acid formed from the administered [^{13}C]benzoic acid indicates the combined functions of hippuric acid synthesis and renal hippuric acid excretion, whereas that of the administered [^{13}C]hippuric acid indicates only the renal function of hippuric acid excretion. Thus, the capacity to synthesize hippuric acid, a direct indication of the liver function of the individual, is considered to be estimated from these excretion data for both hippuric acids. In this paper we report the feasibility of the co-administration methodology using normal and liver-injured rats.

Materials and Methods

Chemicals

[7-^{13}C]Benzoic acid (99 atom% ^{13}C) was purchased from Daiichi Pure Chemical (Tokyo, Japan). [1-^{13}C]Sodium acetate (99 atom% ^{13}C) was purchased from Nippon Sanso (Tokyo, Japan). [Glycine carbonyl-^{13}C]Hippuric acid ([gly-^{13}C]hippuric acid) was synthesized by benzoylation of [1-^{13}C]glycine with benzoyl chloride (Akira et al 1994). [7-^{13}C]Hippuric acid was synthesized by reaction of [7-^{13}C]benzoic acid with glycine benzyl ester using dicyclohexylcarbodiimide and the subsequent catalytic reduction. Other reagents were purchased from Kanto Chemical (Tokyo, Japan).

Animals

Male Wistar rats, 350–450 g, were anaesthetized with pentobarbital (40 mg kg^{-1}, i.p.) and maintained at 37°C by means of a heating lamp. The right jugular vein was cannulated with 3 Fr. polyethylene catheter (Atom, Tokyo, Japan). Both ureters were cannulated with SP 10 polyethylene tubing (Natsume, Tokyo, Japan) for urine collection. [7-^{13}C]Benzoic acid (0·20 mmol) and [gly-^{13}C]hippuric acid (0·20 mmol) dissolved in saline (0·5 mL) containing sodium hydroxide equimolar with the labelled compounds were injected into the jugular vein through the catheter at doses ranging from 0·4 to 0·6 mmol kg^{-1}. After injection urine excreted through the ureters was collected in 5-min periods for the first 30 min, 10-min periods for the second 30 min and then for the next hour while infusing 5% mannitol at 1 mL h^{-1} to stimulate urine excretion. The volume of urine samples for each 5-min or 10-min period was 150–300 μL; that for 1–2 h period was 0·6–1·6 mL. Urine was stored at −20°C until analysis. Whole urine collected in each 5-min or 10-min period and samples (200 or 300 μL) of 1–2 h post-dose urine were analysed by NMR.

Induction of liver damage

A solution of CCl_4 in olive oil (20%, v/v) was administered orally to rats at a dose of 5 mL kg^{-1} after overnight fast. The cirrhotic condition was characterized after 24h by measurement of the activities of GOT and GPT in plasma with specific kits (GOT-UV and GPT-UV tests; Wako Pure Chemical, Osaka, Japan) followed by the immediate co-administration experiments.

Sample preparation

[1-^{13}C]Sodium acetate aqueous solution (12·05 mg mL^{-1}, 50 μL) as the internal standard for quantitation and aqueous sodium hydroxide solution (10 M, 35 μL) were added to each urine sample. The volume of the sample was diluted to approximately 0·5 mL by addition of water, then vortex-mixed. The sample was then transferred to a 5-mm NMR tube containing 50 μL of a solution (100 mg mL^{-1}) of sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP) in ^2H_2O.

NMR measurements

Proton-decoupled ^{13}C NMR (^{13}C{^{1}H}) measurements were performed at 300 K on a Bruker AM400 (9·4T) spectrometer. 216 free-induction decays (10 min accumulation time) were collected, after 75° (5-μs) pulses, using a relaxation delay of 2 s and a data-acquisition time of 0·655 s. Free induction decays were Fourier-transformed with 10·0-Hz line broadening. Chemical shifts were relative to TSP ($\delta^{13}C = 0$).

Quantitation

A mixture of [7-^{13}C]hippuric acid (4·30 μmol), [gly-^{13}C]hippuric acid (5·46 μmol) and the internal standard (14·52 μmol) dissolved in alkaline control urine was analysed in quadruplicate by ^{13}C NMR spectroscopy under the conditions used for the post-dose urine samples. The relative sensitivity for equal numbers of C7 (δ^{13}C 173·2) of [7-^{13}C]hippuric acid, glycine carbonyl carbon (δ^{13}C 179·5) of [gly-^{13}C]hippuric acid and C1 (δ^{13}C 184·1) of the internal standard was calculated to be 1·887 ± 0·051 (C7 of hippuric acid/C1 of internal standard) and 1·948 ± 0·027 (glycine carbonyl carbon of hippuric acid/C1 of internal standard), respectively, from the resonance heights. The amounts of the labelled hippuric acids in the post-dose urine were calculated on the basis of the relative sensitivity, the ratios of the resonance heights to the internal standard, and the amount of the internal standard added. The amounts calculated for the samples of 1–2 h post-dose urine were corrected for urine volume.

Pharmacokinetic analyses

The urinary excretion data for [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid were analysed according to equations 1-3 based on Michaelis–Menten elimination for benzoic acid and first-order elimination for hippuric acid:

$$dX_1/dt = -V_{max}X_1/(K_m + X_1) \quad (1)$$

$$dX_2/dt = V_{max}X_1/(K_m + X_1) - K_{re}X_2 \quad (2)$$

$$dX_3/dt = K_{re}X_2 \quad (3)$$

where X_1 and X_2 are the amounts of benzoic acid and hippuric acid, respectively, in the body, X_3 is the amount of hippuric acid in the urine, V_{max} is the maximum rate of biotransformation of benzoic acid to hippuric acid expressed as

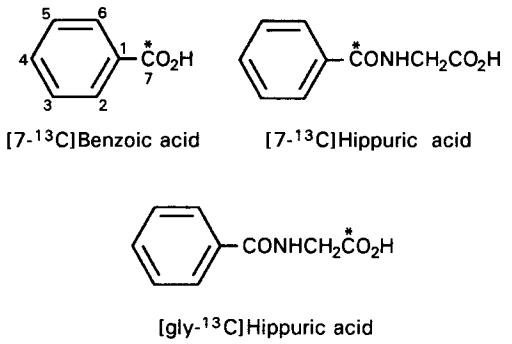

FIG. 1. The structures of the ^{13}C-labelled compounds. *Labelled position.

amount time^{-1}, K_m is the Michaelis–Menten constant expressed as amount, and K_{re} is the apparent first-order elimination rate constant of hippuric acid. The differential equations were solved by the Runge-Kutta method (Yamaoka & Nakagawa 1983).

Results and Discussion

When NMR spectroscopy is applied to drug metabolism studies, sufficient sensitivity and spectral resolution are required for effective detection and separation of the metabolite signals. In our previous study (Akira et al 1993) the ^{13}C resonances of C7 (δ^{13}C 173·2) and glycine carbonyl carbon (δ^{13}C 179·5) of hippuric acid and C7 (δ^{13}C 178·3) of benzoic acid were found to be satisfactorily separated in water. Although protonated carbons are generally preferable for ^{13}C-labelling because of the higher ^{13}C{^{1}H}NMR sensitivity (Akira et al 1993), the sensitivity of the above nonprotonated carbons was considered to be sufficient in this research because of the relatively high dose. Thus, [7-^{13}C]benzoic acid and [gly-^{13}C]hippuric acid (Fig. 1) with high incorporation levels (99%) were used as substrates for the co-administration.

The labelled benzoic acid and hippuric acid were intravenously administered to normal rats and the excreted urine was analysed directly by ^{13}C{^{1}H}NMR. The dose (ca 0·5 mmol kg^{-1}) of benzoic acid was similar to that in the hippuric acid test designed for man (Quick 1933, 1940) and the dose of hippuric acid was the same as that of benzoic acid. There was no appreciable resonance from endogenous benzoic acid and hippuric acid in the spectra of control urine under the NMR conditions used although the resonances of ~1% natural abundance of the infused mannitol (δ^{13}C 66, 74 and 75) and endogenous urea (δ^{13}C 165) were observed. [1-^{13}C]Sodium acetate was added to the urine samples as an internal standard for quantitation because it gave a resonance at δ^{13}C 184·1 separate from the resonances of benzoic acid, hippuric acid, mannitol and urea. In the ^{13}C{^{1}H}NMR spectra of urine after administration of the labelled compounds, the resonances of C7, glycine carbonyl carbon and other carbons (natural abundance) of hippuric acid were also observed at the same chemical shifts as in water. Very minor resonance from C7 (δ^{13}C 178·3) of benzoic acid was also detected in some spectra (Gregus et al 1992). The typical time-course of the spectra is shown in Fig. 2, where the resonances of C7 and glycine carbonyl carbon of hippuric acid were detected with favourable signal-to-noise ratios over 2 h using the 10-min

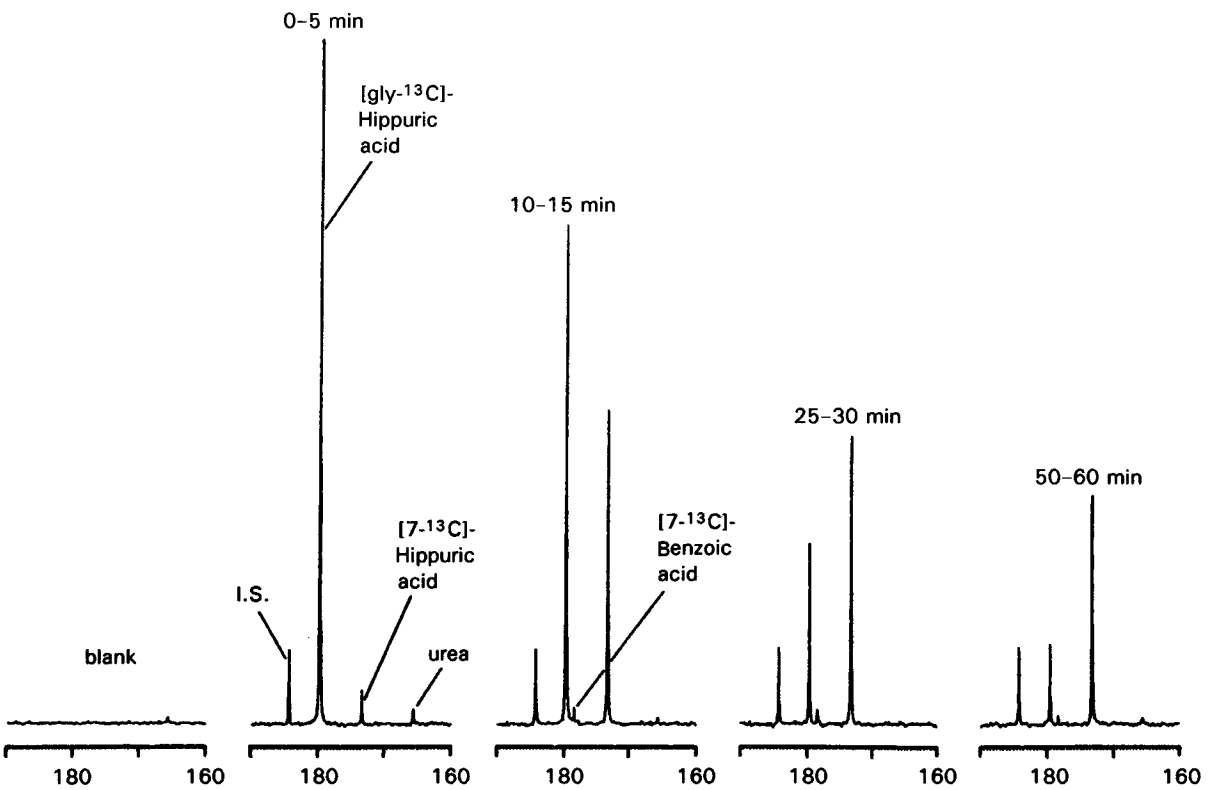

FIG. 2. Partial ^{13}C{^{1}H}NMR spectra of urine obtained from a rat intravenously administered [7-^{13}C] benzoic acid and [gly-^{13}C] hippuric acid simultaneously. Collection periods are indicated above the resonances. All spectra were plotted relative to the fixed height of the resonance of the added internal standard (603 μg).

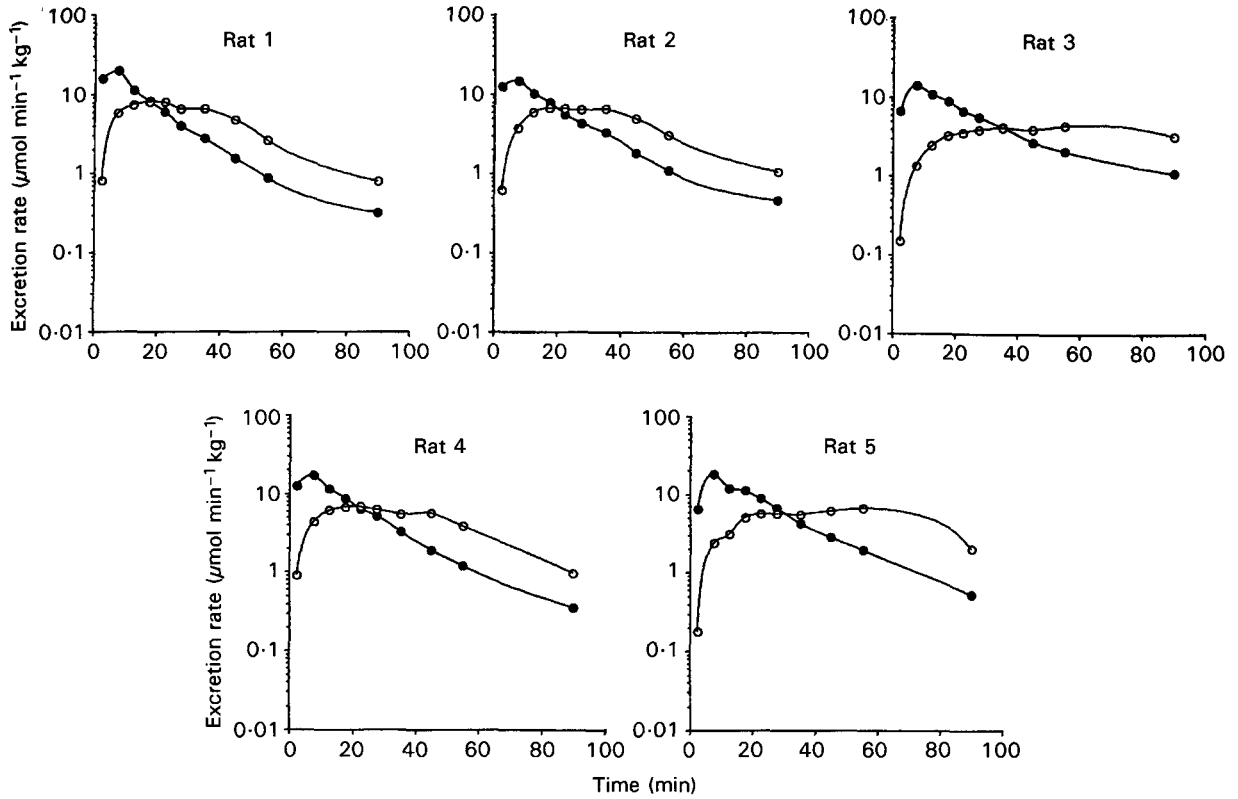

FIG. 3. Urinary excretion of [7-^{13}C] hippuric acid (○) and [gly-^{13}C] hippuric acid (●) after intravenous co-administration of [7-^{13}C] benzoic acid and [gly-^{13}C] hippuric acid to normal rats.

accumulation. The urinary excreted [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid were quantitated on the basis of the resonance heights of the labelled compounds and the internal standard, as described in the experimental section.

The plots of urinary excretion rate against time of both [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid in the individual normal rats were obtained as shown in Fig. 3. Pronounced differences were observed between the [7-^{13}C]hippuric acid excretion as a combined indication of hippuric acid synthesis and renal excretory function and the [gly-^{13}C]hippuric acid excretion as an indication of renal excretory function. The excretion rates of [gly-^{13}C]hippuric acid peaked (14–19 μmol min^{-1} kg^{-1}) at 5–10 min after administration and then apparently followed linear log rate against time curves. In contrast, the excretion of [7-^{13}C]hippuric acid remained at a plateau (4–8 μmol min^{-1} kg^{-1}) from the 10th or 15th min to the 40th or 60th min after administration, and then decreased before the end of the 2-h period at a rate similar to that of [gly-^{13}C]hippuric acid. The cumulative excretion of the labelled hippuric acids in the individual rats is shown in Table 1. [7-^{13}C]Hippuric acid was found to be excreted more slowly than [gly-^{13}C]hippuric acid in each rat although the cumulative excretion of both labelled hippuric acids amounted to 80–90% in 120 min. Thus, liver function can be evaluated by cumulative [7-^{13}C]hippuric acid excretion as in the previous hippuric acid test, monitoring renal excretory function for hippuric acid itself. Although the reliability of the test is improved by this method, exact evaluation is still impossible in renal failure. Thus the capacity to synthesize hippuric acid, i.e. a direct indication of liver function which is not affected by the renal function, needs to be estimated from these excretion data.

Table 1. Cumulative urinary excretion of [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid after intravenous co-administration of [7-^{13}C]benzoic acid and [gly-^{13}C]hippuric acid to normal rats.

	[7-^{13}C]Hippuric acid			[gly-^{13}C]Hippuric acid		
Rat	30 min	60 min	120 min	30 min	60 min	120 min
1	40	70	81	71	82	86
2	34	67	81	62	76	82
3	15	41	79	55	73	87
4	35	68	81	68	83	87
5	21	55	78	60	77	83
Mean ± s.e.m.	29 ± 4·6	60 ± 5·5	80 ± 0·5	63 ± 2·8	78 ± 1·8	85 ± 1·0

The values show cumulative percentage of dose.

Benzoic acid is apparently eliminated with Michaelis–Menten kinetic behaviour (Oyanagi et al 1987; Kubota & Ishizaki 1991; Gregus et al 1992). The biotransformation of benzoic acid to hippuric acid is a saturable process as represented by the [7-^{13}C]hippuric acid excretion patterns in Fig. 3. The capacity to synthesize hippuric acid is shown by the maximum rate of in-vivo formation of hippuric acid (V_{max}) Because the rate of excretion of [gly-^{13}C]hippuric acid at the peak is much faster than that of [7-^{13}C]hippuric acid at the plateau, as shown in Fig. 3, renal transport capacity does not apparently limit the rate of excretion of endogenously formed hippuric acid (Gregus et al 1992). Thus, the rate of excretion of [7-^{13}C]hippuric acid at the plateau might reflect the V_{max} value (Kubota & Ishizaki 1991).

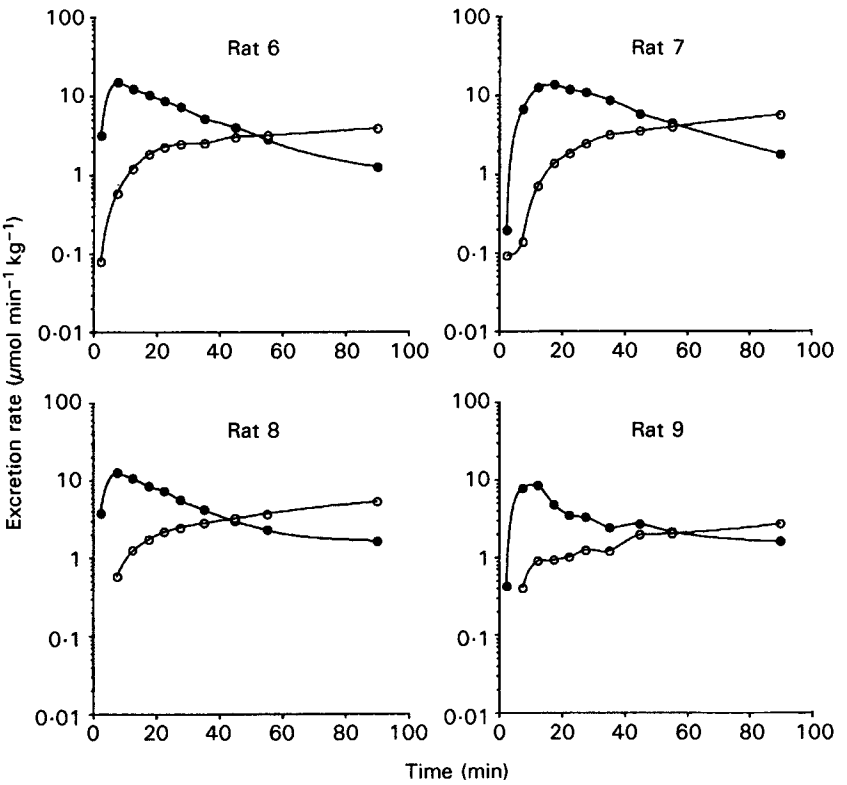

FIG. 4. Urinary excretion of [7-^{13}C] hippuric acid (○) and [gly-^{13}C] hippuric acid (●) after intravenous co-administration of [7-^{13}C] benzoic acid and [gly-^{13}C] hippuric acid to CCl_4-treated rats.

Table 2. Pharmacokinetic parameters for elimination of benzoic acid and hippuric acid by normal rats.

	Rat no.					
	1	2	3	4	5	Mean ± s.e.m.
Renal elimination rate constant of hippuric acid (min^{-1})	0·045	0·034	0·026	0·040	0·029	0·035 ± 0·0032
Maximum rate of metabolism ($\mu mol\ min^{-1}\ kg^{-1}$)	11·4	11·2	6·7	11·2	11·8	10·5 ± 0·96
Michaelis–Menten constant ($\mu mol\ kg^{-1}$)	4·3	4·2	2·5	4·2	4·5	4·0 ± 0·37

Table 3. Cumulative urinary excretion of [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid after intravenous co-administration of [7-^{13}C]benzoic acid and [gly-^{13}C]hippuric acid to CCl_4-treated rats.

	[7-^{13}C]Hippuric acid			[gly-^{13}C]Hippuric acid		
Rat	30 min	60 min	120 min	30 min	60 min	120 min
6	7	23	63	49	70	84
7	5	23	78	46	77	95
8	8	27	89	47	65	85
9	4	15	46	28	42	61
Mean ± s.e.m.	6 ± 0·8	22 ± 2·6	69 ± 9·3	43 ± 4·9	64 ± 7·6	81 ± 7·2

The values show cumulative percentage of dose.

Although this idea seems useful, it is difficult to apply to liver-injured rats whose [7-^{13}C]hippuric acid excretion has no clear plateau, as shown in Fig. 4. Thus, the kinetic parameters were computed from the urinary excretion data based on Michaelis–Menten elimination for benzoic acid and first-order elimination for hippuric acid (Gregus et al 1992), as shown in Table 2. The V_{max} and Michaelis–Menten constants (K_m) showed relatively small variation among the individuals except for rat 3. The renal elimination rate constants (K_{re}) were relatively varied, which possibly affected [7-^{13}C]hippuric acid excretion. In practice, the rate of excretion of [7-^{13}C]hippuric acid for rat 5 was relatively low (see Table 1) despite it having the highest V_{max} value. This discrepancy might be due to the low K_{re} of the individual. Thus the V_{max} value is considered to be a reliable indicator of liver function that is not affected by the renal excretory function. Also, the K_{re} value is a useful indication of renal excretory function (Schwei & Quick 1942).

To investigate whether the decrease in liver function can be practically evaluated by the co-administration methodology, [7-^{13}C]benzoic acid and [gly-^{13}C]hippuric acid were simultaneously administered to CCl_4-treated rats and urinary excreted [7-^{13}C]hippuric acid and [gly-^{13}C]hippuric acid were quantified in the same manner as for normal rats. The time-courses of the urinary excretion rates of the labelled hippuric acids are depicted in Fig. 4. The excretion rates of [gly-^{13}C]hippuric acid peaked (8–15 $\mu mol\ min^{-1}\ kg^{-1}$) at 5–20 min after administration and then apparently followed linear log rate against time curves with appreciably shallower slopes than those for normal rats. In

Table 4. Pharmacokinetic parameters of benzoic acid and hippuric acid elimination in CCl_4-treated rats.

	Rat no.				
	6	7	8	9	Mean ± s.e.m.
Renal elimination rate constant of hippuric acid (min^{-1})	0·021	0·017	0·020	0·010	0·017 ± 0·0025
Maximum rate of metabolism (μmol min^{-1} kg^{-1})	4·9	5·8	5·6	4·8	5·3 ± 0·27
Michaelis–Menten constant (μmol kg^{-1})	4·9	2·7	3·1	3·3	3·5 ± 0·48

contrast, the excretion of [7-^{13}C]hippuric acid was initially very low, but kept increasing with time during the 2-h period. The cumulative excretion of the labelled hippuric acids in the individual rats is shown in Table 3. [7-^{13}C]Hippuric acid was found to be excreted much more slowly than [gly-^{13}C]hippuric acid in each rat. The liver-injured rats were found to excrete the labelled hippuric acids more slowly than the normal rats, and the extent of delay in [7-^{13}C]hippuric acid excretion seemed to be more remarkable than that in [gly-^{13}C]hippuric acid excretion. These results suggest that the capacity to synthesize hippuric acid was greatly reduced by CCl_4 liver injury, whereas the renal function of hippuric acid excretion was impaired to a lesser extent. The kinetic parameters for the individual liver-injured rats were computed as shown in Table 4. The V_{max} and K_{re} values were obviously lower than those of the normal rats although the K_m values were not appreciably reduced. The decrease in V_{max} and K_{re} clearly revealed impairment of the capacity to synthesize hippuric acid, i.e. liver function, and the renal excretory function of hippuric acid, respectively. These results have demonstrated that the decrease in liver function for the individual rats can be evaluated by the V_{max} values even though the renal function of hippuric acid excretion (K_{re}) is impaired.

In conclusion, the co-administration methodology has been demonstrated to be feasible for rats. This simple and convenient approach might be useful for investigation of the liver toxicity of xenobiotics and the effects of therapeutic agents on liver diseases. The co-administration methodology can greatly improve the previous hippuric acid test because it can be used to evaluate liver function even in renal failure. The results could form the basis for a more convenient and reliable hippuric acid test for man.

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