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Studies on Drug Metabolism by Use of Isotopes. XIV.[1] Mass Spectrometric Quantification of Urinary Metabolites of Deuterated *l*-Ephedrine in Rabbits

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As part of problems on the applicability of a drug labeled with deuterium to metabolic studies in man, an analytical method for urinary metabolites by deuterium-tracer technique was investigated and its reliability was compared with that of ^{14}C-tracer technique. *l*-Ephedrine[arom.-$^{2}H_5$] or *l*-ephedrine[α-^{14}C] was subcutaneously injected to rabbits and the main urinary metabolites (1-phenyl-1,2-propanediol and hippuric acid) excreted for 24 hr after dosing were analyzed. A deuterated metabolite such as the diol absent in normal urine was submitted to the inverse isotope dilution analysis using the unlabeled compound both as an internal standard and as a carrier. The molar ratio of the unlabeled to the deuterated compound was determined by mass spectrometry. The amount of deuterated hippuric acid diluted with the endogenous material present in urine was determined by using both the direct isotope dilution analysis and the inverse isotope dilution analysis. It was found that reliability of the deuterium-tracer technique was comparable with that of the ^{14}C-tracer technique on the basis of data obtained from both techniques.

One important problem in the evaluation of safety for new drugs is to gather informations about their metabolic fate in man. In recent years, the metabolic studies of drugs in animal species have been aided by the use of radioactive isotope (RI)-tracer technique. Although species differences in the metabolism have been demonstrated for many drugs,[3] no procedure to extrapolate animal data to man has yet been established. Besides, administration of an RI-labeled compound to man is under the regulations of the International Commission on Radiological Protection. For these reasons, it is of great importance to develop a stable isotope (SI)-tracer technique applicable to man.

As the metabolic patterns of *l*-ephedrine (I) differ considerably among animal species,[4] we chose this drug as a model for establishing a tracer technique with deuterium and have reported a series of work. *l*-Ephedrine [arom.-$^{2}H_5$] (I-d_5) was synthesized from $C_6{}^{2}H_6$.[5] The stability of hydrogen isotope labeled on the benzene ring in biological systems was investigated by using ^{3}H- and ^{14}C-labeled I.[6] Mass spectral problems in the quantitative analysis of deuterated benzoic acid and hippuric acid as model metabolites were studied in detail.[7] Furthermore, deuterium-isotope effect which might be observed in the metabolism was investigated by using *l*-ephedrine[α-^{14}C] (I-^{14}C) and *l*-ephedrine[α-^{14}C, arom.-$^{2}H_5$].[1] In the present paper, an analytical method for urinary metabolites, which were excreted by rabbits given I-d_5, is reported. In addition, reliability of the deuterium-tracer technique is compared with that of the ^{14}C-tracer technique using I-^{14}C.

1) Part XIII: K. Kawai and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **22**, 2372 (1974).
2) Location: *20–1, Kitashinjuku 3-Chome, Shinjuku-ku, Tokyo.*
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Experimental

Labeled Compounds——*l*-Ephedrine[arom.-$^2H_5$] hydrochloride (I-d_5·HCl)[5] and *l*-ephedrine[α-^{14}C] hydrochloride (0.358 μCi/mg) (I-^{14}C·HCl)[8] were samples previously prepared from $C_6{}^2H_6$ (99.5 atom% ^{2}H) and sodium propionate[1-^{14}C], respectively, in this laboratory. Hippuric acid [carbonyl-^{14}C] was purchased from Daiichi Pure Chemicals Co. (Tokyo) and diluted to a specific radioactivity of 2.31×10^{-3} μCi/mg with the unlabeled compound.

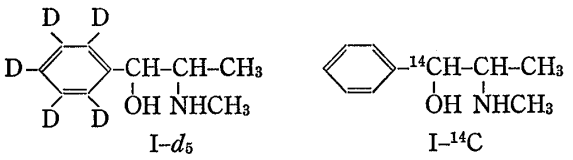

Fig. 1. Structure of Isotopically Labeled *l*-Ephedrines

Unlabeled Compounds——Hippuric acid of GR grade was purchased from Tokyo Kasei Kogyo Co. (Tokyo). 1-Phenyl-1,2-propanediol was prepared according to the method of Zincke, *et al.*[9]

Animals and Dosing——To three male white rabbits (3.0±0.2 kg), I-d_5·HCl and I-^{14}C·HCl (10 mg/kg) were subcutaneously injected at an interval of 3 days. Subsequent 24-hr urine was collected and diluted to 500 ml with water. Control urine was collected during 24 hr before the administration of I-d_5.

Isolation and Purification of Main Urinary Deuterated Metabolites——An aliquot (100 ml) of each urine sample from the rabbits receiving I-d_5 was acid-hydrolyzed in order to liberate the diol as a metabolite from its conjugate, as described previously.[1] After being cooled, the hydrolysate was neutralized with 5 N NaOH. Hippuric acid and the diol (50 to 100 mg) as carriers were dissolved in the hydrolysate, which was then concentrated at 40° under a reduced pressure. The residue was transferred to a 50 ml-centrifuge tube using 10 ml of water and $(CH_3)_2CO$ (20 ml) was added to the solution for deproteinization. After centrifugation, the supernatant was evaporated at 40° under a reduced pressure, the residue was dissolved in 5 ml of water, and the diol dissolved in the solution was extracted with $(C_2H_5)_2O$ at pH 7—8, and then hippuric acid with AcOEt at pH 1. The diol and hippuric acid were recrystallized from AcOEt–benzine and water, respectively.

Oxidation of 1-Phenyl-1,2-propanediol to Benzoic Acid——To a solution of the diol (20 mg) in 1.0 ml of water, 5% $KMnO_4$ (2 ml) was added and the mixture was boiled in a water bath for 30 min. After acidification with conc. HCl, the solution was extracted with $(C_2H_5)_2O$, the extract was evaporated, and the residue was recrystallized from water.

Measurement of Deuterated Metabolites——All mass spectra were obtained with an ionization voltage of 30 eV on a Hitachi RMU-7L Mass Spectrometer. Benzoic acid and the diol, or hippuric acid, which were diluted with the respective carrier and recovered from the urine, were introduced into an ion source from the indirect inlet system or the direct inlet system, respectively. While a total ion current was being maintained at constant, partial mass spectra were repetitively scanned at a slow speed over a narrow mass range covering mass peaks to be determined.

Isolation, Purification, and Determination of ^{14}C-Metabolites——Hippuric acid and the diol (50 to 100 mg) as carriers were dissolved in the acid-hydrolysate of an aliquot (5 ml) of the urine sample, which were separately extracted. Each ^{14}C-metabolite recovered was recrystallized to a constant specific radioactivity, which was determined conventionally with a liquid scintillation counter (Aloka Model 502, Japan Radiation and Medical Electronics, Inc., Tokyo).

Direct Isotope Dilution Analysis of Urinary Hippuric Acid——^{14}C-Hippuric acid (50 to 100 mg) was added to an aliquot (100 ml) of the 24-hr urine sample before or after dosing of I-d_5. The solution was subjected to deproteinization as described above for the deuterated metabolites and a specific radioactivity of the purified compound was determined.

Results and Discussion

Chemical characteristics of a drug labeled with SI and its metabolites should be identical with those of the corresponding unlabeled compounds but the difference in mass should make it easy to distinguish between the labeled molecule and its unlabeled counterpart. SI-labeled compounds have been chiefly used as internal standards for the quantification of prostaglandins,[10,11] nortriptyline,[12] homovanillic acid,[13] and amphetamine[14] in biological fluids by the

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combination of a gas chromatograph with a mass spectrometer (GC-MS). However, when an unlabeled drug and its metabolites are analyzed by these methods, each SI-labeled internal standard must be prepared and it is impossible to distinguish between an endogenous compound (*e.g.*, hippuric acid) present in biological samples and the exogenous metabolite derived from a drug. In contrast to the administration of an unlabeled drug to animal species or man, that of an SI-labeled drug would enable unlabeled compounds to serve as the internal standard for the metabolites to be analyzed and permit a distinction between an endogenous and exogenous metabolite.

It is necessary to isolate and purify completely a minute amount of SI-labeled metabolites from a large amount of endogenous components in urine for their quantitative analysis. This problem can be solved by the inverse isotope dilution analysis in which unlabeled compounds serve both as a carrier and as an internal standard for the SI-labeled metabolites.

Determination of Molar Ratio of Unlabeled to Deuterated Compound

The mass spectra of unlabeled hippuric acid, 1-phenyl-1,2-propanediol, and benzoic acid are shown in Fig. 2. As an isotope effect of a few percent was observed in the formation of the phenyl ion and $C_4{}^2H_3{}^+$ ion from deuterated benzoic acid or hippuric acid,[7] the benzoyl ion at *m/e* 105 (*m/e* 110 for the labeled compound) of hippuric acid, and the fragment ions at *m/e* 107 and 108 (*m/e* 112 and 113 for the labeled compound) of the diol were chosen as major peak(s) for quantitative mass spectrometry. In the mass spectrum of an unlabeled compound, the prominent molecular ion and fragment ions are inevitably accompanied with ions at 1 and 2 atomic mass units (amu) higher and, in some cases, with ions at 1 and/or 2 amu lower than the *m/e* value of the major peak; the former is mainly derived from naturally occurring heavy isotopes and the latter is due to an ion losing one or two hydrogen atoms. In the mass spectrum of a compound having a benzene ring which contains more than 99 atom% ^2H, peaks due to a d_4-species and a d_3-species contaminated in the labeled compound should additionally appear at lower amu than the *m/e* value of the major ions (d_5-species) carrying the ring. A d_4-species may be also formed by an intramolecular exchange between deuterium and hydrogen during a fragmentation process. It has been proved that an accuracy better than 3% was obtained in the quantification of deuterated benzoic acid or hippuric acid even if peaks at +2 and −2 amu of a major peak were neglected.[7] The molar ratio was, therefore, calculated from the total peak intensities in the region of the ±1 amu of the major peak(s) in both variants.

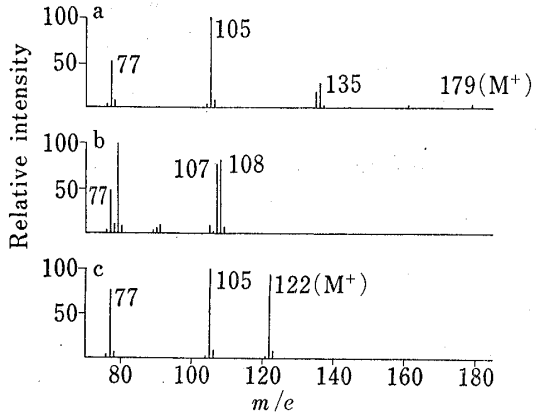

Fig. 2. Mass Spectra of Hippuric Acid (a), 1-Phenyl-1,2-propanediol (b), and Benzoic Acid (c)

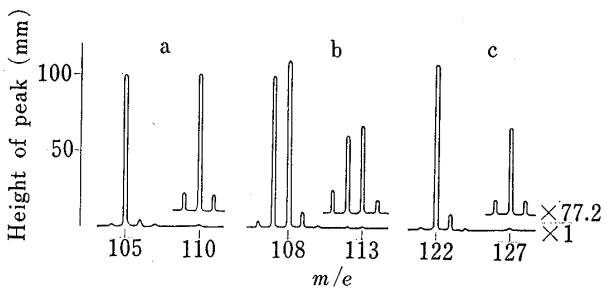

Fig. 3. Examples of Partial Mass Spectra of Deuterated Metabolites, Diluted with Unlabeled Compound and Recovered from Urine of Rabbit Administered I-d_5

a) hippuric acid
b) 1-phenyl-1,2-propanediol
c) benzoic acid obtained from the diol in b) by $KMnO_4$ oxidation

The mass spectrum of a deuterated metabolite diluted with several hundred-fold amounts of the unlabeled compound was measured at two scales (×1 and ×100) in the region of *m/e*

103 to 112 for hippuric acid and *m/e* 105 to 115 for the diol. Examples of the partial mass spectra of the deuterated metabolites which were diluted with the unlabeled compound are shown in Fig. 3. The intensities of amplified peaks of a deuterated metabolite were corrected both for the background observed before introduction of the sample into the mass spectrometer and for the contribution derived from the unlabeled compound as described previously.[7]

As it was shown that tritium attached to the benzene ring of I was stable to $KMnO_4$ oxidation,[6] the deuterated diol which was diluted with the carrier was oxidized to benzoic acid and also determined quantitatively in this chemical form. The amount of the deuterated diol in the urine determined in both chemical forms agreed closely (*cf.*, Table II). This finding suggests that this method would be useful for quantifying a compound such as I, in the mass spectrum of which the molecular ion does not appear and the fragment ions carrying the benzene ring are of very low intensity.

Calculations

The amount of deuterated metabolites can be calculated as follows. For a metabolite such as 1-phenyl-1,2-propanediol absent in normal urine, the molar ratio of a carrier (C_1 mole) to the deuterated metabolite (D_1 mole) is equal to the peak intensity ratio (R_1) of both variants. Hence,

$$D_1 = C_1/R_1 \quad (1)$$

The amount of a metabolite such as hippuric acid diluted with the endogenous material present in urine can be calculated according to the following simultaneous equation. The total amount (T mole) of the endogenous (E mole) and the exogenous compound (D_2 mole) is determined by the direct isotope dilution analysis using the RI-labeled compound and given by

$$E + D_2 = T \quad (2)$$

The ratio, E/D_2, is calculated from peak intensities of both variants in a sample recovered from urine without the carrier. However, when the amount of the endogenous compound is so small as to require the carrier (C_2 mole), the peak intensity ratio (R_2) is given by

$$(C_2+E)/D_2 = R_2 \quad (3)$$

Combination of Eq. 2 and 3 leads to

$$D_2=(C_2+T)/(R_2+1) \quad (4)$$

As a large amount of urinary endogenous hippuric acid was excreted (*cf.*, Table I), the compound may be recovered from urine without the carrier. However, in this experiment the carrier was used in order to recover a sufficient amount of the compound for subsequent purification. The amount of the diol and hippuric acid in the urine excreted by the rabbits given I-d_5 was calculated by using Eq. 1 and 4, respectively.

Distinction between Endogenous and Exogenous Hippuric Acid

The excretion of hippuric acid during 24 hr before or after dosing of I-d_5 was determined by the direct isotope dilution analysis. Table I shows that the normal output of hippuric acid was considerably variable both individually and daily. It was reported that 20—30% of I·HCl (10 mg/kg) administered to a rabbit was excreted as hippuric acid in the urine.[1] Although urinary exogenous hippuric acid derived from I-d_5 is, therefore, calculated to be 6—7 mg, it would be impossible to estimate the amount of exogenous hippuric acid as an increment over the normal output before dosing. However, the amount of deuterated hippuric acid can be determined on the basis of the difference in mass with the aid of the mass spectrometer. This distinction between an endogenous and exogenous metabolite is an advantage of our method.

Comparison of Deuterium- and ^{14}C-Tracer Techniques

The main urinary metabolites of I in rabbits are 1-phenyl-1,2-propanediol and hippuric acid.[4] On the basis of the amount of two main metabolites which were analyzed by the

Table I. Urinary Hippuric Acid Levels before and after Administration of Deuterated *l*-Ephedrine in Rabbits

No. of rabbits	Hippuric acid in 24-hr urine (mg)[a]	
	before dosing	after dosing
1	886.3	607.2
2	683.1	590.1
3	650.4	765.5

I-d_5 • HCl was injected *s.c.* at a dose of 10 mg/kg to rabbits.
a) determined by the direct isotope dilution analysis using ^{14}C-hippuric acid

deuterium- and ^{14}C-tracer techniques, the reliability of the former technique was compared with that of the latter (Table II). In the previous paper,[1] no significant difference in the amount of these metabolites was observed between administration of I-^{14}C and deuterated I-^{14}C. In addition, it was found that the amount of the metabolites of I in rabbits varied individually and daily to a small extent. Judging from these findings, it can be seen that there was no significant difference in the results obtained by the two tracer techniques. Therefore, it is suggested that reliability of this deuterium-tracer technique is comparable with that of the ^{14}C-tracer technique.

Table II. Comparison of Deuterium- and ^{14}C-Tracer Techniques in Quantification of Urinary Metabolites of *l*-Ephedrine

No. of rabbits	Metabolite	Deuterium-tracer technique % of dose	^{14}C-tracer technique % of dose
1	1-phenyl-1,2-propanediol	16.7[a] 16.6[b]	17.6
	hippuric acid	27.6	28.5
2	1-phenyl-1,2-propanediol	16.7[a] 16.7[b]	18.4
	hippuric acid	23.7	20.4
3	1-phenyl-1,2-propanediol	8.7[a] 8.7[b]	14.4
	hippuric acid	22.5	28.0
Mean±S.D.	1-phenyl-1,2-propanediol	14.0±3.8[a] 14.0±3.7[b]	16.8±1.7
	hippuric acid	24.6±2.2	25.6±3.7

I-d_5• HCl and I-^{14}C•HCl (10 mg/kg) were injected s.c. to the same rabbits at an interval of 3 days.
a) determined as 1-phenyl-1,2-propanediol
b) determined as benzoic acid being a $KMnO_4$-oxidation product of the diol

In this deuterium-tracer technique, one-fifth of 24-hr urine was used for one analysis. The use of recrystallization for purification of deuterated metabolites lowered the sensitivity of this technique. This technique is, however, sensitive enough for the metabolic study. Because a gas chromatograph can purify a minute amount of components, the use of GC-MS equipped with an accelerating voltage alternator[15] which can determine the molar ratio of an unlabeled to its SI-labeled compound would make the SI-tracer technique more sensitive.

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