[Chem. Pharm. Bull. 24(11)2728—2732(1976)]

UDC 547.435.08 : 543.51.062

Studies on Drug Metabolism by Use of Isotopes. XVII.[1] Mass Spectrometric Quantification of Urinary Metabolites of Deuterated *l*-Ephedrine in Man[2]

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(Received February 24, 1976)

In order to develop a practical technique useful for metabolic studies in man by the use of drugs labeled with a stable isotope, *l*-ephedrine-d_5 was administered to man and the urinary metabolites were quantitatively determined by mass spectrometry. In this method, unlabeled compounds were used as carriers and internal standards for deuterated metabolites of interest. It was thereby found that 75.1% of the administered *l*-ephedrine was excreted unchanged, 3.6% as norephedrine, and 3.5% as hippuric acid.

It is well known that the metabolism of a drug differs remarkably from species to species.[4–6] In order to undertake reliable toxicological investigations on a drug, it is necessary to select an animal species in which the metabolic fate of the drug in question is most similar to that observed in man. The metabolism of drugs in man has been studied by the use of unlabeled drugs or drugs labeled with radioactive isotopes. However, these methods have inherent limits. The former procedure has a disadvantage that it is impossible to distinguish between exogenous metabolites such as hippuric acid derived from the administered drug and endogenous compounds present in the biological samples. On the other hand, application of the latter to man is largely restricted by radiation hazards to the living system.

For these reasons, we attempted to develop a practical technique which is applicable to the metabolic studies in man by the use of drugs labeled with a stable isotope. As the metabolic patterns of *l*-ephedrine differ considerably among animal species,[7] this compound was chosen as a model drug to be labeled for our purpose and a number of fundamental problems were examined. Firstly, *l*-ephedrine-d_5 was prepared in a reaction sequence starting with benzene-d_6.[8] Secondly, mass spectrometric problems in the quantification of benzoic acid-d_5 or hippuric acid-d_5 as model metabolites were examined in detail, and the molar ratios of the deuterated to its unlabeled compound were accurately determined in the range of 1:80 to 1:400 with a mass spectrometer.[9] Thirdly, deuterium isotope effect, which might be observed in the metabolism of *l*-ephedrine-d_5, was investigated by the use of ^{14}C-*l*-ephedrine and ^{14}C-*l*-ephedrine-d_5. The *in vivo* studies in rats and rabbits showed no significant difference in the metabolism between the deuterated and the protiated drugs.[10] Fourthly, an analytical method for deuterated metabolites in the urine from rabbits administered *l*-ephedrine-d_5 was reported, and reliability of this deuterium tracer technique was found to be comparable

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2) This work was abstracted in part from a thesis submitted by K. Kawai to Tokyo College of Pharmacy in partial fulfillment of the Doctor of Philosophy degree requirements.
3) Location: *1432–1, Horinouchi Hachioji, Tokyo, 192–03, Japan.*
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to that of the ^{14}C-tracer technique using ^{14}C-*l*-ephedrine.[11] Lastly, the deuterium label in *l*-ephedrine-d_5 was found to be stable in rats under the *in vivo* experimental conditions.[12] The present paper describes the metabolic fate of *l*-ephedrine in man by the use of *l*-ephedrine-d_5. Part of the work has been reported briefly.[13]

Experimental

Labeled Compounds——*l*-Ephedrine-d_5 hydrochloride was the same as the sample previously prepared from benzene-d_6 (99.5 atom % ^2H) in our laboratory.[8] Benzoic acid[carboxyl-^{14}C] and hippuric acid[*carboxyl*-^{14}C] were purchased from Daiichi Pure Chemicals Co. (Tokyo) and diluted to specific radioactivities of 1.53 mμCi/mg and 2.31 mμCi/mg, respectively, with their unlabeled compounds.

Unlabeled Compounds——*l*-Ephedrine hydrochloride of JP grade was purchased from Sanko Seiyaku Kogyo Co. (Tokyo). Benzoic acid and hippuric acid, which are GR grade, were purchased from Tokyo Kasei Kogyo Co. (Tokyo). *dl*-Norephedrine hydrochloride and 1-phenyl-1,2-propanediol were synthesized according to the methods of Hurtung, *et al.*[14] and of Zinke, *et al.*,[15] respectively. *p*-Hydroxyephedrine hydrochloride was supplied from Hoechst Japan Ltd. (Tokyo).

Drug Administration and Urine Collection——One healthy male subject, aged 25 years and weighing 60 kg, received orally 49.5 mg of *l*-ephedrine-d_5 in a capsule, and subsequent 24-hr urine was collected and diluted to 1500 ml with water.

Isolation and Purification of Urinary Metabolites——Unlabeled *l*-ephedrine hydrochloride, 1-phenyl-1,2-propanediol, benzoic acid, and hippuric acid (50—100 mg) were added as carriers to an aliquot (300 ml) of the urine. Unlabeled *dl*-norephedrine hydrochloride, unlabeled *p*-hydroxyephedrine hydrochloride, benzoic acid[carboxyl-^{14}C], and hippuric acid[carboxyl-^{14}C] (50—100 mg) were added to another aliquot (300 ml). Aliquots of the urine sample were concentrated and deproteinized in order to facilitate the subsequent extraction procedure as described previously.[11] Each deuterated metabolite, diluted with its carrier, was extracted with an organic solvent by adjusting the solution obtained above to various pH values and then purified by recrystallization[11,12] except for norephedrine. Based on the report of Beckett, *et al.*,[16] the extract for norephedrine was assumed to contain 7—8 mg of unchanged *l*-ephedrine-d_5, which would be recrystallized concomitantly with norephedrine. The extract was spotted on a TLC plate (20×20 cm, 0.5 mm in thickness) coated with silica gel (Wakogel B-5F, Wako Pure Chemical Ind. Ltd., Osaka) containing a fluorescent indicator. The plate was developed with chloroform–acetone–diethylamine (5: 4: 1),[17] and the *Rf* values of norephedrine and ephedrine were 0.8—0.9 and 0.4—0.5, respectively. The zone corresponding to norephedrine on the plate, which was located by ultraviolet absorption, was scraped off and eluted with ether, and the eluted compound was recrystallized from ethanol–ether as its hydrochloride.

Oxidation of Ephedrine, Norephedrine, and *p*-Hydroxyephedrine——The purified ephedrine or norephedrine hydrochloride (20 mg) was oxidized to benzoic acid with potassium permanganate as described for 1-phenyl-1,2-propanediol in our previous paper.[11] The purified *p*-hydroxyephedrine hydrochloride (20 mg) was converted into *p*-hydroxybenzaldehyde with sodium metaperiodate, which was then oxidized to *p*-hydroxybenzoic acid with silver oxide.[12]

Mass Spectrometry——All mass spectra were recorded at an ionization voltage of 30 eV on a mass spectrometer (Hitachi model RMU-7L, Hitachi Ltd., Tokyo). Deuterated metabolites, diluted with their respective carriers, were determined as follows: Benzoic acid, 1-phenyl-1,2-propanediol, and ephedrine or norephedrine which had been converted into benzoic acid were introduced into an ion source from the indirect inlet system. Hippuric acid and *p*-hydroxyephedrine which had been converted into *p*-hydroxybenzoic acid were directly introduced. Partial mass spectra were repeatedly scanned at a slow speed[18] over a mass range of *m/e* 120—130 for benzoic acid, *m/e* 105—115 for 1-phenyl-1,2-propanediol, *m/e* 103—113 for hippuric acid, and *m/e* 136—146 for *p*-hydroxybenzoic acid. During the scanning, the total ion current was maintained constant.

Measurement of ^{14}C-Activity——Specific activities of ^{14}C-benzoic acid and ^{14}C-hippuric acid, recovered from the urine, were determined with a liquid scintillation counter (Aloka model 502, Japan Radiation & Medical Electronics, Inc., Tokyo).[7]

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17) P.G. Welling, K.P. Lee, J.A. Patel, J.E. Walker, and J.G. Wagner, *J. Pharm. Sci.*, **60**, 1629 (1971).
18) It took about 40 sec to scan a single partial mass spectrum over a mass range described above.

Results and Discussion

Various metabolites of ^{14}C-*l*-ephedrine in animal species have been quantitatively determined.[7] Among these metabolites, ephedrine, norephedrine, *p*-hydroxyephedrine, 1-phenyl-1,2-propanediol, benzoic acid, and hippuric acid were analyzed as the metabolites in man by a deuterium tracer technique in this work.

A certain quantity of unlabeled compounds were added as carriers and as internal standards for deuterated metabolites of interest to an aliquot of the 24 hr urine before the extraction and recrystallization. The partial mass spectra of each purified compound were recorded at a scale of ×1 for unlabeled ions and at a scale of ×10 or ×100 for deuterated ions over a narrow mass range covering mass peaks to be determined.[19] The molar ratio of the unlabeled to its deuterated compound in each purified sample can be calculated from the total peak intensities of an appropriate major ion carrying the benzene ring and its two satellite ions at one atomic mass unit lower and higher in both compounds as described in our previous paper.[9]

The amount of deuterated metabolites such as ephedrine, norephedrine, *p*-hydroxyephedrine, and 1-phenyl-1,2-propanediol in the absence of their endogenous compounds in normal urine can be calculated from the molar ratio and a known amount of the added carrier; this method is known as the inverse isotope dilution analysis. However, the amount of deuterated benzoic acid or hippuric acid can not be estimated by the inverse isotope dilution analysis alone because of the presence of its unlabeled endogenous compound in normal urine. The total amount of the deuterated metabolite and its endogenous compound in the urine was, therefore, determined by comparing the specific activity of its ^{14}C-compound added to the urine with that of the compound recovered from the urine; this method is known as the direct isotope dilution analysis. The amount of the deuterated benzoic acid or hippuric acid can be calculated from the data obtained by using both the inverse isotope dilution analysis and the direct isotope dilution analysis. The equations to be used for the calculation were described in our previous paper more in detail.[11]

The molar ratios in the purified 1-phenyl-1,2-propanediol, benzoic acid, and hippuric ac d were determined from their partial mass spectra.[9,11] Since ephedrine, norephedrine, and *p*-hydroxyephedrine reveal no intensive ion carrying the benzene ring suitable for the mass spectral quantification,[8,12] mixtures of their deuterated and unlabeled compounds were oxidized to benzoic acid or *p*-hydroxybenzoic acid, from whose molecular ion the molar ratio w s determined as described previously.[11]

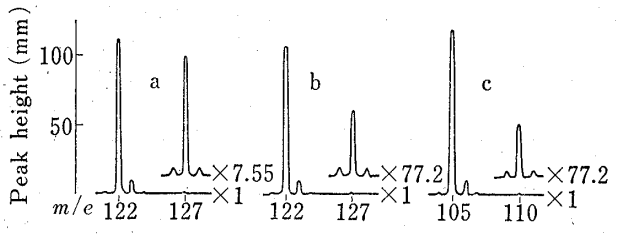

Fig. 1. Examples of Partial Mass Spectra of Deuterated Metabolites, Diluted with Unlabeled Compound and Recovered from Urine of Man Administered *l*-Ephedrine-d_5

a) ephedrine, determined as benzoic acid
b) norephedrine, determined as benzoic acid
c) hippuric acid

Table I. Urinary Metabolites of *l*-Ephedrine in Man

Metabolite	% of dose
Ephedrine	75.1
Norephedrine	3.6
p-Hydroxyephedrine	0
1-Phenyl-1,2-propanediol	0
Benzoic acid	trace[a]
Hippuric acid	3.5[b]
Total	82.2

a) endogenous benzoic acid, 23.4 mg/day
b) endogenous hippuric acid, 155.3 mg/day

19) On the basis of the method described previously,[9] the actual multiplying ratios were 1: 7.55 for the scale setting of 1: 10 and 1: 77.2 for 1: 100, respectively.

Examples of the partial mass spectra of the deuterated metabolites, diluted with the corresponding unlabeled compounds, are shown in Fig. 1. The peaks due to the deuterated metabolites appeared with an intensity suitable for the determination of the molar ratio at the scale of ×10 in the case of ephedrine, and at the scale of ×100 in the case of norephedrine and hippuric acid when the peaks corresponding to the unlabeled compounds were recorded at the scale of ×1. On the other hand, the peak from the deuterated benzoic acid was negligible at the scale of ×100, and the peaks from the deuterated *p*-hydroxyephedrine and 1-phenyl-1,2-propanediol were not observed at all.

The concentrations of *l*-ephedrine-d_5 and of its metabolites excreted in the 24 hr urine are shown in Table I. In man, 75.1% of the administered *l*-ephedrine was excreted unchanged, 3.6% as norephedrine, 3.5% as hippuric acid, and less than 1% as benzoic acid. The unchanged drug and norephedrine determined in the urine by our method were similar in the percentage of the dose to the values reported by Beckett, *et al.*[16] and Welling, *et al.*,[17] who used gas chromatography. By this deuterium tracer technique, it was found that the exogenous hippuric acid (1.56 mg), derived from *l*-ephedrine-d_5, was diluted with a large amount of its endogenous compound (155.3 mg) in the urine. In the case of the administration of the unlabeled drug, the amount of the exogenous metabolite can not be estimated because of a large difference in the quantity between the exogenous and endogenous compounds and because of daily variation in the excretion of the latter. As described above, however, our technique can distinguish between the deuterated metabolite and its endogenous compound on the basis of the difference in mass with a mass spectrometer. Benzoic acid derived from *l*-ephedrine-d_5 in man was excreted almost entirely as hippuric acid. This is in agreement with the results by Bridges, *et al.*[20] using ^{14}C-benzoic acid.

The sum of metabolites listed in Table I amounts to 82.2% of the administered dose. The major metabolites of *l*-ephedrine in various animal species, *p*-hydroxyephedrine (rats), 1-phenyl-1,2-propanediol (rabbits), and benzoic acid (guinea pigs), were not detected in a significant amount in the urine of man. It had been reported that 70—90% of the ^{14}C-activity administered were excreted in the first 24 hr urine from the animal species studied after dosing ^{14}C-*l*-ephedrine.[7] From these facts, there would be little possibility for any metabolite other than the metabolites listed in Table I to be excreted in an appreciable amount.

There are marked differences in the metabolism of *l*-ephedrine among mice, rats, guinea pigs, rabbits, and dogs.[7,21] It is of great interest which animal species most closely resembles man with regard to the metabolism of this drug. Comparison of the present data with the previous results indicates that the metabolic pattern of *l*-ephedrine in man is most similar to that observed in mice.

Stable isotopes are proved to be useful as a means for studying drug metabolism; for identification of unknown metabolites and for quantitative determination of drugs or biological compounds. Such use of stable isotopes has been reviewed.[22,23] In the quantification of a drug in biological fluids, compounds labeled with stable isotopes, particularly with deuterium because of its availability in the widest variety of forms, have been used as carriers and as internal standards. Such a technique has the disadvantage that labeled compounds must be prepared as carriers and internal standards for a drug and all its metabolites. In addition, an exogenous metabolite derived from an unlabeled drug can not be distinguished from its endogenous compound such as hippuric acid in biological samples. These difficulties can be overcome by a technique described in this paper, in which a deuterated drug is administered to man or to animals. Drugs labeled with stable isotopes would be useful for studies on drug

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metabolism in man as a substitute for radioactive drugs because of the absence of possible radiation hazards.

Acknowledgement This work was supported in part by the Kanae Foundation of the New Medicine Research Association.