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QUANTITATIVE DETERMINATION OF THE β-ADRENOCEPTOR STIMULANT FORMOTEROL IN URINE BY GAS CHROMATOGRAPHY MASS SPECTROMETRY

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SUMMARY

A method for the quantitative determination of the β-stimulant formoterol in urine, using a gas chromatograph—mass spectrometer, is described. Formoterol can be analyzed after the addition of a deuterium-labelled internal standard and conversion to a mixed bis-pentafluoropropionyl-methyl derivative for selected ion monitoring. The detection limit was 5 ng/ml.

Urinalysis after the oral administration of formoterol fumarate, using a combined enzymic hydrolysis method, revealed that the drug was conjugated with glucuronic acid in rats, dogs and humans.

INTRODUCTION

A catecholamine analogue formoterol fumarate, 2-hydroxy-5-[(1*RS*)-1-hydroxy-2-{[(1*RS*)-2-(*p*-methoxyphenyl)-1-methylethyl]amino}ethyl]-formanilide fumarate dihydrate, has been introduced as a potent β-adrenoceptor stimulant with high selectivity for β_2-receptor [1—3]. Because its effective dose is small (0.5—10 μg/kg), we can expect formoterol concentrations to be very low in biological samples. For drug measurements we employed selected ion monitoring. Our method involves the detection of nanogram levels of urinary formoterol as the corresponding bis-pentafluoropropionyl-methyl derivative, using a deuterium-labelled internal standard.

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EXPERIMENTAL

Chemicals

Formoterol fumarate was synthesized in our laboratory by the method of Murase et al. [1]. Deuterium-labelled formoterol used as internal standard was prepared by the same method, except that for some raw materials we substituted deuterium compounds as follows. *p*-Methoxyphenylacetone, which was the source of the 2-(*p*-methoxyphenyl)-1-methylethyl function of formoterol, was boiled in a mixture of 1 *N* NaO^2H in ^2H_2O and $C^2H_3O^2H$ (1:1) and then extracted with diethyl ether. The *p*-[$^2H_5$]methoxyphenylacetone obtained was mixed with benzylamine in $C^2H_3O^2H$ and the solution was hydrogenated in the presence of platinum. In this step, deuterium from $C^2H_3O^2H$ was partially introduced into the product and a mixture of 2-benzylamino-1-(*p*-methoxyphenyl)-[1,1,3,3,3-$^2H_5$]propane and -[1,1,2,3,3,3-$^2H_6$]-propane was obtained. The mixture was subjected to the next process. For the reduction of the ketone to the alcohol with $NaBH_4$ [1], we used NaB^2H_4 instead, and finally obtained a mixture of [$^2H_6$]- and [$^2H_7$]formoterol. 2-Hydroxy-[3-^{3}H]-5-[(1*RS*)-1-hydroxy-2-{[(1*RS*)-2-(*p*-methoxyphenyl)-1-methylethyl]amino}ethyl]-formanilide fumarate dihydrate ([^{3}H]formoterol fumarate, radiochemical purity > 99%) was synthesized by Shinloihi Co. (Kanagawa, Japan).

Pentafluoropropionic anhydride (PFPA) was purchased from Tokyo Kasei (Tokyo, Japan). The ethereal solution of diazomethane was prepared from *p*-toluenesulfonyl-N-methyl-N-nitrosamide (Tokyo Kasei). β-Glucuronidase and arylsulphatase were from Boehringer Mannheim Yamanouchi (Tokyo, Japan).

Gas chromatograph—mass spectrometer

An Hitachi RMU-6MG gas chromatography—mass spectrometry (GC—MS) system fitted with an accelerating voltage alternator was used. Gas chromatography was on a glass column (50 cm × 3 mm I.D.) packed with 3% OV-1 on Chromosorb W (AW DMCS, 80—100 mesh) with a helium flow-rate of 30 ml/min. The temperatures were 260°C for the injection and interface, and 240°C for the column. The operating conditions were: electron energy, 20 eV; emission current, 80 μA; ion source temperature, 160°C; accelerating voltage, 2.2—2.4 kV; entrance and collector slits, 0.4 mm. Mass spectra were obtained with the GC—MS system in the scanning mode under the above conditions, except that the entrance and collector slits were adjusted to 0.1 mm and the accelerating voltage was 1.5 kV.

Procedures

To each 2 ml urine sample were added 2 ml of an aqueous solution containing 200 ng of internal standard and 0.5 g of sodium hydrogen carbonate; the mixture was extracted with 4 ml of ethyl acetate. The organic layer was then extracted with 3 ml of 0.1 *N* hydrochloric acid and aspirated off. After adding 0.8 g of sodium hydrogen carbonate, the aqueous layer was extracted with 4 ml of ethyl acetate. The organic layer was evaporated to dryness under reduced pressure. To the residue were added 10% pyridine in methylene chloride (100 μl), and 25% PFPA in methylene chloride (250 μl). After 30 min

at room temperature, the solvent and reagent were evaporated under a nitrogen stream; subsequently, 0.8 g of sodium hydrogen carbonate, 4 ml of distilled water and 4 ml of diethyl ether were added to the residue. The mixture was shaken thoroughly for 30 sec, centrifuged and the ether layer was evaporated to dryness. The residue was admixed with 100 μl of ethereal diazomethane and allowed to stand for 5 min at room temperature. After eliminating the solvent and reagent, the residue was dissolved in 50 μl of ethyl acetate, then 1—2 μl of the solution were injected for GC—MS. The molecular ions of the bis-PFP-methyl derivative of formoterol and [$^2H_7$]formoterol (*m/z* 604 and *m/z* 611, respectively) were monitored, and the peak height ratio was used to calculate the amount of formoterol in each sample by referring to the standard curve, prepared by subjecting control urine spiked with known amounts of formoterol (5—100 ng/ml) to the above procedure.

Extraction recoveries

Control urine samples containing 20 ng/ml [^{3}H]formoterol (0.01 μCi/ml) were extracted with ethyl acetate after adjusting the pH to between 1 and 10, and the radioactivity in the organic layer was counted on a Packard liquid scintillation counter (Model 3255). Concurrently, the urine samples spiked with [^{3}H]formoterol were carried through the assay procedure and the radioactivity extracted in each step of the procedure was measured.

Animal and human experiments

After an overnight fast, two male rats (200 and 210 g) and two male beagles (12 and 13 kg) received an oral dose of 60 and 500 μg/kg formoterol fumarate, respectively, in an aqueous solution; the 0—8 h urine was then collected. In the human study, three male volunteers (50—62 kg) received 40 μg of formoterol fumarate diluted with lactose after an overnight fast; their 0—6 h urine was collected. The urine samples (2 ml) were incubated at 37°C for 20 min, either with β-glucuronidase (9000 units, *p*-nitrophenyl glucuronide as substrate) at pH 7.0, or with arylsulphatase (7500 units, *p*-nitrophenyl sulphate as substrate) at pH 6.2. To inhibit the activity of contaminating β-glucuronidase, before incubation 10^{-3} *M* saccharo-1,4-lactone was added to the arylsulphatase medium. Control samples were analyzed without enzymic hydrolysis.

RESULTS AND DISCUSSION

Derivatization of formoterol for GC—MS

Formoterol has many reactive hydrogen atoms available for substitution (Fig. 1, I). The trimethylsilylation of formoterol with various silylating reagents did not give uniform products, owing to incomplete silylation of the amino or amido group. When N-trimethylsilylimidazole (TSIM) and N,O-bis-(trimethylsilyl)acetamide (BSA) were used as silylating reagents, the products obtained were a major bis-TMS derivative with a minor tris-TMS derivative, and a mixture of almost equal amounts of bis-TMS and tris-TMS derivatives, respectively. When a mixture of TSIM, BSA and trimethylchlorosilane (3:3:2), one of the most active combinations of silylating reagents, was used, a mixture of tris-TMS and tetrakis-TMS derivatives was obtained. Moreover, the ratio of

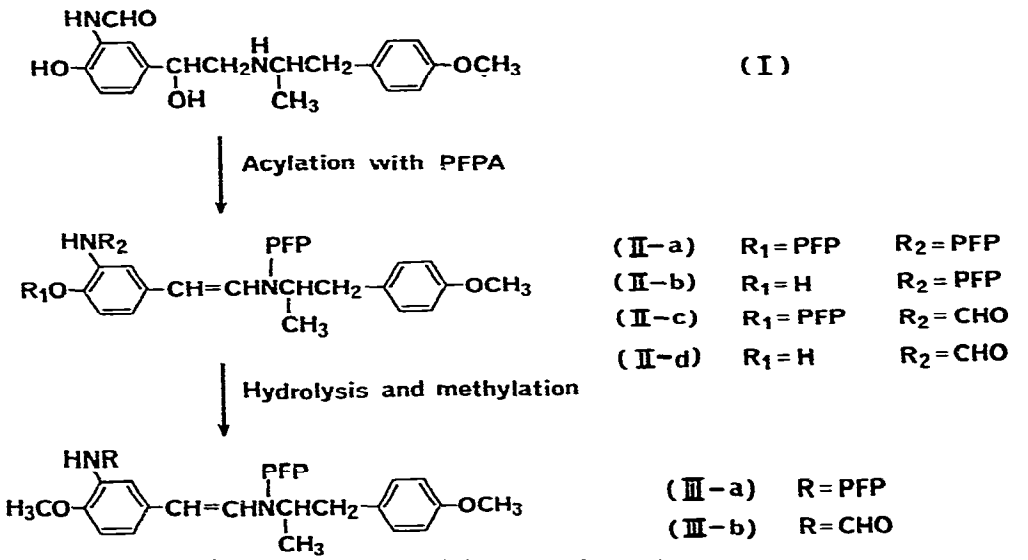

Fig. 1. Derivative-forming reaction of formoterol. Acylation of formoterol (I) with pentafluoropropionic anhydride (PFPA) in the presence of pyridine produces II-a and II-b resulting, after hydrolysis and methylation, in a single product (III-a). When formoterol is acylated with PFPA alone, the reaction is thought to proceed as I → II-c and/or II-d → III-b.

these derivatives was affected by the temperature of the sample; it also varied with the time course.

When formoterol was acylated with PFPA in the presence of pyridine and injected into a GC column (OV-1 3%, oven temperature 220°C), a sharp peak at the retention time of 1.7 min and a broad peak at 2.6 min were obtained. MS analysis revealed that the former peak was the tris-PFP derivative with a molecular ion at *m/z* 736 (Fig. 1, II-a) and the latter was the bis-PFP derivative with a molecular ion at *m/z* 590 (Fig. 1, II-b). Thus, acylation of formoterol did not produce a uniform product either. However, in alkaline solution, the phenolic ester group of II-a was preferentially hydrolyzed thereby converting II-a to II-b. Moreover, treatment of the unified derivative II-b with diazomethane gave bis-PFP-methyl formoterol (Fig. 1, III-a) which produced a well-shaped peak on GC. The derivative, dissolved in ethyl acetate, was stable for at least five days at room temperature. At the acylation step of I to II, the benzylic hydroxy group was lost; this type of hydroxy group of catecholamines has been acylated with PFPA [4]. Whether the loss of the hydroxy function occurs during the reaction, or thermally in the GC—MS system, is uncertain; however, dehydroxylation by perfluoroacylation has been reported by Knapp et al. [5]. The optimal acylation condition is shown in Fig. 2 and was verified by selected ion monitoring. When 10 μl of pyridine were added to the reaction mixture, recovery of III-a was highest and the yield reached a plateau after 30 min at room temperature (ca. 22°C). In the absence of pyridine, the yield of III-a was less than 10% of that in the presence of 10 μl of pyridine. We examined the side-reaction when pyridine was absent by thin-layer chromatography (TLC). Formoterol (20 μg) was reacted with PFPA without adding pyridine, hydrolyzed and methylated as described above and then subjected to TLC on silica gel 60 F_{254} plates (Merck, Darmstadt, G.F.R.), using the solvent system *n*-hexane—ethyl acetate—benzene (5:2:2). The product gave a major spot of

R_F 0.62 with a minor spot of R_F 0.74; acylation of formoterol with 10 µl of pyridine gave a major spot of R_F 0.74. The derivatives of R_F 0.74 and 0.62 were, as was suggested from mass spectra, III-a and the mono-PFP-methyl derivative (Fig. 1, III-b), respectively. Thus, pyridine causes deformylation and the main route of reaction without pyridine is thought to be I → II-c and/or II-d → IIIb.

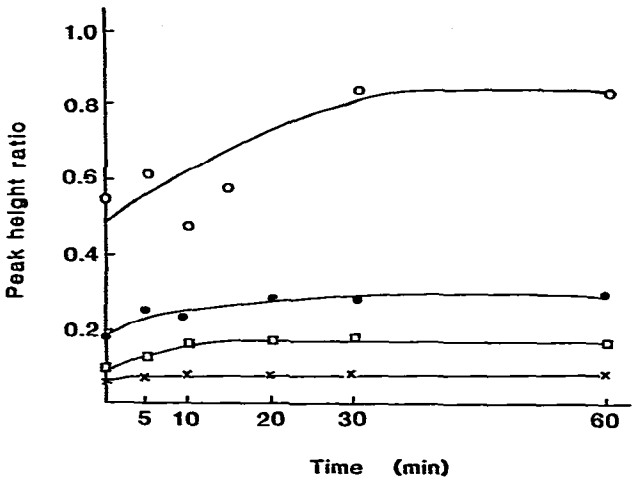

Fig. 2. Peak height ratio of III-a formed to internal standard versus the acylation time course. Acylation was at room temperature with 50 µl (●), 10 µl (○), and 2 µl (□) of pyridine and without pyridine (X). The mixture was admixed with acylated deuterium-labelled formoterol and analyzed by GC–MS as described in the text.

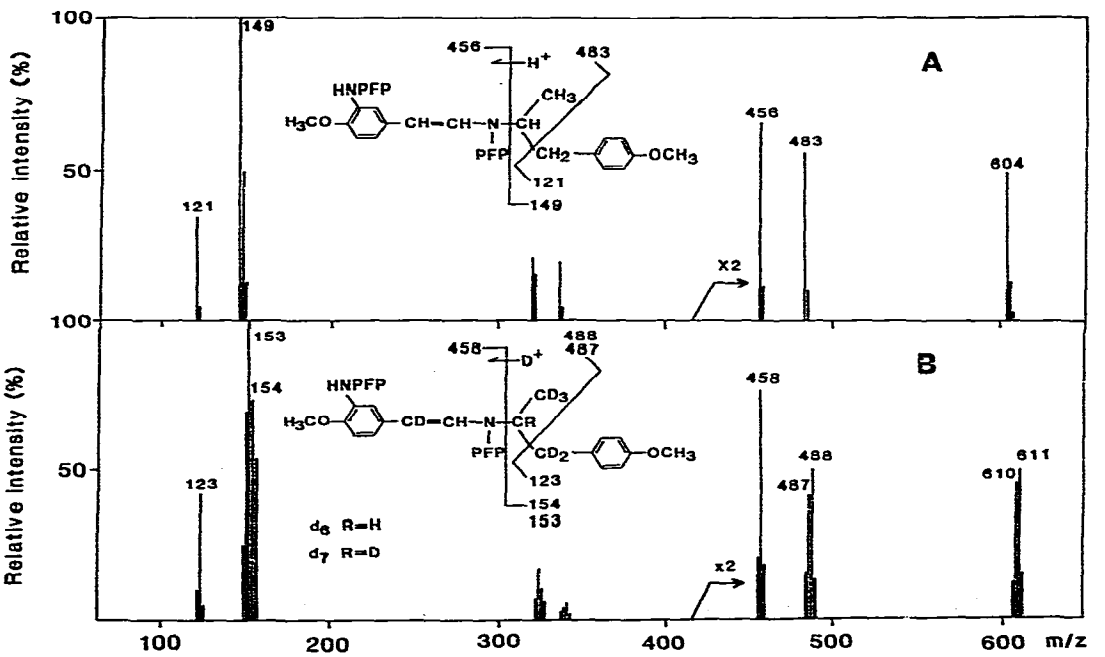

Fig. 3. Mass spectra of III-a (A) and of deuterium-labelled formoterol (B).

Fig. 3 shows the mass spectra of III-a and of deuterium-labelled formoterol. The molecular ions at *m/z* 604 were used for quantitation with selected ion monitoring. Although the ions at *m/z* 483, 456 and 149 were more intense than that at *m/z* 604, the latter was chosen for analysis because background ions at *m/z* 604 were much smaller than the others. As internal standard, we monitored the molecular ions from [$^2H_7$]formoterol at *m/z* 611. Initially, the acylation of formoterol was examined using trifluoroacetic anhydride (TFAA) as the acylating reagent; exactly the same acylation pattern as with PFPA was obtained. However, the molecular ions of the bis-TFA-methyl derivative (*m/z* 504) coincided in mass number with high background ions rising from the dimethyl silicone phase of the GC column [6], resulting in an extreme reduction of analytical sensitivity.

Assay procedure

The extraction of formoterol with ethyl acetate was pH-dependent. Urinary formoterol was extracted efficiently at pH 8—9 and remained about 90% unextracted in the aqueous layer at pH values below 5 (Fig. 4). Therefore, we extracted formoterol under alkaline conditions and back-extracted with diluted hydrochloric acid. To simplify the procedure of making the urine or the acid layer alkaline, we added excess sodium hydrogen carbonate and achieved a good result. The pH values at each step are listed in Table I with the recovery of urinary formoterol determined by radioactive counting. After the three-step extraction, the extract was derivatized for GC–MS by the method described above. As shown in Fig. 5, no significant interference was observed on the chromatograms obtained from control human urine and human urine to which 25 ng/ml formoterol and 200 ng of internal standard had been added. About 10% of the added [^{3}H]formoterol was lost by back-extraction with hydrochloric acid (Table I), and when this procedure was omitted, no interfering peaks were seen on the chromatograms. However, the background level increased with repeated sample injection, necessitating the occasional cleaning of the column and the ion source when a large number of samples was run.

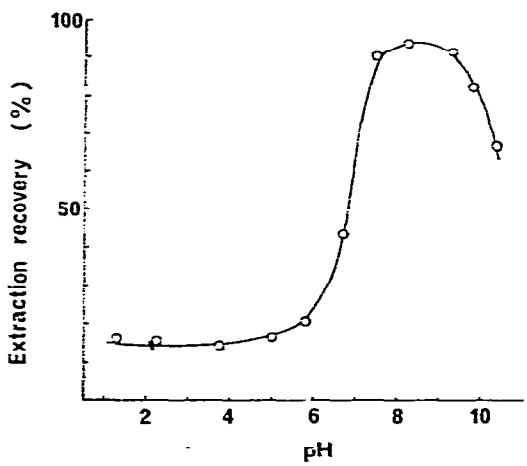

Fig. 4. Effect of pH on the extraction of formoterol from urine.

TABLE I

RECOVERY TEST OF [^{3}H]FORMOTEROL FROM URINE

The recoveries are expressed as the mean ± S.E.M. from three experiments.

	pH of aqueous layer	Recovery of [^{3}H]formoterol (%)
Initial extraction with ethyl acetate	8.1	94.0 ± 0.2
Back-extraction with hydrochloric acid	1.3	84.8 ± 0.6
Re-extraction with ethyl acetate	8.0	83.1 ± 1.3
After derivatization	—	57.7 ± 1.8

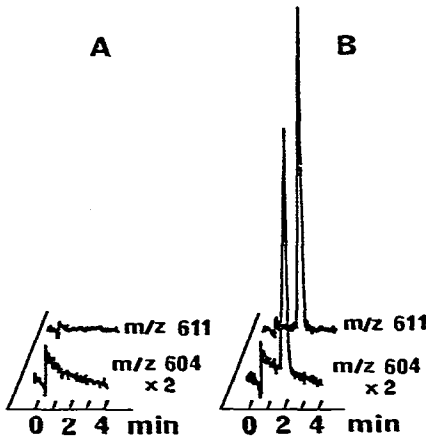

Fig. 5. Chromatograms obtained from (A) control human urine, and (B) human urine spiked with 25 ng/ml of formoterol and 200 ng of internal standard.

The precision of our method at formoterol concentrations from 0 to 100 ng/ml is shown in Table II. Regression analysis of these data gave a slope of 0.013, an intercept of 0.032 and a correlation coefficient of 0.9985. As the internal standard contained a small percentage of protium formoterol, the calibration graph did not pass through the origin. At a concentration of 5 ng/ml, the formoterol peak was about three times higher than in the control; this concentration apparently represents the limit of detection. The signal-to-noise ratio at that concentration was about five.

Specificity

Since deformylation and dehydration of formoterol occur during analysis, deformylated or dehydrated formoterol can be expected to decrease the specificity of the method if these compounds are present in the sample as metabolites. Our study in which we used [^{3}H]formoterol in rats and dogs showed that the only major metabolite was a highly polar conjugate; it was not extracted with ethyl acetate at any pH value examined. However, further study on the metabolism of formoterol in humans is necessary.

Application of the method

The nature of conjugated formoterol was investigated using the present

TABLE II

PRECISION AND REPRODUCIBILITY OF THE METHOD

The results are from four experiments.

Standard concentration of formoterol (ng/ml)	Ratio of peak height of formoterol to peak height of internal standard		C.V. (%)
	Mean	± S.E.M.	
0	0.032	0.004	22.2
5	0.093	0.003	6.0
10	0.157	0.006	7.9
25	0.350	0.006	3.3
50	0.684	0.011	3.2
100	1.300	0.029	4.5

TABLE III

ANALYSIS OF URINE SAMPLES WITH AND WITHOUT ENZYMIC HYDROLYSIS AFTER ORAL ADMINISTRATION OF FORMOTEROL TO RATS, DOGS AND HUMANS

	Urinary concentration of formoterol (ng/ml)						
	Rat samples		Dog samples		Human samples		
	A	B	A	B	A	B	C
Control	69.8	61.6	250.7	227.0	19.5	16.8	6.8
Plus β-glucuronidase (9000 units)	334.6	343.0	676.1	757.9	41.8	34.3	19.9
Plus arylsulphatase (7500 units)	67.2	54.5	221.7	262.7	19.5	13.2	7.0

method. For the treatment of asthma, various sympathomimetic amines have been synthesized, taking adrenaline as a prototype [7]. Among these drugs, isoproterenol, in which the catechol function is not substituted, was conjugated with glucuronic acid in the rat and with sulphate in the dog and human beings [8—10]. As pointed out by Conway et al. [9], this conjugation pattern is the same as that of orally administered adrenaline or some other related catecholamines. However, salbutamol, which is a salicyl alcohol derivative, instead of catechol, was conjugated with glucuronic acid in the dog and the rat and with sulphate in human beings [11,12]. A non-catechol β-stimulant, procaterol, was primarily conjugated with glucuronic acid in all three species [13,14]. It was of interest for us to determine whether these species utilize glucuronidation or sulphation in the metabolism of the non-catechol β-stimulant formoterol. As shown in Table III, formoterol was conjugated with glucuronic acid in rats, dogs and humans.

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REFERENCES

1 K. Murase, T. Mase, H. Ida, K. Takahasi and M. Murakami, Chem. Pharm. Bull., 25 (1977) 1368.
2 H. Ida, Arzneim.-Forsch., 26 (1976) 839.
3 K. Murase, T. Mase, H. Ida, K. Takahashi and M. Murakami, Chem. Pharm. Bull., 26 (1978) 1123.
4 E. Änggård and G. Sedvall, Anal. Chem., 41 (1969) 1250.
5 D.R. Knapp, T.E. Gaffney, R.E. McMahon and G. Kiplinger, J. Pharmacol. Exp. Ther., 180 (1972) 784.
6 D.M. Taylor, Finnigan Applications Tips No. 54, Finnigan Corp., Sunnyvale, CA, June, 1974.
7 K.N. Leifer and H.J. Witting, Ann. Allergy, 35 (1975) 69.
8 G. Hertting, Biochem. Pharmacol., 13 (1964) 1119.
9 W.D. Conway, H. Minatoya, A.M. Lands and J.M. Shekosky, J. Pharm. Sci., 57 (1968) 1135.
10 M.E. Conolly, D.S. Davies, C.T. Dollery, C.D. Morgan, J.W. Paterson and M. Sander, Brit. J. Pharmacol., 46 (1972) 458.
11 L.E. Martin, J.C. Hobson, J.A. Page and C. Harrison, Eur. J. Pharmacol., 14 (1971) 183.
12 C. Lin, Y. Li, J. McGlotten, J.B. Morton and S. Symchowicz, Drug Metab. Dispos., 5 (1977) 183.
13 T. Shimizu, H. Mori, E. Tabusa, S. Morita, G. Miyamoto, Y. Yasuda and K. Nakagawa, Xenobiotica, 8 (1978) 349.
14 T. Shimizu, H. Mori, E. Tabusa and K. Nakagawa, Xenobiotica, 8 (1978) 705.