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Analysis of [^{14}C]-saterinone and its metabolites in dog plasma, urine and bile by high-performance liquid chromatography with radioactivity and fluorescence detection and by mass spectrometry

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Abstract: An HPLC method was developed for the direct on-line radioactivity determination of [^{14}C]-saterinone and its metabolites in biological canine fluids after intravenous and intraduodenal administration. After direct injection of 200 µl of sample, the metabolites were separated on a semi-preparative reversed-phase column. The metabolites were identified by HPLC reference standards, enzymatic hydrolysis and mass spectrometry. Besides a small amount of unchanged saterinone, six metabolites could be detected, both in bile and urine. The main fraction (about 80–90% of the sum of detected metabolites) contained the phase II metabolites of saterinone, the sulphate and glucuronide. Ring hydroxylated saterinone and three metabolites that were not identified made up about 1–4% each. In plasma, only the major compounds could be detected because of the lower absolute concentrations. The metabolic pathway of saterinone in dogs was elucidated and compared with other species. Results from previous studies concerning a first-pass metabolism could be confirmed.

Keywords: *[^{14}C]-saterinone; metabolism; dog bile; dog urine; semi-preparative LC; on-line radioactivity detection; enzymatic hydrolysis.*

Introduction

Saterinone, 1-[(4-cyano-1,2-dihydro-6-methyl-2-oxo-pyridinyl-5)-phenoxy]-3-[4-(2-methoxy-phenyl)-piperazinyl-1]-propan-2-ol (a, Fig. 1), is a novel drug for treatment of chronic cardiac insufficiency and is administered as the racemate in clinical studies [1]. Previous studies on the biotransformation of saterinone in animal and man have shown that saterinone was metabolized extensively. The route of excretion was mainly biliary.

The investigations described here were carried out to obtain detailed information on the excretion and metabolism of saterinone in an animal species, subjected to toxicological investigations. After intraduodenal (i.d.) and intravenous (i.v.) administration of radio-

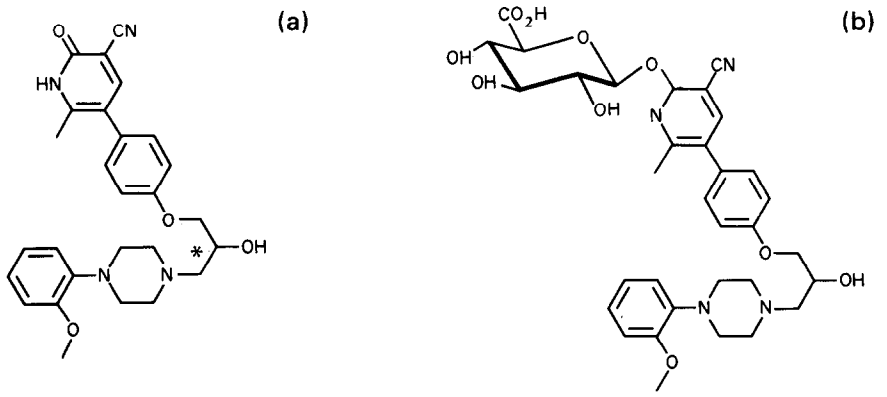

Figure 1
Structures of (a) [^{14}C]-saterinone and (b) of side chain glucuronidated saterinone. The asterisk indicates the position of [^{14}C]-labelling.

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labelled saterinone to dogs, plasma, urine and bile samples were collected and the radioactivity was measured. After chromatographic analyses the metabolites were identified by an on-line radioactivity detector, reference substances for the HPLC system, enzymatic hydrolysis and mass spectrometry.

The metabolic pathway of saterinone in the dog and possible correlations to human metabolism were proposed based on the results obtained.

Experimental

Reagents

Sulphatase type H-2 from *Helix pomatia* and β-D-glucuronidase type VII-A from *E. coli* (Sigma, Deisenhofen, Germany) were used. [^{14}C]-labelled saterinone [specific activity: 0.18 GBq mmol^{-1} base, radiochemical purity >99% (by radio-TLC)] and side chain glucuronidated-saterinone (according to ref. 2) were synthesized in our biochemical and chemical departments. Ring-hydroxylated saterinone [purity >95% (by HPLC)] was obtained as a HPLC reference standard after isolation and chromatographic purification from rabbit bile. All other chemicals used were of analytical grade.

Animal samples

Six male beagle dogs weighing 11.5–14.5 kg received an i.v. or i.d. (three dogs each) dose of 4.41 mg (1.72 MBq) in 10 ml or 8.83 mg (3.44 MBq) in 20 ml respectively of [^{14}C]-saterinone-base ethanol, propylene glycol, lactic acid and water solution (5:10:0.4:84.6, w/w/w), respectively. The catheterization of the bile, the i.d. and i.v. application and the sample collection were performed under narcosis.

Plasma samples were collected 0 (prior to administration), 0.5, 3 and 6 h after administration. In addition, bile and urine were collected in 1 h fractions up to 6 h via a catheter. All samples were stored in a refrigerator at −25°C until analysis.

Apparatus

The chromatographic system consisted of an L-6200 programmable gradient pump, an F-1000 fluorimeter with a 12 μl flow-cell (both from E. Merck, Darmstadt, Germany), an LKB 2150 UV detector with an 8 μl flow-cell (Pharmacia, Freiburg i.Br., Germany) and a Model Ramona D radioactivity detector with a 400 μl glass scintillator flow-cell (Raytest, Straubenhardt, Germany). The samples were injected by means of a Rheodyne injection valve (1.0 ml sample loop) and collected in 2 ml fractions with a Model 202 fraction collector (Abimed, Langenfeld, Germany). A 10 μm LiChrosorb select-B™ column (250 × 4 mm i.d.) (E. Merck) with a self-packed precolumn (20 × 4.6 mm i.d., 10 μm Matrex™ RP-18 — Amicon, Witten, Germany) was used.

All data were acquired and calculated with a computer system using the Ramona software version 11.2 (Raytest). It consisted of a IBM Model PS/2 70 with an 80286 processor and an 80287 co-processor (Stuttgart, Germany).

Chromatographic conditions

In Fig. 2 a scheme of the chromatographic

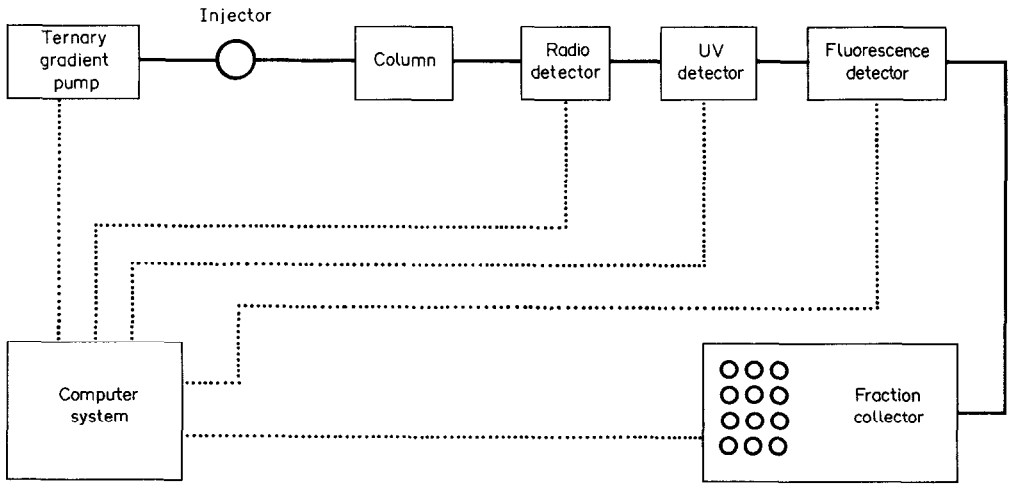

Figure 2
Schematic diagram of the HPLC system used for on-line radioactivity, UV and fluorescence detection of [^{14}C]-saterinone.

system is shown. The solvents for the gradient elution were water (eluent A) and methanol–3% aqueous acetic acid (1:1, v/v) (eluent B). The elution profile was (from 0 to 10 min) 80% A and 20% B and linear to 20% A and 80% B (from 10 to 30 min). These conditions were then maintained for 20 min. The column was reconditioned to 80% A and 20% B (from 50 to 55 min), and these conditions maintained for 10 min. The flow rate was 4 ml min^{-1}. The sample injection volume was 200 µl. The detectors were set in series, first the radioactivity detector, then the UV detector at 244 nm and finally the fluorimeter with an excitation wavelength of 345 nm and an emission wavelength of 435 nm. The total run time was 65 min. The temperature of the whole system was 22 ± 2°C.

Sample preparation and enzymatic hydrolysis

After thawing in a water bath (15°C), the plasma, urine and bile samples were centrifuged (1500*g*, 15 min). The upper layers were filtered through a 0.2 µm Anotop™ 10 filter (E. Merck) and aliquots were injected into the chromatograph.

The glucuronides were analysed according to ref. 3. To 200 µl of a thawed sample 100 µl of a 0.1 molar phosphate buffer (pH 6.8) and 100 µl β-D-glucuronidase (1000 Sigma units dissolved in 1 ml phosphate buffer) were added. The solution was mixed on a vortex for 1 min, incubated for 1.5 h at 37°C and the whole solution was injected. The sulphates hydrolysed by incubation of 200 µl of sample with 100 µl of phosphate buffer (pH 5.0) and 200 µl of sulphatase solution for 15 h under the conditions described above.

Measurement of the total radioactivity

The radioactivities of the samples were measured with a Model LS 3801 liquid scintillation counter (Beckmann Nuclear Systems Operations, Fullerton, CA, USA) for 10 min. A 2 ml volume of a mixture of Soluene-350™ tissue solubilizer (Packard Instruments, Downers Grove, IL, USA) and propan-2-ol (1:1, v/v) were added to 200 µl plasma. After shaking on a vortex for 1 min, 15 ml of Hionic–Fluor™ scintillation cocktail (Packard Instruments) were added and the radioactivity content determined. Urine (100 µl) and bile (50 µl) samples were mixed directly with 15 ml of cocktail. The measured counts were converted into dpm, the zero-counts subtracted and the results expressed as percentage of the administered dose.

Mass spectrometry

The mass spectra were obtained on a Finnigan MAT SSQ-70 quadrupole mass spectrometer (Bremen, Germany) using a direct inlet system, maintaining the temperature at 50°C for 0.5 min and then increasing to 450°C over a 2 min period. The collected fractions were evaporated to dryness. The residues were derivatized with bis-(trimethylsilyl) trifluoroacetamide (E. Merck) to give the volatile silylethers of saterinone and its metabolites. The electron-impact ionization mass spectrometer conditions were as follows: ion source temperature, 150°C; ionizing voltage, 70 eV; emission current, 200 µA; secondary electron amplifier, 1.7 kV with 10^{-8} A/V.

Calculations

The excreted amounts of each metabolite, expressed as percentage of the administered dose, were calculated from the measurements of the total radioactivities. Plasma radioactivities were expressed as percentage of the kg-dose g^{-1}.

Results and Discussion

Identification of the metabolites

The [^{14}C]-radioactivity traces for bile and urine were similar (Fig. 3a and c) and showed six main peaks, following saterinone administration: M1, M4, M2, M3, saterinone and M5. The UV chromatograms were contaminated by the matrix and are not shown here.

The decrease in the peak area of M3 and increase in saterinone after treatment with β-glucuronidase indicated that M3 was glucuronidated saterinone. After enzymatic hydrolysis with sulphatase, M3 and M2 both decreased while the saterinone peak increased correspondingly (Fig. 3b). The sulphatase used contains glucuronidase as well and for that reason take both sulphates and glucuronidates into account.

Therefore, M2 seemed to be the sulphate conjugate of saterinone. The position of conjugation was probably the isopropyl-moiety of saterinone, because the synthesized side-chain glucuronide of saterinone (b, Fig. 1) had another elution profile and excitation and emission wavelength different from the chosen wavelength. The mass spectra provided proof,

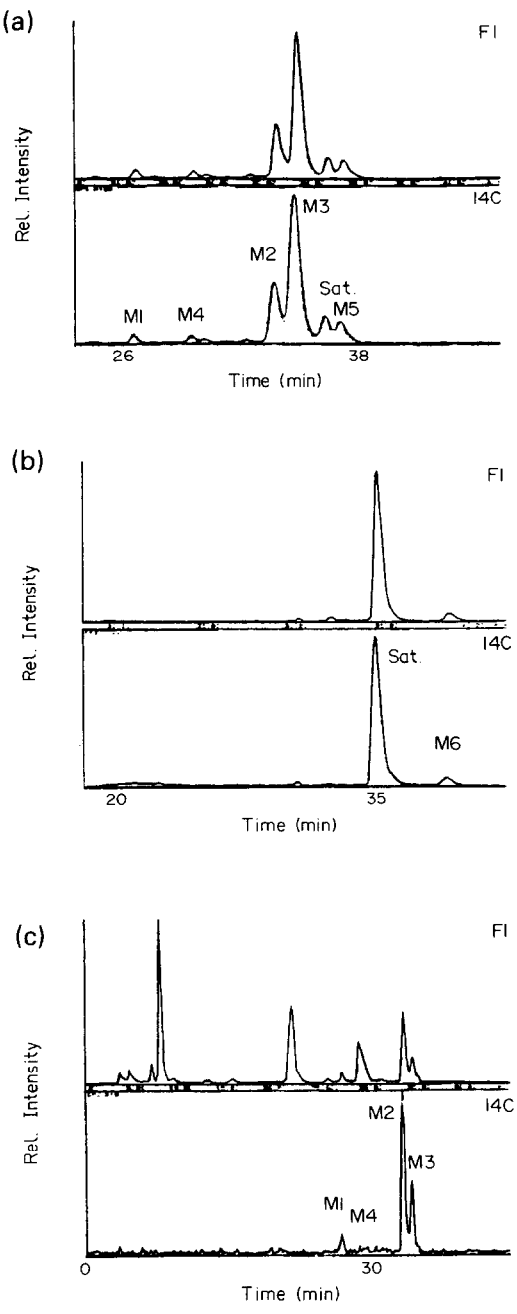

Figure 3
Fluorescence and [^{14}C]-radioactivity chromatograms of saterinone (Sat.) and its metabolites (M1–M6) obtained by injecting samples from dog no. 04 fraction 1–2 h (a) after i.d. administration of 200 μl of bile, (b) after incubation of bile with sulphatase (for conditions see text) and (c) of 200 μl of urine. Conditions: 10 μm LiChrosorb select-B™ column (250 × 4 mm i.d.); gradient elution with water and methanol–3% aqueous acetic acid (see text) at 4 ml min^{-1}.

that the main peak in the chromatogram after enzymatic hydrolysis with sulphatase was saterinone (Fig. 4a and b).

M6 was obtained mainly after enzymatic hydrolysis (Fig. 3b). It accounted for about 2–4% of the administered dose and was eluated in the chromatogram before M2 (Fig. 3a). The increase in the M6-peak after addition of the standard from rabbit bile (see above) and the mass spectra obtained showed (Fig. 4c) that M6 was ring-hydroxylated saterinone.

The absolute concentrations of M1, M4 and M5 were too low for mass spectra to be obtained. Therefore, the substances were tested for their extractability with diethyl ether and stability against acid and alkali to obtain information about their relative polarity. The amounts of all three metabolites fell below the detection limits after incubation with sulphatase. At the same time M6 increased and a new peak occurred in the chromatogram (at about 39 min) (Fig. 3a). From acidified (citric acid) bile samples no radioactivity could be extracted with diethyl ether, while from alkaline (sodium bicarbonate) samples only saterinone was extracted. After incubation of bile samples (100 μl) with 0.1 M phosphoric acid (50 μl) and 0.1 M sodium hydroxide solution (50 μl) at 50°C for 1 h, M4 decreased, while the amounts of M1 and M5 were unchanged. From these results it could be concluded that M1 and M5 were probably phase II metabolites having higher polarities than M2 and M3 (see the elution order Fig. 3a) and that one of them could be the glucuronide of M6.

Quantitative results

The quantitative results of the determination of saterinone and its metabolites are summarized for bile and urine samples in Table 1, for plasma samples in Table 2. The amounts for M2 and M3 excreted were combined if the chromatographic resolution was not sufficient. The accumulation [^{14}C]-radioactivities excreted in bile and urine samples are presented graphically in Fig. 5.

After i.v. administration 61.1, 64.2 and 46.9% of the administered dose was excreted with bile and 8.3, 5.0 and 4.2% with urine. The main amounts were measured in the first three fractions. The phase II metabolites M2 and M3 accounted for 40–56% of the administered dose in bile and 3.2–6.2% in urine, corresponding to 80–90% of the sum of the detected metabolites both in bile and urine. Unchanged saterinone, M1, M4, M5 and M6 made up about 1–4% each of the detected metabolites.

The percentages of the administered dose recovered after i.d. application were 52.4, 25.6 and 53.0 for bile, and 9.6, 5.2 and 3.3 for urine.

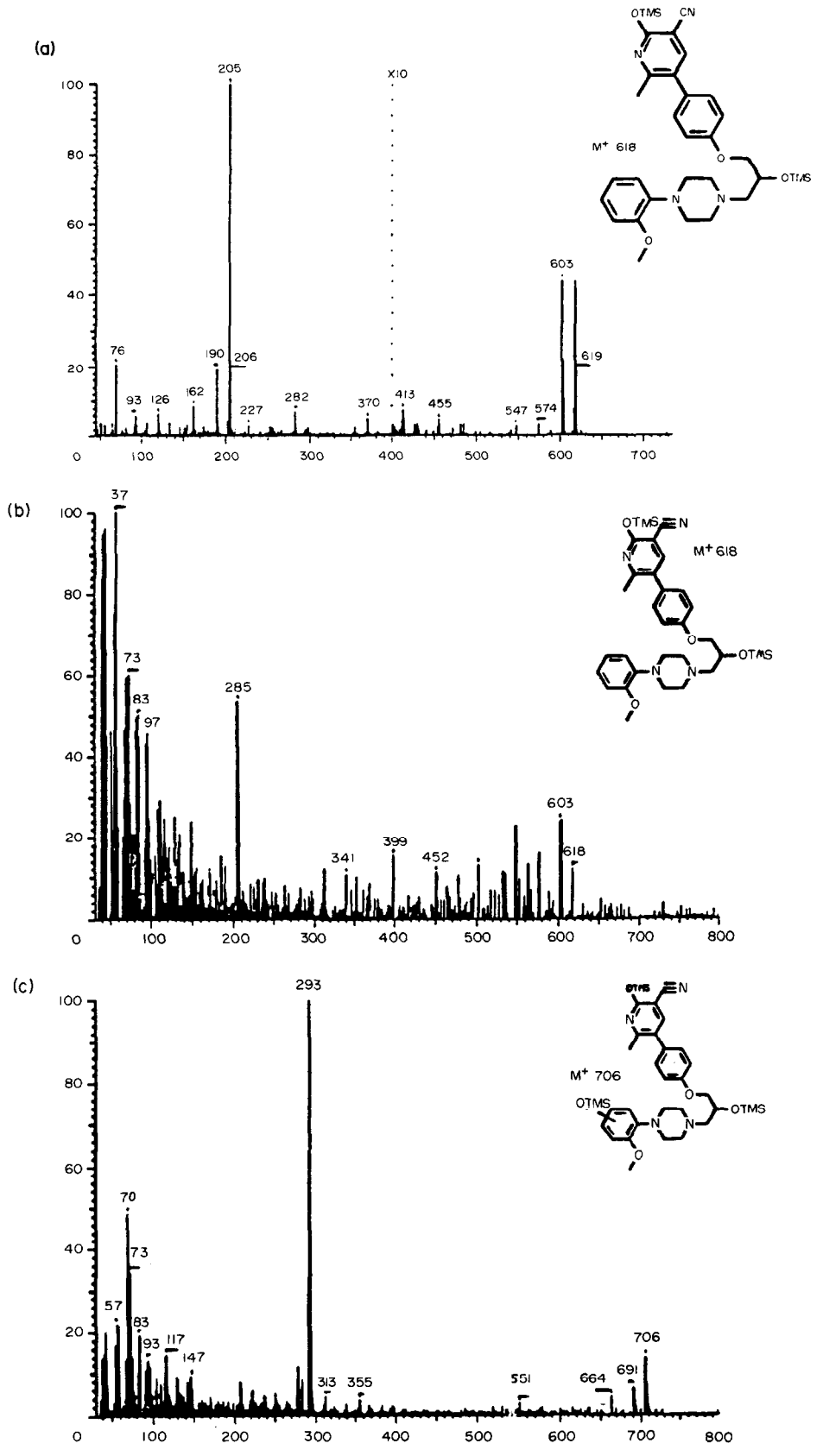

Figure 4
Mass spectra of (a) saterinone, (b) the main peak after sulphatase incubation (see Fig. 3b) and (c) M6.

Table 1
Bile and urine contents of saterinone and its metabolites determined after HPLC with on-line radioactivity detection and external measurement of the total radioactivity by a liquid counter. Each amount was expressed as the percentage of the administered activity

Dog		M1	M4	M2	M3	Sat.	M5	M6	Sum
					(% of administered dose)				
Bile									
i.v.	01	2.22	2.13	24.30	23.10	1.00	0.34	8.01	61.10
	02	1.83	2.41	55.72		3.97	0.29	n.d.	64.22
	03	1.20	1.50	10.17	29.85	2.96	1.22	n.d.	46.90
i.d.	04	1.24	1.92	13.44	28.50	4.32	2.71	0.24	52.37
	05	1.18	1.01	10.68	11.34	0.59	0.72	0.09	25.61
	06	3.50	3.41	44.17		n.d.	n.d.	1.89	52.97
Urine									
i.v.	01	0.83	0.67	4.84	1.40	0.34	n.d.	0.26	8.34
	02	0.52	0.54	2.17	1.55	n.d.	n.d.	0.25	5.03
	03	0.27	0.28	1.93	1.31	0.30	n.d.	0.06	4.15
i.d.	04	0.87	0.80	5.61	1.69	0.33	n.d.	0.27	9.57
	05	0.56	0.35	1.91	1.55	0.54	n.d.	0.25	5.16
	06	0.56	0.57	1.00	0.67	0.20	n.d.	0.34	3.34

Table 2
Plasma concentrations of saterinone, M2 and M3 determined after HPLC with on-line radioactivity detection and external measurement of the total radioactivity by a liquid counter. Each amount was expressed as the percentage of the kg-dose g^{-1}

Dog		Time (h)	M2	M3	Sat.	Sum
			(% of kg dose g^{-1})			
i.v.	01	0.5	0.006	0.035	0.036	0.077
		3.0	0.004	0.015	0.013	0.032
		6.0	n.d.	0.002	0.002	0.004
						0.113
	02	0.5	0.002	0.012	0.026	0.040
		3.0	n.d.	0.003	0.005	0.008
		6.0	n.d.	0.002	0.003	0.005
						0.053
	03	0.5	0.010	0.004	0.029	0.081
		3.0	0.002	0.006	0.006	0.014
		6.0	n.d.	0.001	0.002	0.003
						0.098
i.d.	04	0.5	0.007	0.031	0.015	0.053
		3.0	0.001	0.004	0.003	0.008
		6.0	n.d.	0.001	0.001	0.002
						0.063
	05	0.5	0.007	0.019	0.005	0.031
		3.0	0.001	0.002	0.002	0.005
		6.0	n.d.	n.d.	n.d.	
						0.036
	06	0.5	0.007	0.019	0.005	0.031
		3.0	0.001	0.002	0.002	0.005
		6.0	n.d.	n.d.	n.d.	
						0.036

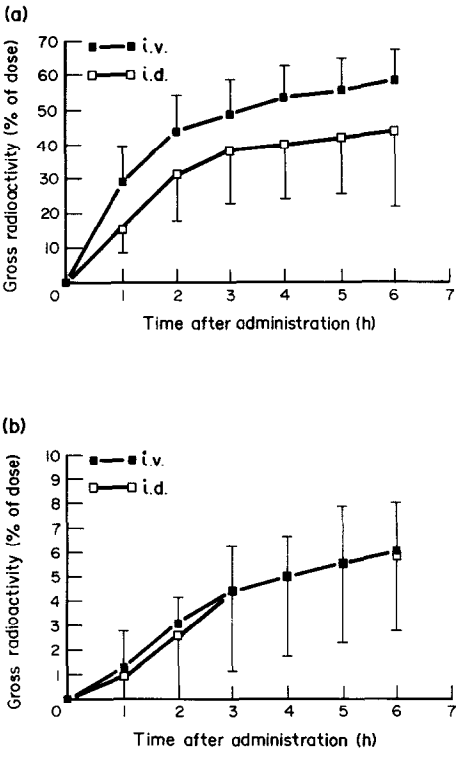

Figure 5
Mean accumulated [^{14}C]-levels in (a) bile and (b) urine samples after administration of [^{14}C]-saterinone in dogs.

Even after this route of administration, results comparable with i.v. administration were obtained, except that the peak values were found in bile in the 0–1 h fractions. M2 and M3, the main metabolites, made up about 80% of the sum of the detected metabolites both in bile and urine, whereas unchanged saterinone and the other metabolites amounted to 0–5% each.

The total bile excretion of dog 05 was 25.6% of the administered dose and thus remarkably low. In the same dog the total urine excretion was 5.2% of the administered dose and comparable to that in the other dogs. No satisfactory explanation could be given for the low amount, as sample collection was correct. However, the mean excreted radio-

activity in bile of 43.7% ± 15.6% after i.d. administration was comparable to the mean of 57.4% ± 9.2% after i.v. administration.

In plasma, only M2, M3 and saterinone could be detected because of the lower absolute concentrations. The highest values for the total radioactivity were measured after 0.5 h and equalled 0.077, 0.040 and 0.081% of the kg-dose g^{-1} for i.v. administration and 0.053, 0.019 and 0.031% for i.d. administration. The values for i.d. and i.v. administration were comparable due to the twofold i.d. dose and despite plasma-level time-curves based on only three measured time points. The saterinone fraction of the detected metabolites, e.g. after 0.5 h, amounted to about 50 and 22% of the total plasma-radioactivity after i.v. and i.d. administration, respectively. These results agree with previous investigations, where the saterinone plasma levels of dogs after i.d. application were about half those after i.v. application.

Chromatography

Recently, an isocratic HPLC method was described for the quantitation of saterinone in biological fluids [4]. This method provided insufficient separation for determining saterinone together with its metabolites, even when the polarity of the eluent was increased. Therefore, a gradient elution was attempted to improve resolution. By using water–methanol–acetic acid in different proportions, saterinone and six metabolites could be separated and analysed in a single run in both bile and urine samples (Fig. 3a and c). The flow rate was set at 4 ml min^{-1} to achieve the shortest retention times possible. An injection volume of 200 µl of sample was chosen to obtain sufficient signal to noise ratios in the radioactivity detector.

After enzymatic hydrolysis the entire solution of 400 or 500 µl was injected with no loss of resolution (Fig. 3b), to obtain [^{14}C]-signals comparable to those of pure samples. Every 15–20 injections the pre-column had to be repacked, because matrix components contaminated the semi-preparative column and interfered with the separation.

Excretion and metabolism

The main route of saterinone excretion in dogs was found to be via biliary elimination. Less than 10% of the administered dose was excreted in urine. These results were in good agreement with the saterinone elimination in man and other animal species, where about 5–10% of the oral dose was eliminated via urine. The metabolic pattern in dog differed from that in previous investigations with other species in the quantitative amounts of the individual metabolites. The main metabolites detected in dog were the glucuronide and sulphate conjugates of saterinone (up to 90% of the metabolites), whereas in man saterinone glucuronide, ring-hydroxylated saterinone and its glucuronide could be determined in about the same proportions. The ring-hydroxylated metabolite, which is the major metabolic route of saterinone in rabbits, seemed characteristic for the methoxyphenyl-moiety and was also detected in related compounds [5, 6]. The hydroxy- group of the isopropyl-moiety was preferred for phase II reactions [7, 8]. In some cases, only glucuronic acid was used for conjugation in man, while, in the dog, sulphate conjugation was also present [9]. The three metabolites, M1, M4 and M5, which were determined in amounts of about 1–4% of the sum of detected metabolites, played a minor role in the metabolic pattern and were not identified.

As expected, the relative amounts of unchanged saterinone were higher in plasma than in bile and urine. The saterinone fractions after 0.5 h were about twice as large after i.v. than after i.d. administration. These results indicated a higher metabolism of saterinone after the oral route of administration than after i.v. administration. In addition the high amount of biliary excretion of saterinone indicated a first-pass metabolism [10], confirming results of a previous study.

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