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PHARMACOKINETIC STUDIES OF A POTENT GLUCOCORTICOID (BUDESONIDE) IN DOGS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

To evaluate the pharmacokinetic profile of a new highly potent non-halogenated glucocorticoid [^{3}H]-budesonide (a one to one mixture of two epimers, A and B) in Beagle dogs, an analytical method specific for budesonide was developed. Plasma samples were extracted with methylene chloride followed by high-performance liquid chromatography on a Spherisorb ODS column with water–ethanol (60:40, V/V) as the mobile phase. By analysis of simulated plasma samples an overall recovery of 95.4 ± 4.2 (X ± S.D.) % was obtained. [^{3}H]-budesonide was administered both iv and po at two doses (23 and 230 nmol/kg). At the low dose $t_{1/2\beta}$ ranged from 1.9–2.1 h, Vd_β from 5.2–7.7 l/kg, Cl from 1.8–2.5 l/h/kg and systemic bioavailability from 9.2–16.2%. The corresponding figures at the high dose were 1.8–3.0 h, 2.5–6.9 l/kg, 1.0–1.6 l/h/kg and 18.3–18.8%. A separate iv study with epimer A (23 nmol/kg) was made and the corresponding figures were 1.3 h, 4.8 l/kg, and 2.6 l/h/kg. Of the excreted radioactivity about 80% was recovered in faeces irrespective of route of administration in all studies.

INTRODUCTION

Glucocorticoids with high local anti-inflammatory activity but low systemic effects are of interest both in the treatment of diseases of the respiratory tract and the skin. In screening tests for glucocorticoids, a non-halogenated compound was found with local anti-inflammatory potency comparable to that of fluocinolone acetonide but the systemic glucocorticoid activity was 4–7 times lower [1]. This new compound, budesonide, is a mixture of two epimers both seeming to have the same pharmacological profile [1]. The improved ratio between local and systemic effects may be due to rapid and extensive enzymatic inactivation after absorption. To elucidate this, the pharmacokinetic profile of budesonide was evaluated in dogs, a species used in pharmacological and toxicological investigations of the compound. To analyze the expected low plasma concentrations an analytical method of high sensitivity should be required. The selected method also should be of high selectivity due to the expected biotransformation of the drug.

In previous studies with other potent glucocorticoids the problem with low concentrations was overcome by the use of radioactively labelled compounds. Various degrees of specificity were obtained by measuring the drug, e.g. as total radioactivity [2, 3] as extractable radioactivity [4] and in a few cases by the use of more specific methods such as radioimmunoassay [5]. Since the selectivity of these methods varied, adequate comparisons of the pharmacokinetics of the drugs can hardly be made.

Since the use of extraction methods, column chromatography or radioimmunoassay methods are either inadequate or laborious, high-performance liquid chromatography (HPLC) has become an attractive method. HPLC with U.V.-detection was used to analyze e.g. prednisone, prednisolone, dexamethasone and cortisol in plasma [6–9]. In these studies the detection limit was in the range 25–250 nmol/l (10–100 ng/ml). However, in our case with budesonide, measurements of total radioactivity in plasma indicated concentrations as low as 0.1 nmol/l (0.05 ng/ml). In order to achieve high separation capacity, high quantification ability, high sensitivity and acceptable time for analysis, HPLC in combination with tritiated drug was used.

MATERIALS AND METHODS

Chemicals. Tritiated budesonide (16α,17α-(22R,S)-propylmethylenedioxy-pregna-[1,2-^{3}H]-1,4-diene-11β,21-diol-3,20-dione) was obtained from the Radiochemical Centre, Amersham, England. Two batches of budesonide were used with the specific activities 4.2 and 16.0 Ci/mmol, respectively. According to HPLC (column: μ-Bondapak C_{18}, mobile solvent: ethanol–water (43:57, V/V), the radiochemical purity of the compound from the two batches were >98% and >92%, respectively. The epimeric ratios (A:B) were 57:43 and 51:49, respectively. Tritiated epimer A was obtained from [^{3}H]-budesonide by preparative liquid chromatography. The specific activity was 3.7

Ci/mmol, the radiochemical purity >94% and the epimeric ratio 98:2 (A:B). Non-labelled budesonide as well as the separate epimers were supplied by A. Thalén, AB Draco. Water and p.a. Merck methylene chloride were distilled, ethanol was of spectroscopic grade and other chemicals were of p.a. quality.

HPLC. The liquid chromatograph was a Waters Associates involving a M 6000 A pump, a U6K injector system and a M 440 U.V. detector (254 nm) connected to a Varian A25 recorder. The outlet of the detector was interconnected with a Gilson fraction collector, model B 100 with a timer drop counter model TDCA, via a teflon capillary tube. The volume of tube was about 100 μl. The collector was used in the drop calculation mode and fractions in the range 0.3–1.0 ml were collected.

Columns of 3.9 mm inner diameter and 100 mm long were packed with Spherisorb ODS (10 μm). The particles were suspended in a mixture of tetrabromethane and tetrachlorethylene and a Haskel pump, model DST-150A, was used for packing. HETP (height equivalent of a theoretical plate) values for the corticosteroids studied were about 250 μm which was about twice as high as the values obtained for smaller molecules like benzene. These discrepancies have been discussed elsewhere [10].

One hundred μl samples or standards, dissolved in the mobile phase, were injected by a SGE syringe, model 100A-RN-GSG. Using UV detection a standard deviation for injections of about 2% was obtained. The flow rate in all experiments was 0.5 ml/min. The mobile phase was composed of a mixture of water–ethanol (60:40, V/V). The experiments were carried out at ambient temperature.

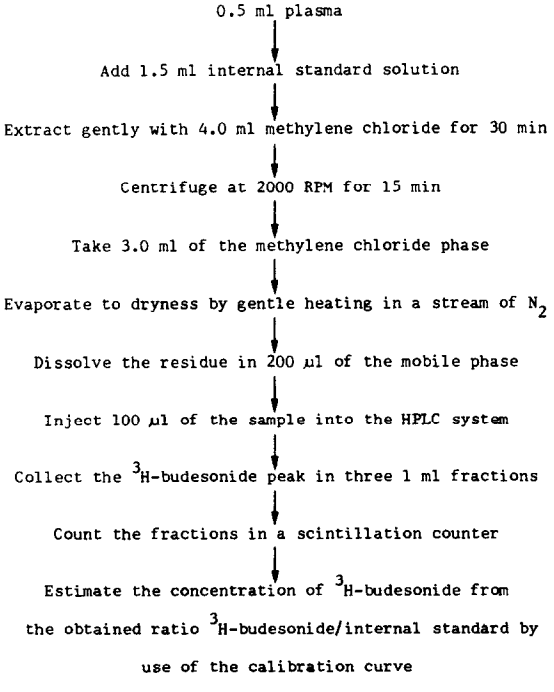

Fig. 1. Assay method for [^{3}H]-budesonide in plasma.

Measurement of radioactivity. Radioactivity was measured in a liquid scintillation counter Packard Tricarb, model 2425, using 10 ml Instagel (Packard) which was added to the chromatography fractions or plasma samples. The counting efficiency was estimated by the external standard channels ratio procedure. Standard deviations for counting were in the range of 1–5% depending on the radioactivity of the sample.

[^{3}H]-Budesonide assay. The assay method developed for unchanged [^{3}H]-budesonide in dog plasma is schematically shown in Fig. 1. The internal standard solution was prepared by diluting 250 μl of an ethanolic epimer B (nontritiated) solution (2 mg/ml) to 25.0 ml water. Quickfit glass (borosilicate) was used. The samples were protected from light throughout the entire procedure. During the extraction the shaking was performed gently to avoid emulsion formation. Residues obtained after evaporation were not allowed to stand in dryness.

Standardization procedure and recovery experiments. Standardization was performed with each analysis. Selected concentrations of [^{3}H]-budesonide in plasma in the range 0.1–10 nmol/l were prepared. A standard curve was made after calculation of the ratio of radioactivity of the [^{3}H]-budesonide peak to the area of the internal standard U.V. peak obtained during chromatography. By comparing the radioactivity obtained by direct scintillation counting with that from the assay procedure, recovery estimations could be performed.

Animal experiments. Female Beagle dogs, weighing 9–16 kg, were used in these experiments. For both intravenous and oral studies, two doses were investigated, 23 and 230 nmol/kg (10 and 100 μg/kg). With epimer A, only one intravenous study was performed (23 nmol/kg). In all experiments, the radioactive dose was 50 μCi/kg). Budesonide as well as the epimer A were dissolved in ethanol and these solutions were diluted with physiological saline, so that the final concentration of ethanol was about 30% (w/w). The orally dosed animals received the drug solution in gelatine capsules. The intravenously administered animals received 0.20 ml of drug solution per kg body mass. After administration, the dogs were housed separately in metabolism cages with food given once a day (~4 h after administration) and with free access to water. Blood samples were taken into heparinized test tubes at 0, 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, and 8 h after administration. Plasma was prepared. Urine and faeces were sampled during 24 h periods for three days after administration. All samples were kept in a frozen state until assayed.

Determination of radioactivity in biological samples. Aliquots from urine samples were dissolved in Instagel® and the radioactivity was determined by liquid scintillation counting using a Packard Tri-Carb liquid scintillation spectrometer. The faeces samples were homogenized with two parts of distilled water and then aliquots of these slurries were combusted as well

as plasma samples in a Packard Sample Oxidizer 306 before they were subjected to analysis for radioactivity. Scintillation solution was Monophase®.

Kinetic calculations. The terminal slope (β) of the plasma concentration time curve was determined by linear regression analysis. The area under the plasma concentration time curve (AUC_{0-8h}) was determined by the trapezoidal rule. The remaining area ($AUC_{8-\infty h}$) was calculated as $Cp_{(8h)}/\beta$. The plasma clearance (Cl) of the drug was calculated from intravenous studies as dose/AUC_{tot} and the distribution volume (Vd_{β}) as Cl/β. The systemic availability was calculated as the ratio between $AUC_{0-\infty h}$ obtained after oral to intravenous administration.

RESULTS AND DISCUSSION

Analytical studies

Preliminary determinations of the distribution ratio for budesonide between water and chlorinated hydrocarbons and between serum and methylene chloride gave values of the size 10^3 and 10^2, respectively. It has been found that the binding of [^{3}H]-budesonide to plasma proteins of rat, dog and man is about 90% [11]. This high degree of protein binding may contribute to the decrease in distribution ratio. The ratio will probably also be time dependent. However, experiments indicate that equilibrium is almost attained after 30 min and this extraction time was considered sufficient. By use of the extraction procedure described in Fig. 1, the distribution ratio would correspond to a recovery of 99.6%.

Injections of 100 μl of fourteen plasma [^{3}H]-budesonide standards prepared according to the assay method gave a chromatographic recovery of 101.8% with a relative standard deviation of 4.45. The amounts of [^{3}H]-budesonide injected were in the range of 0.04–1.8 pmol corresponding to the plasma concentrations found in the animal studies. The standard deviation obtained is of the same level as that found for scintillation counting of corresponding amounts of radioactivity.

Figure 2 illustrates a standard curve for [^{3}H]-budesonide obtained according to the assay method. A correlation coefficient of 0.9998 and an intercept of 0.03 calculated for the calibration line shows that the

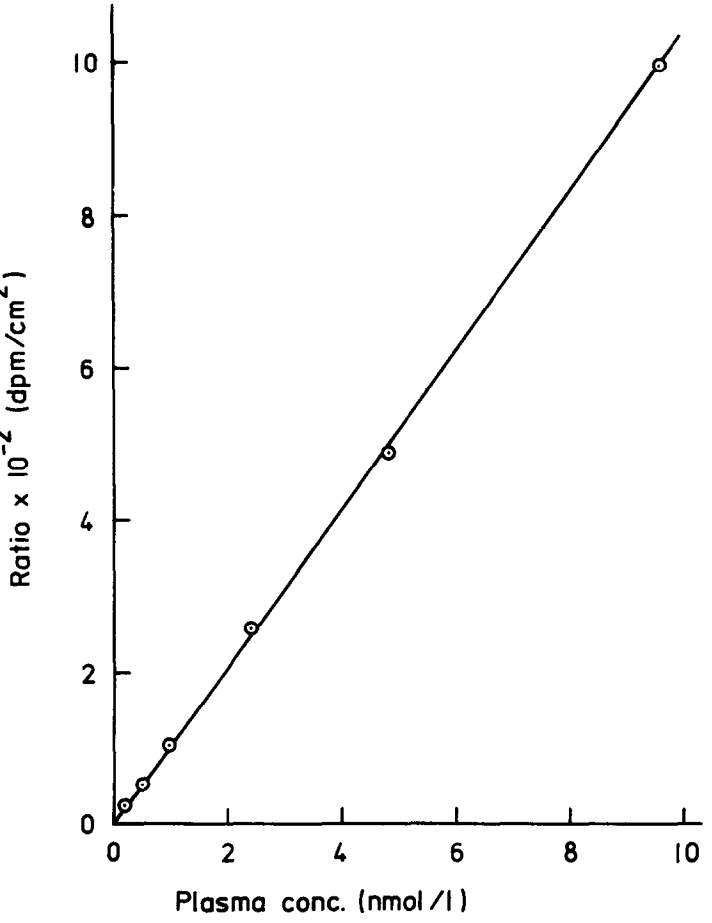

Fig. 2. The ratio of [^{3}H]-budesonide (d.p.m.) to the internal standard (cm^2) plotted versus the concentration of [^{3}H]-budesonide. Standardization was made according to the assay method after additions to blank plasma.

Table 1. Recovery of various amounts of [^{3}H]-budesonide added to 0.5 ml dog plasma prior to the assay procedure

Date	[^{3}H]-Budesonide added (pmol)	[^{3}H]-Budesonide found (pmol)	Recovery (%)	Remaining in plasma phase (%)
76/12/15	0.100	0.107	107.0	—
	0.237	0.230	9.7.1	—
	0.490	0.467	95.3	—
	1.17	1.17	100.0	—
	2.37	2.26	95.4	—
	4.74	4.60	97.1	—
77/01/17	0.268	0.236	88.1	6.1
	1.36	1.31	96.3	2.3
77/01/20	0.522	0.488	93.4	2.1
	2.63	2.66	101.1	4.9
77/01/24	0.512	0.510	99.6	2.4
	2.58	2.61	101.2	1.4
77/02/16	0.510	0.518	101.6	1.9
	0.510	0.492	96.5	2.5
77/06/13	0.504	0.468	92.9	3.2
	0.997	0.942	94.5	3.3
	2.53	2.39	94.5	2.9
77/06/16	0.486	0.454	93.4	2.8
	0.974	0.908	93.2	2.4
	0.974	0.906	93.0	2.3
77/06/20	0.477	0.442	92.7	3.6
	0.959	0.882	92.0	3.0
77/06/28	0.528	0.489	92.6	4.2
	1.06	1.02	96.2	3.5
	1.06	1.00	94.3	3.6
77/06/30	0.483	0.449	93.0	4.2
	0.983	0.901	91.7	3.9
	0.983	0.866	88.1	3.4
Mean			95.4	3.2
Standard deviation			±4.16	±1.07

analytical system behaves ideally even at low concentrations.

Recovery for the internal standard referring to the extraction procedure was found to be 101.5% with a standard deviation of 1.39 ($n = 6$). Recovery for [^{3}H]-budesonide obtained according to the assay method is shown in Table 1. As might be expected from the distribution experiments and from the chromatographic results given above, recoveries close to 100% were obtained. However, for [^{3}H]-budesonide about 3% remained in the plasma phase after the extraction procedure. This is probably due to the presence of non-extractable polar components of [^{3}H]-budesonide. The relative standard deviation of 4.16 for [^{3}H]-budesonide is of the same order as found for scintillation counting of radioactivity in the chromatographic fractions. Thus variations in the assay method mostly originate from this step. The main advantage of using the internal standard probably is its functions as a carrier of [^{3}H]-budesonide during extraction, evaporation and chromatography.

Figure 3 shows a chromatogram illustrating the separation of [^{3}H]-budesonide from co-extracted metabolites in dog plasma. As can be seen a distinct peak of activity is eluted before the [^{3}H]-budesonide peak, representing a fraction of more polar metabolites. The [^{3}H]-budesonide peak appears about 0.2 ml after the internal standard peak and this delay is mostly due to the volume of the teflon tube interconnecting the U.V. detector and the fraction collector. The band broadening of the latter peak is larger than that of the internal standard peak, since there is a slight separation of the two epimers of [^{3}H]-budesonide.

Pharmacokinetic studies

Plasma concentrations of total radioactivity and unchanged drug after intravenous and oral administration are given in Figs. 4 and 5. A summary of pharmacokinetic parameters is given in Table 2. In the intravenous studies, the kinetics of both budesonide and the epimer A were investigated. By kinetic evaluation of the plasma concentration time curve for

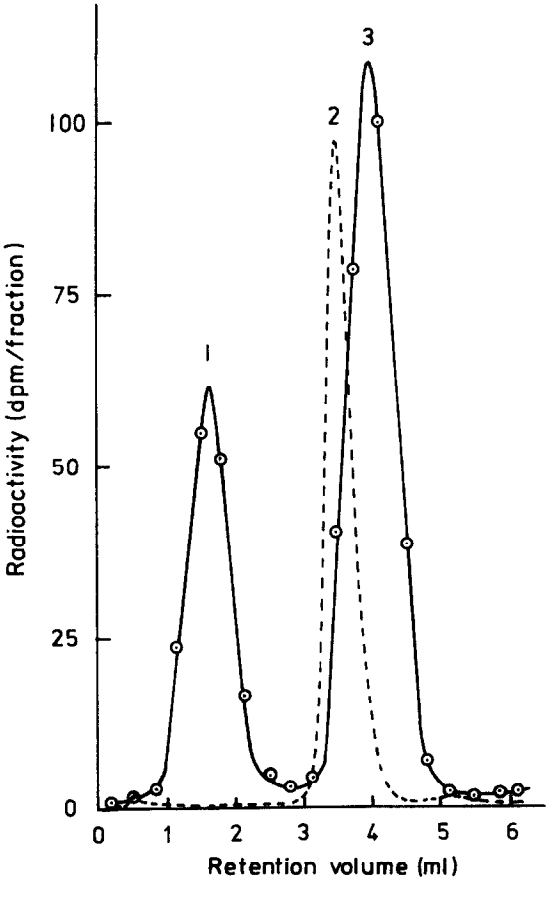

Fig. 3. Chromatogram illustrating the separation of [^{3}H]-budesonide from co-extracted metabolites in dog plasma. Support: Spherisorb ODS (10 μm). Mobile phase: Ethanol–water (40:60, V/V). Flow rate: 0.5 ml/min. Fraction volume: 0.3 ml. Solute: Peak 1, co-extracted metabolites; Peak 2, (UV-detection), internal standard; Peak 3, [^{3}H]-budesonide.

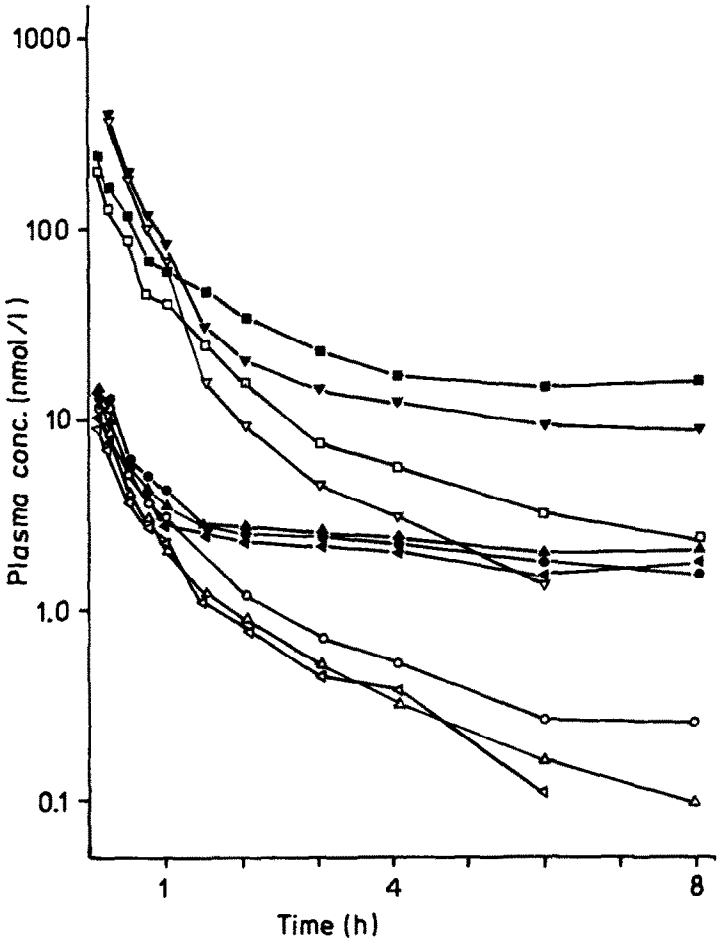

Fig. 4. Plasma concentrations of total radioactivity and unchanged drug after intravenous administration of [^{3}H]-budesonide and epimer A to female Beagle dogs. The doses were 23 (○, △) and 230 (□, ▽) nmol/kg for budesonide and 23 nmol/kg for epimer A (◁). Filled symbols represent total radioactivity and open symbols unchanged drug. Total radioactivity is expressed as unchanged drug.

unchanged budesonide the plasma half-life ($t_{1/2\beta}$) was found to range from 1.9 to 2.1 h at the low dose (23 nmol/kg) and from 1.8 to 3.0 h at the high dose (230 nmol/kg). In the single study performed with the epimer A (23 nmol/kg) the plasma half-life was found to be 1.3 h. This indicates that the plasma half-life of the epimer A is comparable to that of budesonide and no apparent difference in the elimination between the two epimers prevails. This is also indicated by the plasma clearance values which ranged from 1.8 to 2.5 l/h/kg for budesonide and the plasma clearance value for epimer A was 2.6 l/h/kg. For budesonide the distribution volume (Vd_β) ranged from 2.5 to 7.7 l/kg and no difference between the low and high dose was found. That of the epimer A was 4.8 l/kg. This indicates that the compound is distributed to a rather high extent into tissues. Concerning other synthetic glucocorticoids few pharmacokinetic data are available. Mostly the plasma half-life for the total radioactivity or radioactivity extractable into some organic solvent after intravenous injection of the radioactively labelled compound is given [e.g. 3, 4]. The plasma half-life for triamcinolone in the dogs as estimated from radioactivity extracted into ethylacetate was found to be 116.7 min [4] and the half-life for triamcinolone acetonide in rats using radioim-

Table 2. Pharmacokinetic data evaluated from plasma concentration time curves of unchanged drug after intravenous and oral administration of [^{3}H]-budesonide and [^{3}H]-labelled epimer A to female Beagle dogs

	Budesonide				Epimer A
	23 nmol/kg		230 nmol/kg		23 nmol/kg
Parameter	Dog A	Dog B	Dog A	Dog C	Dog B
$t_{1/2\beta}$(h)	1.9	2.1	3.0	1.8	1.3
Vd_β(l/kg)	5.2	7.7	6.9	2.5	4.8
Cl (l/h/kg)	1.8	2.5	1.6	1.0	2.6
Bioavail (%)	16.2	9.2	18.3	18.8	—

Table 3. Cumulative excretion of radioactivity in urine and faeces after administration of [^{3}H]-budesonide and [^{3}H]-labelled epimer A to female Beagle dogs

Time (h)	Activity excreted (% of administered doses)																	
	iv (23 nmol/kg)*				iv (230 nmol/kg)*				iv (23 nmol/kg)†		po (23 nmol/kg)*				po (230 nmol/kg)*			
	Dog A		Dog B		Dog A		Dog C		Dog B		Dog A		Dog B		Dog A		Dog C	
	urine	faeces	urine	faeces	urine	faeces	urine	faeces	urine	faeces	urine	faeces	urine	faeces	urine	faeces	urine	faeces
0–24	8.9	32.8	11.6	0.1	8.9	—	4.6	33.3	4.7	49.7	12.6	—	11.1	47.3	12.1	11.1	13.9	25.4
0–48	12.7	53.8	16.5	50.8	12.9	50.2	6.6	49.9	6.1	66.3	18.9	32.4	13.6	58.3	16.6	55.3	18.6	56.1
0–72	15.1	62.3	18.7	55.1	15.6	65.6	7.7	53.9	7.1	70.4	23.3	54.3	14.6	61.4	19.1	59.9	20.7	57.4
Σ	77.4		73.8		81.2		61.6		77.5		77.6		76.0		79.0		78.1	

* Budesonide. † Epimer A.

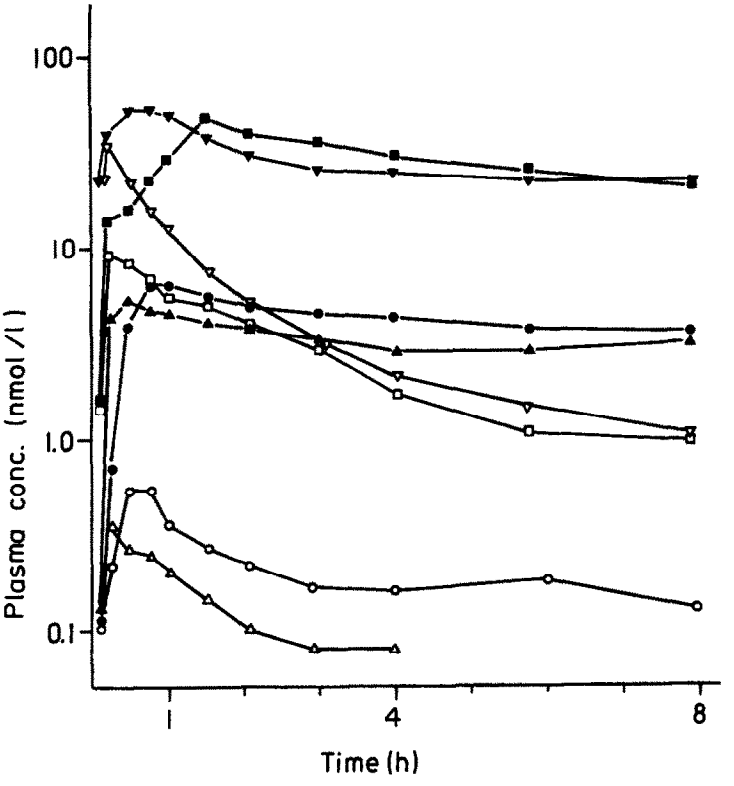

Fig. 5. Plasma concentrations of total radioactivity and unchanged drug after oral administration of [^{3}H]-budesonide to female Beagle dogs. The doses were 23 (○, △) and 230 (□, ▽) nmol/kg. Filled symbols represent total radioactivity and open symbols unchanged drug. Total radioactivity is expressed as unchanged drug.

munoassay was recently reported to range from 75.9 to 81.8 min [5].

In the oral studies with budesonide, the maximal plasma concentrations of unchanged budesonide were noted within 1 h after administration at both dose levels (Fig. 5), indicating a fast absorption. The systemic availability of budesonide ranged from 9.2 to 16.2% at the low dose and from 18.3 to 18.8% at the high dose. This shows that the first-pass metabolism is extensive, however, too few data are available to demonstrate any dose-dependency of the bioavailability. As a consequence of the extensive first-pass metabolism systemic effects should be low. If this also applies to man this might be to advantage, e.g. in inhalation therapy. By this route of administration, the majority of the administered dose is deposited in the oral cavity and then swallowed [2].

The data for the urinary and faecal excretions of radioactivity are given in Table 3. The dominating part of the excreted radioactivity (~80%) was recovered in faeces both after intravenous and oral studies, indicating extensive biliary excretion of the compound and/or formed metabolites. The importance of the biliary excretion in dogs has been documented for several other glucocrticoids such as triamcinolone [4] and beclomethasone dipropionate [2]. The total recovery of administered radioactivity in urine and faeces during 0–72 h sampling period was 75.8 ± 5.69% ($x \pm$ S.D., $n = 9$) of the administered doses.

Because of the incomplete recovery in the balance studies urine samples were analyzed for amounts of tritiated water by distillation. The relative amount of tritiated water to total radioactivity in the urine samples increased with time throughout the sampling period (Table 4). Assuming the same concentration of tritiated water in the bodywater as in urine (48–72 h sample) about 11% of the total amount of radioactivity administered should remain in the body as tritiated water 72 h after administration. By distillation of blank urine samples to which [^{3}H]-bude-

Table 4. Tritiated water (estimated by distillation) in the urine samples obtained from dog B after intravenous administration of [^{3}H]-budesonide (23 nmol/kg)

Sampling period (h)	^3H_2O (% of tot. radioactivity in urine sample)
0–24	4.4
24–48	32.1
48–72	55.3

sonide administering solution had been added, a distillable fraction of 0.4–0.5% was found. Storage of these radioactive urine samples in room temperature for up to 3 days did not increase the distillable fraction. There is also a polar fraction of radioactivity in the body which is not attributable to tritiated water. Since the distribution volume for this activity is not known the amount in the body cannot be calculated. This polar fraction is probably attributable to metabolites which are prone for active elimination e.g., conjugates of budesonide or hydroxylated products of budesonide. Consequently this fraction will decrease in relation to the fraction of tritiated water which is not eliminated by active processes. The biological half-life of tritiated water in the dog is about 5 days [12].

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