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Pharmacokinetic profile of RU 41740, a bacterial immunomodulator, in mice, rats, and monkeys

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Summary

A study of the intestinal transport, tissue distribution, metabolism and excretion of RU 41740 (trade name Biostim), an immunomodulating compound given to patients prone to chronic bronchitis, was carried out in mice, rats, and monkeys. This pharmacokinetic study is of value in determining the mode of action of an orally administered biological response modifier on the immune system. Tissue localisation in the duodenal epithelium of C3H axenic mice was performed by an indirect immunofluorescence method; the plasma kinetics, the tissue distribution, and the excretion were assessed using tritiated RU 41740. The metabolites were examined by gel filtration of the circulating products. RU 41740 was found to be present in the lamina propria of duodenal villi 2 h after oral administration and maximum radioactivity was found in the systemic circulation of rats and monkeys from 1 to 4 h after treatment; small amounts of the administered dose were recovered (0.3%) at that time. The absorption was 6.9% of the administered dose in the rat and 1.8–2.1% in the monkey (from plasma kinetics data collected over 14 days). The elution profile of the tritiated circulating product indicated that RU 41740 was modified. Radioactivity was found associated mainly with molecular weight fragments of less than 10,000. The time course distribution of RU 41740 showed that 3 h after treatment, the radioactivity was mainly in the digestive tract, the liver, and the kidneys; later (24 h), it increased in the lung, spleen, mesenteric lymph nodes, adrenals, and ovaries. The excretion route of the radioactivity was predominantly fecal. The presence of RU 41740 fragments in the intestinal tissue, the systemic circulation, and some other tissues may contribute to direct activation of the immune cells localised in the gut, liver, lungs, and so partly explain the immunomodulatory properties of RU 41740.

Introduction

RU 41740 (trade name Biostim) is a glyco-protein complex extracted from *Klebsiella pneumoniae* (K2 : 01 strain) composed of two fractions of molecular weight (mol. wt.): 350,000 (Eur. Pat. Appl. EP 49,182) and 95,000 (Ger. Offen., 3,029,111) (Kol et al., 1987). In vitro RU 41740 acts as a selective B cell mitogen (Wood and Moller, 1984), induces IL-1 secretion (Guenounou et al., 1985) and activates other macrophage functions (Takada et al., 1982). Experimental studies have shown that RU 41740 protects against bacterial and viral infections (Griscelli et al., 1982). In cancer patients, RU 41740 has been shown to restore cutaneous delayed type hypersensitivity

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(Lang et al., 1980). In chronic bronchitis patients, RU 41740 enhances macrophage engulfment and killing capacities and increments the antibody titer when administered together with a vaccination (Profeta et al., 1987). This drug is used in chronic bronchitis patients to reduce the number and length of infectious episodes (Nielsen and Bonde, 1986; Bonde et al., 1986). These immunomodulating activities are observed in humans when RU 41740 is administered orally. Therefore, it would be interesting to determine whether RU 41740 enters the gut unchanged or in a fragmented form. Proteins have long been shown to be absorbed by an endocytotic process (Warshaw et al., 1977; Silverstein et al., 1977) across columnar epithelial cells and M cells present over Peyer's patches (Owen, 1977). Previous results (Heyman et al., 1987) evaluated the transport of RU 41740 across isolated duodenal epithelium and Peyer's patches of the rabbit. An initial consequence may be an increase of the local production of immune mediators or the migration of immune cells from the gut. The presence of RU 41740, or its fragments in the blood, lymphatic circulation, and immunocompetent tissues, may also increase the local or systemic level of mediators. The aim of the present study was to investigate further the pharmacokinetic profile of RU 41740 after per os administration to mice, rats, and monkeys. Data presented in this report show that RU 41740 and fragments of RU 41740 are recovered mainly in the gastrointestinal tissue, liver, kidneys, and in the blood.

Materials and Methods

Preparation of tritiated-RU 41740 ([^{3}H]RU 41740)

The galactose residues of RU 41740 were tritiated on the carbon-6 position (Van den Eijnden et al., 1977). The lyophilised tritiated RU 41740 was stored at $-30\,^{\circ}$C. Its specific activity was 21 μCi/mg (780 KBq/mg). The toxicological, biochemical, and biological properties of the labelled product were checked.

Pharmacokinetic studies

Plasma kinetic studies were carried out on two adult *Macaca fascicularis* monkeys (no. 1, a 3.9 kg male and no. 2, a 3.4 kg female). Food was withdrawn for 24 h but water was available ad libitum. [^{3}H]RU 41740 was either administered orally at a dose of 2 mg/kg in capsule form or injected into the external saphena 0.5 ml/kg, 16 h after the withdrawal of food. The intravenous treatment was carried out on the same monkeys 3 months after the oral treatment.

Plasma kinetic studies were also carried out on adult male Sprague–Dawley rats (Charles River). Food (UAR feed) and water were available ad libitum throughout the study. [^{3}H]RU 41740 in aqueous solution was administered at 4 mg/kg by gastric intubation ($n = 5$) or by injection at 0.4 mg/kg into the vein of the penis ($n = 5$).

Blood samples were drawn from the retro-orbital sinus of the rats and the inguinal vein of the monkeys and collected in lithium heparin tubes at various intervals over 14 days. The radioactivity of the lyophilised samples was determined by liquid scintillation counting in 10 ml of scintillant (Biofluor, NEN).

Pharmacokinetic parameters for the radioactivity

The kinetic curves of the lyophilised plasma radioactivity were obtained over 14 days in the monkeys and rats. The absorption of radioactivity in the monkeys and rats was determined by comparing the areas under the kinetic curves of the radioactivity following the oral treatment with the corresponding areas following intravenous treatment from time 0 to 14 days. The total plasma clearance of the radioactivity was determined from the ratio of the quantity of intravenously administered radioactivity to the area under the plasma radioactivity concentration curves (days 0–14).

Tissue distribution

The tissue distribution of [^{3}H]RU 41740 was studied in Sprague–Dawley adult female rats (180–200 g, Charles River) by counting the radioactivity in lyophilised tissues expressed as disintegrations per minute (dpm). The animals were kept in cages with grilled floors to avoid radioactive oral contamination by feces. Animals were sacrificed 3 h ($n = 3$) or 24 h ($n = 3$) after oral treatment (10 mg/kg). The tissues were homogenized (Polytron-Kinematica) and then treated with

Soluene 350 (Packard) (2 h, 50 °C). The radioactivity of 100 mg tissue samples was determined by liquid scintillation counting in 18 ml of scintillant (Dimilume 30, Packard). The results were expressed as an index I = dpm per gram of fresh tissue/dpm per gram in the muscle.

Localisation in the gut tissue

RU 41740 was administered by gastric intubation at a dose of 2 mg/mouse to C3H axenic mice (2–4-month-old females, Laboratoire d'Ecologie Microbienne, INRA, France). Food was withdrawn 16 h before the treatment. The animals were killed by cervical dislocation 2 or 6 h after intubation. Blocks of the duodenal tissue were immediately removed, frozen (15 s at −180 °C in isopentane, Rectapur Prolabo) and then stored at −80 °C. RU 41740 was detected in sections of duodenum from RU 41740-treated axenic mice by indirect immunofluorescence using optimal dilutions of rabbit anti-RU 41740 antiserum absorbed with the intestine of normal C3H axenic mice to remove cross-reactivity with mouse tissue. The second antibody was a sheep anti-rabbit antibody labelled with fluorescein isothiocyanate (Institut Pasteur Production ref 74.561). Negative controls were performed on tissue from mice that had not received RU 41740, and on tissue from RU 41740-treated mice using normal rabbit serum. The preparations were examined with a Ploemopak 204 fluor illuminator under a Leitz Dialux 20 microscope.

Disposition studies

Male and female Sprague–Dawley rats (120–140 g, Charles River) were placed in individual metabolism cages, designed for the separate collection of urine and feces. The animals were treated at 4 mg/kg orally ($n = 3$) or intravenously ($n = 3$) with [^{3}H]RU 41740. Unpooled samples of feces and urine were collected 6 h and 24 h after the treatment and every 24 h thereafter for the following 20 days and stored at −30 °C. The lyophilised feces (50 mg) were treated with Soluene 350 (2 h, 50 °C) and then with 0.5 ml isopropanol before 18 ml of scintillant (Dimilume 30) were added in order to count the radioactivity. The radioactivity of the lyophilised urine (1 ml) was determined after the addition of 10 ml scintillant (Biofluor NEN). The disposition is expressed as the percentage of the administered dose.

Chromatographic studies

The plasma of monkeys and rats (0.3 ml) was assayed by liquid chromatography on a Sephacryl S300 (Pharmacia) column (27 × 1 cm) eluted with 0.05 M Tris-HCl; 0.15 M NaCl buffer (pH 8.0). The column was calibrated with Dextran blue 2000 (mol. wt. 2,000,000), ferritin (mol. wt. 440,000), aldolase (mol. wt. 147,000), bovin serum albumin (mol. wt. 66,000), ovalbumin (mol. wt. 45,000), myoglobin (mol. wt. 17,800) and cyanocobalamin (mol. wt. 1350). The radioactivity of 1 ml eluted fractions was measured. Control plasma from an untreated monkey, to which parent [^{3}H]RU 41740 had been added to give a final concentration of 1 μg/ml, was chromatographed under the same conditions.

Samples of rat urine (0.4 ml) from the disposition studies were assayed by liquid chromatography on a Trisacryl GF05 (IBF) column (27 × 1 cm) eluted with 0.05 M Tris-HCl; 0.15 M NaCl buffer (pH 8.0). The column was calibrated with Dextran blue 2000, insulin A (mol. wt. 2532), cyanocobalamin, and the tripeptide Tyr-Tyr-Tyr (mol. wt. 507). The radioactivity of 1 ml eluted fractions was measured.

Results

Plasma kinetics

Maximum plasma radioactivity was found 1 h after oral treatment for monkey no. 1 and after 3–4 h for monkey no. 2 (Fig. 1). This represents 0.27% (monkey 1, 1 h) and 0.23% (monkey 2, 3 h) of the administered dose. Half of the maximal circulating radioactivity was recovered 2.5 and 2.4 h after the peak for the monkeys 1 and 2. Seven days later 9.5% (monkey 1) and 11.2% (monkey 2) of the radioactivity were still present. After intravenous treatment, the radioactivity was eliminated at similar rates in both animals (Fig. 1). 41% of the administered dose was recovered in the circulation at 0.5 h. This may indicate that the radioactivity is mainly localised in the tissues.

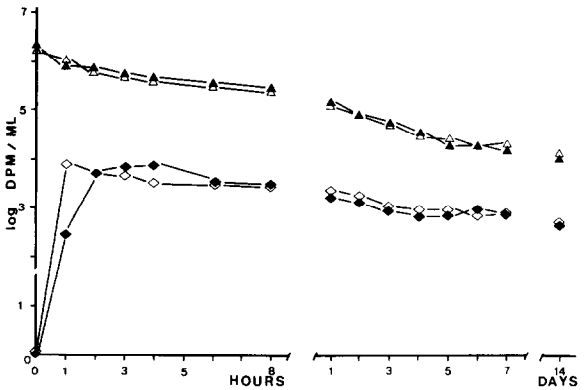


Fig. 1. Plasma kinetics of the radioactivity, after oral (◇———◇, monkey no. 1; ◆———◆, monkey no. 2) and intravenous (△———△, no. 1; ▲———▲, no. 2) administrations of [^{3}H]RU 41740 (2 mg/kg).

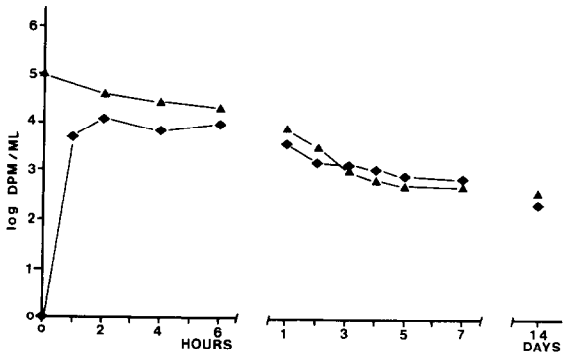


Fig. 2. Plasma kinetics of the radioactivity in the rat after oral (◆———◆) and intravenous (▲———▲) administration of [^{3}H]RU 41740. Mean values of 5 orally treated rats (4 mg/kg) and 5 intravenously treated rats (0.4 mg/kg).

The plasma radioactivity kinetics in rats are shown in Fig. 2. The radioactivity of the lyophilised plasma increased for 2–5 h following oral administration. The maximum quantity of radioactivity present in the circulation was 0.28% of the administered dose. Then it slowly fell off until day 14. After injection to the rat, the circulating radioactivity rapidly decreased during the first 0.5 h, as in the monkey.

Pharmacokinetic parameters

The pharmacokinetic parameters for rats and monkeys are shown in Table 1. The radioactivity absorbed was 2.1% and 1.8% for monkeys 1 and 2, and 6.9% for the rats ($n = 5$). The total plasma clearance of intravenously administered product was 5.9 and 5.8 ml/h/kg for the monkeys and 32.1 ml/h/kg for the rats.

An attempt was made to assess the structural modifications to which RU 41740 is subjected in passing into the blood. The elution profiles of the radioactivity of parent [^{3}H]RU 41740 were compared to those of plasma from orally treated animals. The results (Table 2) reveal that most of the tritiated products eluted had low mol. wt. (< 10,000); high mol. wt. products (> 440,000) were also present in the circulation (4–19% of the eluted radioactivity) at the peak time for both species. The distribution of radiolabelled structures in the circulation after intravenous treatment was similar to that of the control plasma containing parent [^{3}H]RU 41740.

Tissue distribution

The distribution of the radioactivity in the lyophilised tissue samples is given in Fig. 3. The radioactivity was counted in each tissue and com-

TABLE 1

Pharmacokinetic parameters of the [^{3}H]RU 41740 in monkeys and rats

Species	Dose ($\times 10^6$ dpm/kg)		AUC (days 0–14) ($\times 10^6$ dpm · h/ml)		Absorption (%)	Plasma clearance i.v. (ml/h/kg)
	i.v.	p.o.	i.v.	p.o.		
Monkey 1	92.4	92.4	15.7	0.328	2.1	5.9
Monkey 2	92.4	92.4	16.0	0.293	1.8	5.8
Rat ($n = 5$)	19.8 ± 0.8 *	189.5 ± 6.7 *	0.62 ± 0.07 *	0.41 ± 0.05 *	6.9	32.1 ± 4.3 *

Absorption is calculated from the values of the AUC (area under the plasma concentration time curve) i.v. and p.o.
* Mean value + S.D.

TABLE 2

Characterization on Sephacryl S300 of [^{3}H]RU 41740 in the plasma of intravenously or orally treated animals compared to parent [^{3}H]RU 41740

Treatment	Animals	mol. wt. > 440,000	440,000 < mol. wt. < 10,000	mol. wt. < 10,000
Intravenous	Monkeys 1 (3 h)	45	46	9
	2 (3 h)	48	43	9
Oral	Monkeys 1 (1 h) peak	4	33	63
	2 (3 h) peak	8	17	75
	Rats 1 (3 h) peak	19	21	62
	2 (3 h) peak	4	31	65
Parent [^{3}H]RU 41740		55	38	7

pared to that in striated muscle (used as a reference). At 3 h, the radioactivity was principally recovered in the digestive tract (stomach, duodenum, Peyer's patches, jejunum, ileum, large intestine), liver, and kidneys. A lower level of radioactivity was found in the adrenals, bone marrow, mesenteric lymph nodes, ovaries, lungs, and spleen. After 24 h, the radioactivity was found mainly in the kidneys, gut, and liver. The level had increased in the adrenals, ovaries, bone marrow, mesenteric lymph nodes, lungs, and spleen compared to the muscle.

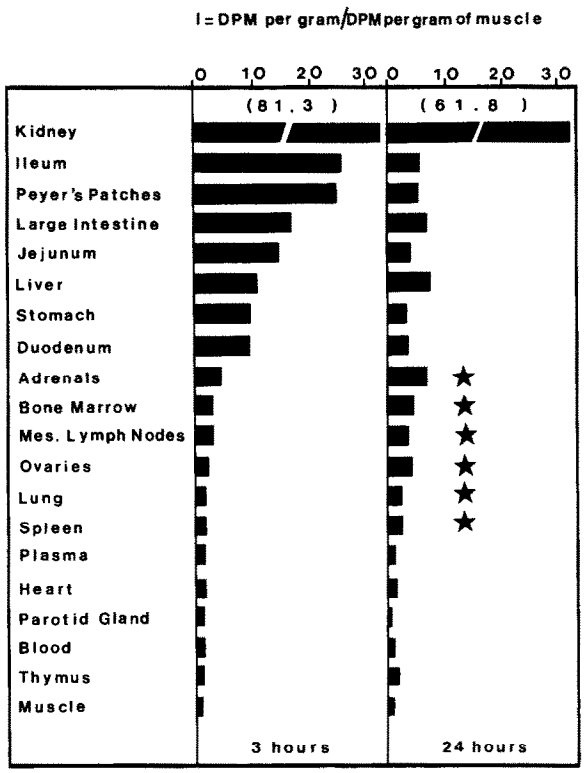


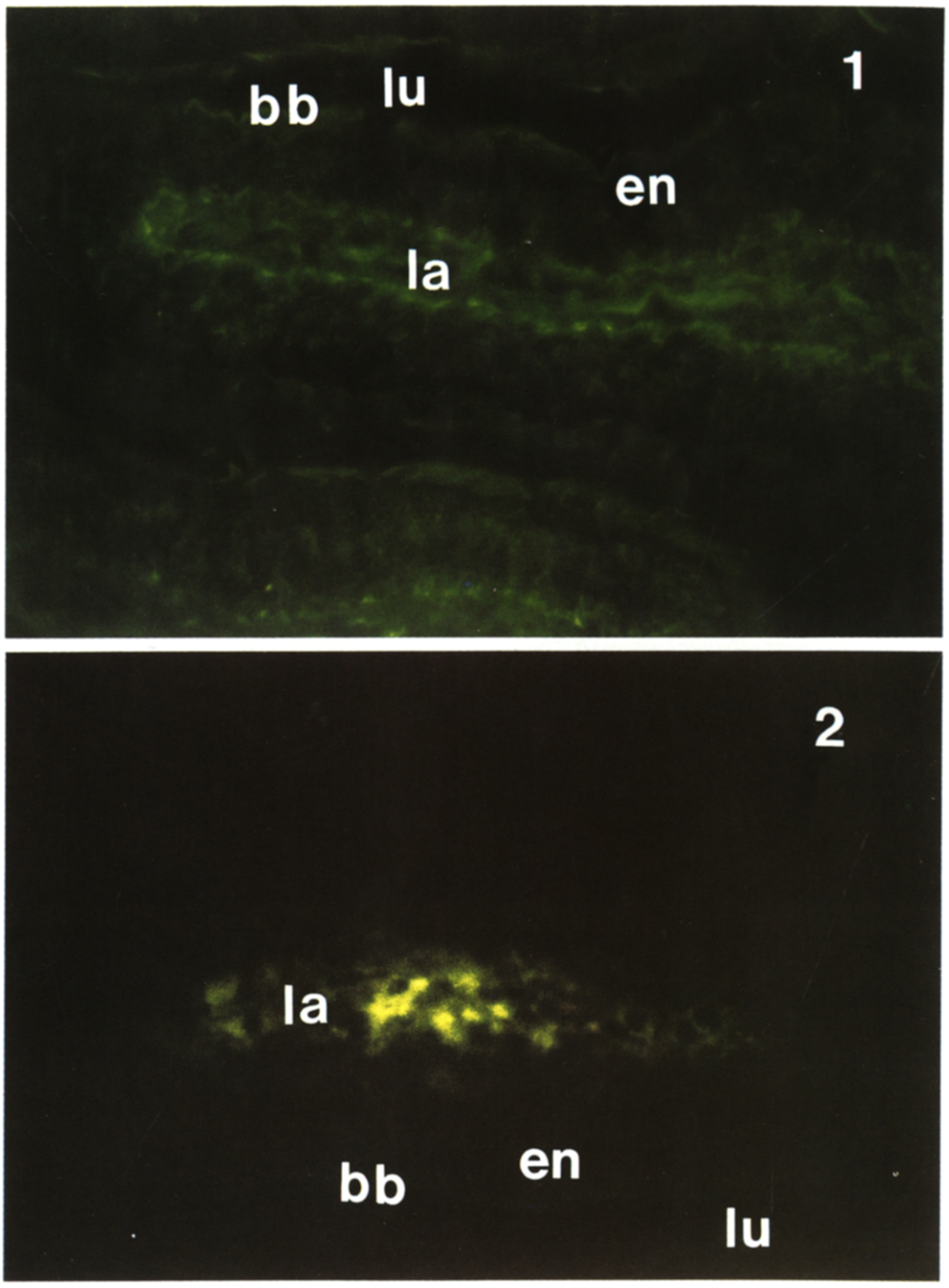
Fig. 3. Distribution of the radioactivity in the tissues of [^{3}H]RU 41740 orally treated rats (10 mg/kg, $n = 3$) compared to the radioactivity present in the striated muscle. ★, I at 24 h > I at 3 h.

Localisation in the gut tissue

The indirect immunofluorescent studies on sections of duodenum from C3H axenic mice 2 h after intubation (Plate 1) showed that fluorescence was found in the lamina propria (particularly in the peripheral area, near the base of the enterocytes and the capillaries), along the brush border of the epithelial cells, and in the intestinal lumen. The fluorescence persisted in the central region of the lamina propria of the intestinal villi 6 h after intubation (Plate 2). These various localisations of the fluorescence show that penetration of RU 41740 determinants occurs across the layer of intestinal epithelial cells.

Disposition studies

Studies on the disposition of [^{3}H]RU 41740 were carried out on rats following oral and intravenous administrations (Table 3). After oral treatment, the major excretion route for the radioactivity in the rats was fecal, accounting for about 59% of the dose. The high values of the standard deviations of the radioactivity excreted in the feces of orally treated rats are due to the intestinal transit variability. Urinary excretion represented 16.4% of the dose and was at a maximum (7.6%)



Plates 1 and 2. Intestinal villi of germ-free C3H mouse. Indirect immunofluorescence (anti-RU 41740 anti-serum × 1/50). Observations occurred 2 h (1) and 6 h (2) after oral administration of RU 41740. bb, brush border; lu, intestinal lumen; en, enterocytes; la, lamina propria.

TABLE 3

Cumulated excretion of radioactivity in the urine and feces following oral or intravenous administration on [^{3}H]RU 41740 (4 mg/kg) to the rat

Percentages of the administered dose recovered on day 0.25, 1, 7 and 20 (mean ± S.D., $n = 3$). Total radioactivity and radioactivity due to tritiated water.

Treatment		% of radioactive administered dose			
		Feces			
		day 0.25	day 1	day 7	day 20
Oral	Total radioactivity	0.04 ± 0.04	34.0 ± 29.5	58.2 ± 6.5	59.1 ± 7.1
	Tritiated water	0.03 ± 0.04	0.5 ± 0.7	2.6 ± 2.1	3.5 ± 1.3
Intravenous	Total radioactivity	0.60 ± 0.90	7.7 ± 4.0	20.2 ± 3.7	24.7 ± 4.2
	Tritiated water	0.08 ± 0.09	0.27 ± 0.28	4.2 ± 2.9	6.0 ± 3.4
		Urine			
		day 0.25	day 1	day 7	day 20
Oral	Total radioactivity	3.1 ± 1.0	7.6 ± 1.6	14.1 ± 2.4	16.4 ± 2.2
	Tritiated water	0.2 ± 0.2	1.4 ± 0.8	6.0 ± 2.5	8.2 ± 1.5
Intravenous	Total radioactivity	12.7 ± 9.5	20.1 ± 11.8	37.7 ± 11.0	45.1 ± 11.0
	Tritiated water	0.2 ± 0.3	0.7 ± 0.8	5.6 ± 1.5	9.3 ± 1.6

during the 24 h following the treatment. A fraction of the excreted radioactivity was eliminated as tritiated water (5% of the radioactivity excreted via the feces and 50% of the radioactivity via the urine). Thus, according to the urinary excretion values, at least 8% of the administered dose was absorbed.

After intravenous injection, the major excretion pathway for the radioactivity was urinary, accounting for about 45% of the dose. Urinary excretion was at a maximum (12.7%) during the first 6 h following the treatment. Fecal excretion represented 25% of the dose. The fraction of radioactivity excreted as tritiated water was 20% in the urine and 24% in the feces.

The chromatographic patterns of the radioactivity excreted in the urine of rats receiving [^{3}H]-RU 41740 orally or intravenously are shown in Fig. 4. The radioactivity in the urine collected in the first 6 h from the orally treated rats was

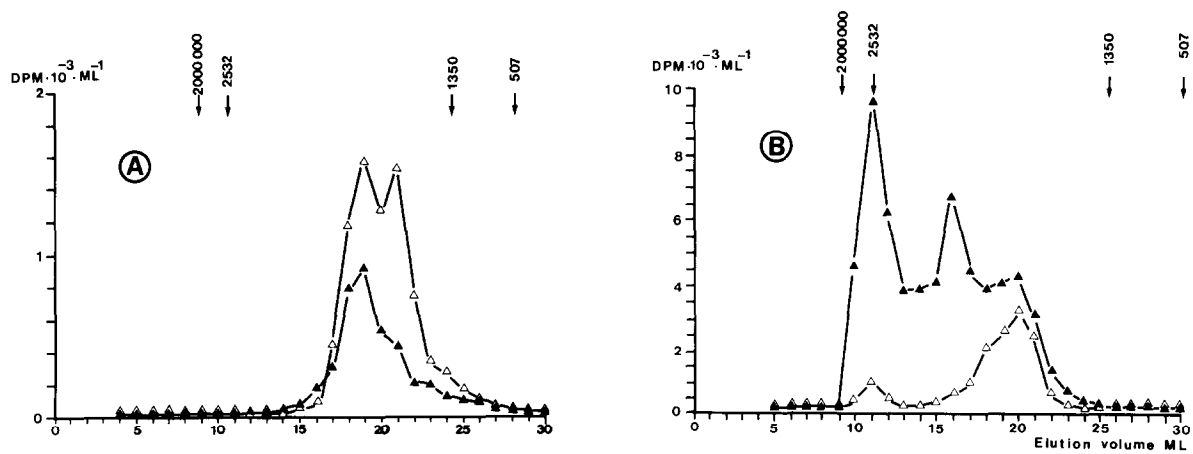


Fig. 4. Chromatographic analysis of the structure of [^{3}H]RU 41740 tritiated fragments in the urine of rats after oral (A) and intravenous (B) treatments (4 mg/kg). ▲———▲, 0–6 h collection; △———△, 6–24 h collection.

associated with mol. wt. products between 1350 and 2500. A second peak of slightly smaller mol. wt. fractions appeared 24 h after the treatment. After intravenous treatment, higher mol. wt. fractions (> 2500) were excreted in the urine in the first 6 h.

Discussion

This report describes the pharmacokinetic profile of RU 41740 in 3 animal species. We have evaluated the absorption of the radiolabelled product, its structure, and localisation after oral administration. Fragments of [^{3}H]RU 41740 were recovered in the blood circulation of monkeys and rats from 1 to 4 h following the oral dose. The maximum concentration of the circulating products was 0.25% and 0.28% of the dose. The total quantity absorbed was higher in the rats (6.9% of the dose) than in the monkeys (2.0%). The structure of the radiolabelled circulating products was similar in both species but differed from the parent product. The modifications of structure occurred in the intestinal epithelium, as previously demonstrated by Heyman et al. (1987). They showed that [^{3}H]RU 41740, which had crossed the duodenal villi or the Peyer's patches, was partly degraded. However these modifications preserved some antigenic structures. For instance, we observed by indirect immunofluorescence a transient presence of RU 41740 in the duodenal villi, 2 and 6 h after the oral treatment. The presence of radioactivity in the digestive tract tissues at this time (duodenum, jejunum, ileum, Peyer's patches, and the large intestine) may be responsible for the local stimulation of the gut-associated lymphoid tissues. A consequence of this may be an augmentation in the migration of the lymphocytes (Reynolds and Pabst, 1984; Pabst and Reynolds, 1986) or an increase in the secretion of GM-CSF (Andreux et al., 1986) and IL-1 like activity (Guenounou et al., 1988).

Once the gut wall was crossed, the radioactivity was mainly recovered in the liver. The radioactivity in the lungs, spleen, mesenteric lymph nodes, adrenals, and ovaries was lower than in the liver but had a slower elimination.

Immunofluorescence data obtained with RU 41740 are in agreement with the results obtained for horseradish peroxidase (HRP) which can cross the basal membrane and can be localised in the lamina propria (Cornell et al., 1971) and between the adjoining absorptive cells (Walker et al., 1972). The presence in the blood of high mol. wt. fragments of other products administered via the digestive tract has been reported for HRP (Warshaw et al., 1971), bovine serum albumin (BSA) (Warshaw et al., 1977), ovalbumin (Beh, 1985) and PSK, a biological compound (mol. wt. 94,000) extracted from Basidiomycetes (Fujita et al., 1983). These products, absorbed via the lymph and the blood, preserved some of their properties. Small amounts (1.7%) of intact tritiated BSA were recovered in the peripheral blood of rats 3 h after intraduodenal infusion (Warshaw et al., 1971, 1974), and fragments of PSK of mol. wt. greater than 10,000 were characterized in the serum (Tsukagoshi et al., 1984).

The relatively high level of radioactivity recovered with [^{3}H]RU 41740 in the liver is in agreement with studies carried out with other extracts from different gram-negative bacteria. Acylglycanic fractions from *Escherichia coli* or *Salmonella minnesota* were localised in the Kupffer cells after intravenous injection (Mathison and Ulevitch, 1979; Freudenberg et al., 1982). Receptors for these fractions have been characterized on macrophages (Wright and Jong, 1986). They are detoxified by the macrophages (Munford and Hall, 1985) and induced a local immunostimulation (Duncan et al., 1986). Accordingly, the presence of fragments of RU 41740 in the liver may provide a similar local immunostimulation. In the lung, the radioactivity compared to the muscle had increased 24 h after the oral treatment with RU 41740. Similar results were obtained with fractions from *Salmonella* detected in the lung parenchyma 24 h after the intravenous injection; however, no acylglycanic fractions were found during the first 7 h (Freudenberg et al., 1982).

The slow elimination rates of [^{3}H]RU 41740 from the adrenals and ovaries indicate that interactions may occur between the circulating fragments of [^{3}H]RU 41740 and plasma lipoproteins since these organs possess high density lipoprotein

binding activity (Gwyne and Mahafee, 1986). Such interactions were previously demonstrated in vivo (Van Lenten et al., 1986; Ulevitch and Johnston, 1978), and a specific uptake by the adrenals was observed (Munford et al., 1981; Mathison and Ulevitch, 1985). They resulted in detoxification (Ulevitch and Johnston, 1978) and longer half-life (Mathison and Ulevitch, 1979). The RU 41740 characteristics and half-life may be modified in the same way after interaction with plasma lipoproteins.

The presence of fragments of RU 41740 in organs such as the spleen, bone marrow, lungs, gut, and lymph nodes may produce a direct activation in the immune cascade which seems to be the cause of the increase in the colony-stimulating activity and in the increased levels of "IL-1-like activity" in the serum. Consequently, an enhancement in the non-specific resistance would explain the protective effect of RU 41740 against infections by intra- and extracellular microorganisms.

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