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Development of a radioimmunoassay for SR 31747A, a new sigma ligand, in human plasma

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Abstract

A specific and sensitive radioimmunoassay was developed for SR 31747A, a new sigma ligand, using a monoclonal antibody. This antibody was produced from spleen lymphocytes of a mouse immunized with SR 31747A coupled to bovine serum albumin via a peptide bond using SR 120684A, a succinamic acid derivative of SR 31747A. Negligible binding occurred when metabolites, obtained by chemical synthesis or by "in-vitro" incubation with hepatic microsomal fraction, were tested for cross-reactivity. A quantitative recovery in serum of the exogenous analyte was obtained for all the concentrations tested and the quantification limit was found to be 0.25 ng ml^{-1} of SR 31747 (the non-salified derivative). Intra- and inter-assay relative standard deviations ranged from 6.3–10.9 and from 5.3% to 15.4% respectively. Furthermore, comparison of results from samples which were assayed by radioimmunoassay and gas chromatography demonstrated an excellent correlation ($r = 0.984$).

Keywords: Correlation between radioimmunoassay and gas chromatography; Human plasma; Metabolites' cross-reactivity; Monoclonal antibody; Radioimmunoassay; Sigma ligand

1. Introduction

SR 31747A, *N*-cyclohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride is a newly characterized ligand for peripheral sigma receptors. High affinity binding for SR 31747A on rat spleen cell membranes and human lymphocytes has already been identified [1]. This ligand is the most potent molecule so far characterized in terms of its ability to compete with all known sigma ligands and is specific for type 1 and type 2 receptors. It is not reciprocally displaced by these molecules suggesting that it might recognize a new site allosterically related to sigma 1 and 2 receptor subtypes. Moreover, potential activity of the ligand on immune responses, probably coupled to the biological function of lymphocytes, has been demonstrated [2–4]. "In-vitro" studies showed that SR 31747A was a potent inhibitor of proliferative response to mitogens on mouse and human lymphocytes, whereas, "in vivo", mice treatment with SR 31747A prevented both graft versus host disease, and delayed-type hypersensitivity granuloma formation. Furthermore, in

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Table 1
Sample protocol[a]

	Standard solution	Unknown samples	RIA buffer	Untreated plasma	Antibody dilution	[^{3}H]SR 31747A
NSB[b]	None	None	150	200	None	50
Zero binding	None	None	100	200	50	50
Standards	100	None	None	200	50	50
Unknown	None	200	100	None	50	50

[a] All values in microliters for a unit test. Volumes were distributed by a processor-controlled sample distributor.
[b] Non-specifically bound

mice, this compound markedly suppresses the LPS-induced production of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α [3].

A gas chromatography (GC) method, with a limit of quantification of 10 ng ml^{-1} has already been developed for determination of SR 31747 (the non-salified derivative) in plasma. However, in order to increase the sensitivity of the assay, necessary to evaluate pharmacokinetic properties of the drug in human clinical studies, the characteristics of a radioimmunoassay (RIA) using a monoclonal antibody (Mab 15D4) for the measurement of SR 31747 in human plasma are described in this paper. The performance of the assay in terms of sensitivity and specificity towards potential metabolites is discussed. As a demonstration of the potential utility of the RIA, human plasma samples were assayed by RIA and GC and results were compared.

2. Materials and methods

2.1. Preparation of the hapten and of the immunogen

The hapten SR120684A (*N*-(4-chloro-5-cyclohexyl-2-[3-cyclohexyl-ethyl-amino)-propenyl]-phenyl-succinamic acid) (26.5 mg); (Recherche, Toulouse, France) was dissolved in 2.5 ml of a 1/1 DMF/ dioxane (v/v) mixture to which was added 100 μmoles of isobutyl chloroformate and 100 μmoles of tributylamine in ice. After 10 min, 67 mg of bovine serum albumin (BSA) dissolved in aqueous solution made alkaline with 30 μl of 0.5 M sodium hydroxide was slowly added to the hapten solution. The mixture was stirred continuously and allowed to react for approximately 20 h at room temperature. The solution was then dialyzed against 10 mM phosphate buffer pH 7.4, 0.9% NaCl (PBS) and stored frozen at − 20°C.

2.2. Immunisation and antibody production

Five Balb/C mice were injected subcutaneously with 50 μg of BSA–hapten conjugate in 100 μg water and 100 μl complete Freunds adjuvant. The animals were boosted at monthly intervals under the same conditions. 3 days before the fusion, the mice received final intravenous and intraperitoneal injections under conditions designed to prevent anaphylactic shock. Hybridomas were produced according to a procedure previously described [5]. Suspensions of fusion cells (150 000 cells ml^{-1}) in selective medium containing hypoxanthine, aminopterin and thymidine (HAT) were transferred into microculture wells (100 μl) containing macrophages as feeder cells.

A sample of medium from each well was screened for its ability to bind [^{3}H]SR 31747A in a RIA procedure described below.

Cells from positive wells were subcloned by limiting dilution in 96-well microculture plates. Selected clones were injected intraperitoneally into Balb/C mice primed 10 days before with 0.5 ml pristane. 8–15 days later, ascites fluids were collected and antibodies purified by affinity chromatography on a protein-A-Sepharose column [6]. Purified antibodies were stored at − 80°C.

2.3. In-vitro metabolite preparation

Hepatic microsomal fractions were prepared from the liver of a beagle dog as previously

described [7]. Protein content was determined using the Bio-Rad protein assay and cytochrome P450 levels were determined by the method of Omura and Sato [8].

For preparation of metabolites, microsomal fractions were incubated for 30 min at 37°C with 60 μg of SR 31747A containing 10^6 dpm of [(1,2)-^{3}H] SR 31747A (synthesized by Sanofi Recherche, specific activity 2109 GBq mmole^{-1} and stocked in ethanol). Enzymatic reaction was initiated by the addition of NADPH (1 mM final concentration) and stopped by the addition of acetonitrile. After a 10 min centrifugation (3000*g*) supernatant fluid was purified on a Bondelut CN column preconditioned with 2 ml of distilled water, 1 ml of methanol and 2 ml of solvent A (0.1% diethylamine and 0.1% tetrahydrofuran in water pH 7.5) successively. The column was washed with 2 ml of solvent A and compounds of interest were eluted with 2 ml of acetonitrile. They were then evaporated to dryness and the residue solubilized in solvent A containing 50% acetonitrile. Metabolites were then separated on a Supelcosil LC-CN column (250 mm × 4.6 mm, 5 μm). Elution was conducted at 0.8 ml min^{-1} using a 0.1% diethylamine, 0.1% tetrahydrafuran in water/acetonitrile (60/40) mixture along a 40–90% acetonitrile gradient for 30 min. According to 250 nm optical density detection, fractions corresponding to metabolites were collected and stored at −20°C. Concentrations of metabolites were determined on the basis of radioactivity.

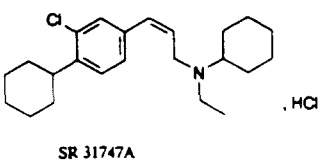
2.4. RIA procedure

Assays were conducted in 5 ml polystyrene tubes. Phosphate buffer (0.1 M KH_2O_4, pH 6.0) containing 0.1% BSA (Sigma) and 0.1% Triton (Sigma) was used as the diluent throughout. The assay tubes for standards, blanks, metabolites and unknown samples were set up according to the format shown in Table 1.

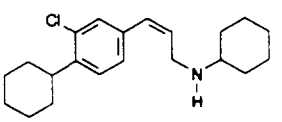
100 μl aliquots of blanks, standards, and metabolites or 200 μl of unknown samples were added to the tubes, followed by 200 μl of untreated plasma (or 100 μl of RIA buffer for unknown samples), 50 μl of [(1,2)-^{3}H[SR 31747A (containing 10^4 dpm) and 50 μl of Mab at the working dilution (1/2000). After 18 h incubation at 4°C, 1 ml of chilled dextran–charcoal suspension (50/500 mg ml^{-1}) was added, mixed, incubated for 30 min at 4°C and centrifuged (15 min, 4000*g*, 4°C). Then, 1200 μl of supernatant was taken and mixed with 5 ml of scintillation fluid and the radioactivity was measured for 2 min on a liquid scintillation counter (Wallac type 1409 equipped with WIACALC software).

2.5. GC assay

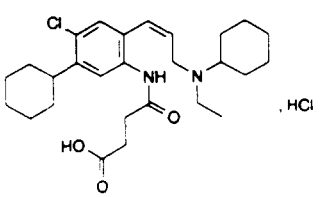
A GC method was developed and validated for SR 31747 in plasma. Briefly, prior to GC analysis, plasma samples were extracted on Extrelut columns, eluted with hexane and dried under a nitrogen stream. Residues were reconstitued with 100 μl methanol and 1 μl aliquots were injected into the GC system. The GC conditions were as follows: detection mode, nitrogen phosphorus; column, RTX 1301 (15 m × 0.53 mm, 1 μm); injection port temperature, 160°C; column temperature programmed from 50°C to 235°C at



SR 31747A



SR 120496



SR 120684A

Scheme 1. Chemical structures of SR 31747A, SR 120496 and SR 120684A.

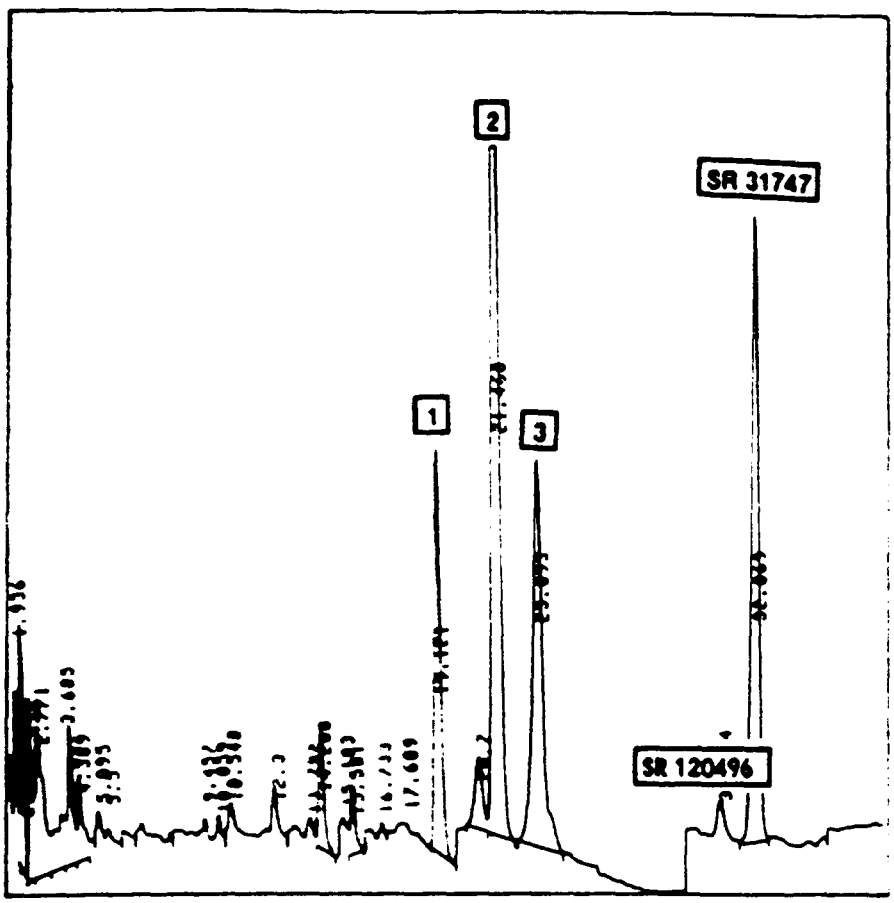


Fig. 1. Biotransformation of SR 31747A with cytochrome P450: HPLC analysis of crude incubation medium. Dog liver microsomal fraction was incubated for 30 min at 37°C with SR 31747A in the presence of NADPH (1 mM). After the prepurification step in a Bondelut CN column, the incubation medium was analyzed by HPLC.

30°C min^{-1}; initial time: 2 min; final time, 17 min. The range of linearity of the method was 10–2000 ng ml^{-1}. The quantification limit was fixed at 10 ng ml^{-1} of SR 31747.

2.6. Plasma samples

Eight healthy volunteers received a single 400 mg oral dose of SR 31747A. Blood samples were collected in heparinized tubes at 0 (predose), 2, 3, 4, 5, and 6 h after dosing. The plasma was immediately separated and stored at −20°C until analysis by the two procedures.

3. Results

3.1. Preparation of the immunogen

The postulated metabolic behavior for SR 31747A described in Scheme 2 has been previously determined [7] using "in-vitro" models such as microsomal fractions and hepatocytes in primary cultures. The main metabolism steps identified were the *N*-deethylation and three hydroxylation levels of SR 31747. Consequently, and in order to obtain antibodies specifically directed against SR 31747, the parent molecule was

coupled to the protein corrier via a peptide bond using SR 120684 (Scheme 1), a succinamic acid derivative of SR 31747A. UV spectroscopy analysis indicated that the final conjugate contained 15 moles of hapten per mole of BSA.

3.2. Production of Mabs against SR 31747

Balb/C mice were immunized with the immunogen described above. After the third immunisation, specific antibody response was observed in mice sera. The mouse displaying the highest titer was selected for hybridoma preparation. After cell fusion, hybridoma grew in 50% of wells. Clones positive for SR 31747 were identified in a RIA screening using [^{3}H] SR 31747A. Among the 30 hybridoma cell lines producing Mabs reactive with SR 31747, three of them were selected for further experiments. They were produced in Balb/C mice and purified as IgG 1 on protein A Sepharose.

3.3. Characterisation of the anti-SR 31747 Mabs

The specificity of anti-SR 31747 Mabs was tested by assessing cross-reactivity with postulated metabolites in RIA carried out without plasma. *N*-deethyl SR 31747 (SR 120496) was obtained by chemical synthesis and hydroxy derivatives by in-vitro biotransformation of SR 31747A by cytochromes P450 of the hepatic microsomal material from beagle dog liver. Dog microsomal fraction was used instead of human fraction because previous studies [9] had demonstrated that if the metabolic pathway was identical whatever the species studied, hydroxy derivatives were obtained in larger quantities with dog then with human microsomal fractions. After incubation in the presence of NADPH, the three peaks corresponding to hydroxy metabolites were isolated by HPLC purification (Fig. 1). RIA results reported in Table 2 show cross-reactivity levels (defined as the ratio of the SR 31747 concentration to that of the test compound needed to attain IC_{50}) lower than 7% with any one of the four metabolites tested with any one of the three Mabs. Yet, for further experiments the Mab 15D4 was selected as it had the weakest level of cross-reactivity.

Table 2
Specificity of Mabs

	Cross-reactivity in %[a]		
Metabolite	14C2	15D4	17B4
SR 120496	0.2	0.2	0.2
P1[b]	0.7	ND[c]	1.7
P2	1.3	2.2	6.6
P3	1.8	1.0	2.5
Total	4.0	3.4	11

[a] Cross-reactivity of compound = [IC_{50} of SR 31747] / [IC_{50} of compound] × 100.
[b] P1–P3 refer to HPLC peaks (see Fig. 1).
[c] Not detectable.

3.4. RIA validation in human plasma with Mab 15D4

3.4.1. Standard curve

A typical standard curve for SR 31747 in the concentration range 0.049–50 ng ml^{-1} in human plasma is shown in Fig. 2. At first, interference due to plasma was examined. Whatever the plasma used, addition of plasma to the reaction medium decreased the binding of tracer to antibody in proportion to the amount of plasma (data not shown). For this reason, the standard series

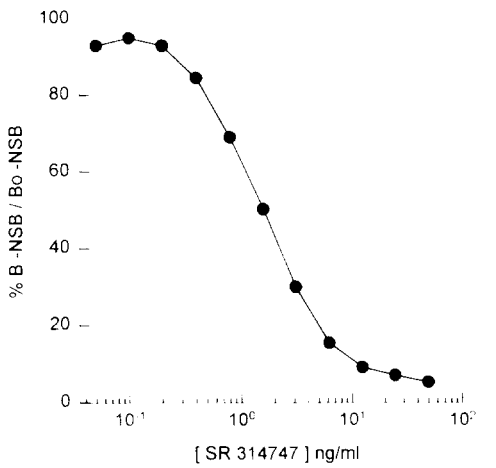


Fig. 2. Standard curve in RIA for SR 31747 in plasma. Calibration curves were constructed using the *B*-NSB/B_0-NSB ratio (linear scale) plotted against SR 31747 concentration (logarithmic scale). Fitting was obtained using WIN'CALC software from Wallac. Samples were analyzed in triplicate.

Table 3
Intra-assay reproducibility

SR 31747 theoretical concentration (ng ml^{-1})	Observed mean[a] (ng ml^{-1})	Standard deviation (ng ml^{-1})	RSD (%)	Accuracy (%)
4	4.22	0.56	13.2	106
1	1.11	0.07	6.3	111
0.5	0.54	0.05	9.3	108
0.25	0.26	0.024	9.2	104

[a] Observed mean from six replicates.

were prepared by adding to each standard solution the same volume of plasma (200 μl from a plasma pool) as that which was to be assayed.

3.4.2. Detection and quantification limit

The detection limit was determined by using the relative standard deviation (RSD) of drug-free plasma blanks and setting the limit at 1.7 times the standard deviation of the mean. Accordingly, the detection limit was calculated to be 0.2 ng ml^{-1}. The limit of quantification was fixed at 0.25 ng ml^{-1} with a precision of 15.4% and an accuracy of 104%.

3.4.3. Reproducibility

Intra- and inter-assay precision were obtained by analysing samples from plasma spiked with different amounts of SR 31747A. To establish intra-assay precision four standard concentrations ranging from 0.25 to 4 ng ml^{-1} were assayed six times. The RSD values were ⩽ 13% and the accuracy was between 104 and 110% (Table 3). For inter-assay evaluation control plasma samples were determined on six different days. The RSD was ⩽ 15% and the accuracy was between 100 and 113% (Table 4).

Table 4
Inter-assay reproducibility

SR 31747 theoretical concentration (ng ml^{-1})	Observed mean[a] (ng ml^{-1})	Standard deviation (ng ml^{-1})	RSD (%)	Accuracy (%)
4	4.01	0.58	14.4	100
1	1.13	0.06	5.3	113
0.5	0.52	0.05	9.6	104
0.25	0.26	0.04	15.4	104

[a] Observed mean from six assays, each in triplicate.

Table 5
Dilution test in plasma spiked with 50 μg ml^{-1} SR 31747A and diluted several times in control plasma

SR 31747 theoretical concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})	Recovery (%)
4.36[a]	3.95	90.0
2.93	3.07	104.8
1.93	2.23	114.4
1.3	1.48	113.8
0.87	1.00	115.0
0.58	0.64	110.3
0.39	0.43	110.2
3.12[b]	3.20	102.6
1.56	1.71	109.6
0.78	0.87	111.5
0.39	0.41	105.1
5.55[c]	5.67	102.2
1.85	2.07	111.9
0.62	0.73	112.1

[a–c] Plasma samples spiked with a large amount of SR 31747A (50 μg ml^{-1}) were diluted several times in control plasma (factor 1.5[a], factor 2[b], factor 3[c]) in order to reach the range of the standard curve. The found concentrations were reported and compared to the theoretical one.

3.4.4. Precision of assay

To determine the precision, dilution and recovery tests were performed with human plasma containing SR 31747A. As shown in Table 5 and 6, nearly quantitative recoveries were observed in all cases. Results obtained (two dilutions) in plasma from subjects who had received SR 31747 confirmed the dilution test (data not shown).

3.4.5. Specificity

The cross-reactivities of the four metabolites are shown in Fig. 3. The percent cross-reaction with SR 120496, P1, P2 and P3 was 0.2, 2, 6, and 4% respectively. These values demonstrated a slight decrease of the specificity when RIA was carried out in the presence of plasma. However, despite a cross-reactivity close to 10%, Mab 15D4 remained suitable for a RIA in plasma.

Table 6
Recovery test with plasma sample spiked with 1, 0.5 and 0.25 ng ml^{-1} of SR 31747A[a]

Initial concentration	Amount added	Theoretical concentration	Measured concentration	Recovery (%)
1.56	1.0	2.56	2.68	104.7
0.78	1.0	1.78	1.74	97.7
0.39	1.0	1.39	1.57	112.9
0.20	1.0	1.20	1.25	104.2
1.56	0.5	2.06	2.29	111.2
0.78	0.5	1.28	1.36	106.3
0.39	0.5	0.89	0.96	107.8
0.2	0.5	0.7	0.83	119.0
1.56	0.25	1.81	2.04	112.7
0.78	0.25	1.03	1.14	110.7
0.39	0.25	0.64	0.65	101.6
0.2	0.25	0.45	0.53	117.5

[a] All values in nanograms per milliliter (except recovery).

3.4.6. Comparative accuracy

In a direct comparative study, 48 samples were assayed by both GC and RIA. The concentrations determined by the two methods were compared by using linear regression and showed a good agreement (Fig. 4). The correlation coefficient was 0.984 and the equation of the regression line was RIA = 1.242 GC + 4.5. The higher RIA values might reflect binding of the antibody to an unknown metabolite different from those assayed in the specificity studies. However, when samples which fell within the more representative clinical range were used, the equation of the regression line became: RIA = 1.06 GC + 16 ($r = 0.94$). These results seem to indicate that the difference is dependent on the concentration range tested and that in the clinically-relevant concentration range the method is suitable for the monitoring of SR 31747 in plasma samples.

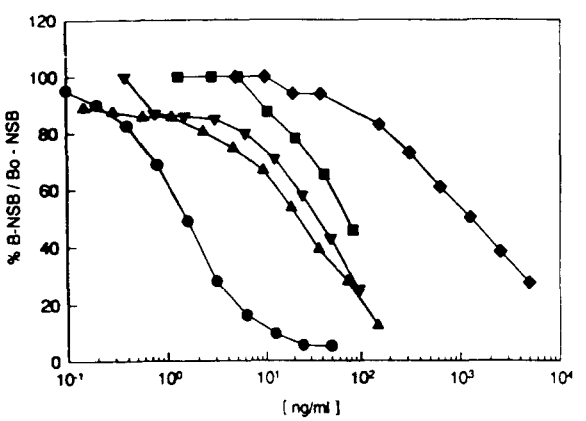


Fig. 3. Specificity of 15D4 antibody in plasma. Cross-reactivity of SR 120696 (◆), HPLC-purified metabolites P1 (■), P2 (▲), P3 (▼), and SR 31747 (●) with Mab 15D4 in the presence of plasma.

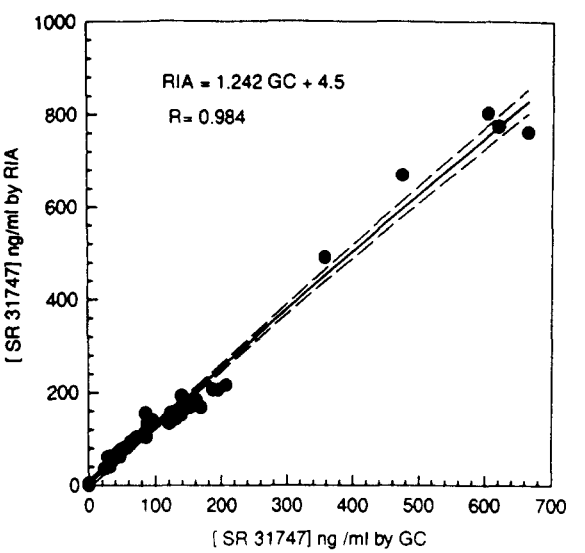
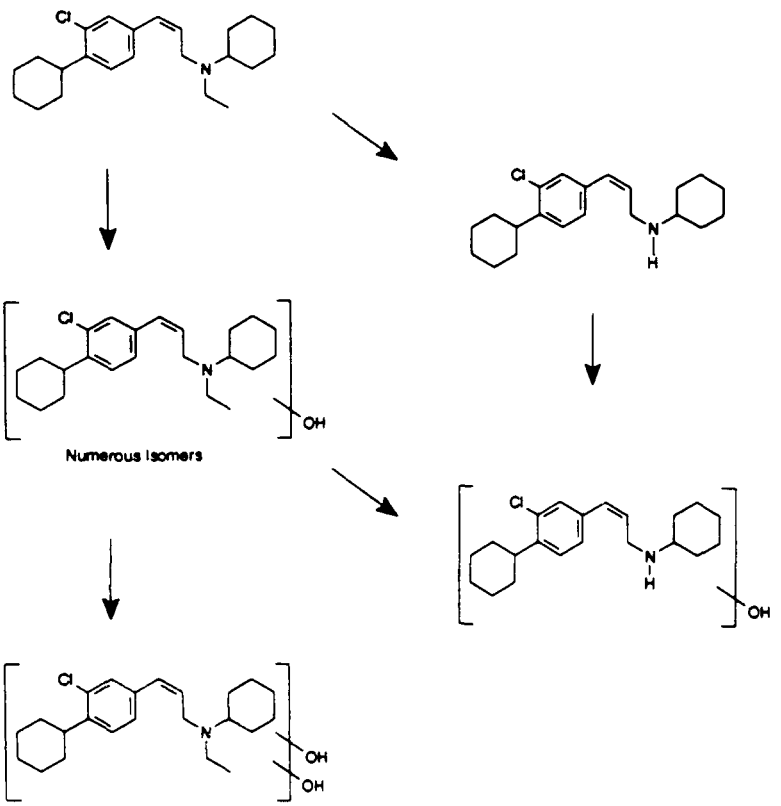


Fig. 4. Correlation of the concentrations of SR 31747 in plasma samples assayed by RIA and GC. SR 31747 concentrations of 48 samples measured by GC assay (x) are plotted against those obtained with the RIA (y). Results of the two methods were related by the regression equation: RIA = 1.242 GC + 4.5 (coefficient of correlation = 0.984).



Scheme 2. Postulated biotransformation pathways for SR 31747A. SR 31747A metabolism was investigated with Human material, using hepatic microsomal fractions and hepatocytes in primary culture. The major metabolic pathways are the *N*-deethylation and mono-hydroxylation processes.

4. Discussion

Preliminary determination of SR 31747 concentrations in human plasma has shown that the available GC method did not have adequate sensitivity to measure SR 31747 in pharmacokinetic studies. A more sensitive RIA method was thus developed using a monoclonal antibody referred to as 15D4. In the development of RIA for drugs, the cross-reactivity of the employed antibody with the metabolites of the drug is the most important determinant of assay specificity. This is why the choice of the immunogen design for immunization requires some previous knowledge of the drug metabolism. In the present case, the postulated metabolism for SR 31747A had been previously investigated using "in-vitro" models [9] on different species. Similar metabolic pathways were observed whatever the species investigated (humans, dogs, baboons, mice). The metabolism of SR 31747A mainly involves hydroxylation at various (although not yet localized) sites of the molecule and *N*-deethylation as determined by mass spectrometry and summarized in Scheme 2. Consequently, the immunogen was prepared by coupling SR 31747A to BSA via a peptide bond using an analogue of SR 31747A bearing a succinamic acid radical in a position opposite the *N*-ethyl residue of the molecule. Cross-reactivity of monoclonal antibodies obtained with such an immunogen was evaluated towards synthetic *N*-deethyl SR 31747 (SR 120496) and three hydroxy derivatives isolated after "in-vitro" incubation of SR 31747A with dog microsomes. Cross-reactivity levels observed were lower than 7% for any one of

the four metabolites tested and for each of the three Mabs.

Cross-reactivity levels of any metabolite for a given antibody should be balanced by the "in-vivo" concentration of the metabolite compared to that of the parent drug. However, in expectation of a fairly well-documented knowledge of the nature and levels of circulating metabolites, Mab 15D4 was selected for further experiments as it presents the weakest level of total cross-reactivity.

Using this Mab a sensitive RIA method was developed which has a detection limit 40-fold lower than that of the GC method. The system is accurate and precise. No sample preparation, such as an extraction or purification step was required, thus making the method suitable for monitoring of SR 31747 in plasma samples from clinical studies.

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