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Determination of a novel angiotensin-AT_1 antagonist CR 3210 in biological samples by HPLC

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Abstract

A simple and sensitive method for the determination of a new angiotensin-AT_1 antagonist i.e. CR 3210, 4-[4-[(2-ethyl-5,7-dimethylimidazo[4,5-*b*]pyridin-3-yl)methyl]phenyl]-3-(2*H*-tetrazol-5-yl)quinoline, is described. The assay was utilised to describe the pharmacokinetic profile of the title compound after intravenous and intraperitoneal administration to Sprague Dawley rats. CR 3210 and the internal standard CR 1505 (loxiglumide, 4-[(3,4-dichlorobenzoyl)amino-5-[(3-methoxypropyl)pentylamino]-5-oxopentanoic acid) were isolated from rat plasma by solid-phase extraction. The sorbent extraction material along with the pH in the conditioning solution and the washing volume were considered pivotal parameters for the optimisation of the procedure. The separations were performed by reversed-phase high-performance liquid chromatography with ultraviolet detection. The samples were injected onto the analytical column (Tracer Extrasil ODS1) and detected at 238 nm, giving a retention time of 6.19 min for CR 3210 and 4.39 min for the internal standard, respectively. The selectivity of the method showed to be satisfactory. The mean recovery of CR 3210 from spiked rat plasma was 80.3 at 1 μg/ml and 79.9 at 2 μg/ml. The lower limit of detection (LOD) was taken as 0.014 μg/ml in plasma samples. The lower limit of quantification (LOQ) was taken as 0.02 μg/ml, the lowest calibration standard using 500 μg rat plasma. The procedures were validated according to international standards with a good reproducibility and linear response from 0.02 to 2 μg/ml. The sensitivity of the method allowed for its application to pharmacokinetic studies. The maximal concentration was detected 5′ after the IV administration, whereas no significant absorption was evident after IP administration of CR 3210 to Sprague–Dawley rats. Our study suggests the absence of extensive bio-transformation of the drug in vivo, supported by the evidence that no metabolites were detected in plasma samples.

Keywords: Angiotensin-AT_1 antagonist; RP-HPLC; Pharmacokinetic

1. Introduction

Angiotensin II (AII) is the biologically active component of the renin-angiotensin system (RAS) and is responsible for most of the peripheral effects of this system. In fact, AII is a major regulator of blood pressure, aldosterone secretion, and fluids homeostasis [1], and is also an important etiological factor in hypertension and other cardiovascular disorders. There are three commonly described classes of effective inhibitors of RAS: renin inhibitors, angiotensin converting enzyme (ACE) inhibitors and AII receptor antagonists. In recent years, renin inhibitors with high specificity and affinity for human renin have been reported [2], but they have yet to be marketed. ACE inhibitors such as Captopril, Enalapril, and others are

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very effective for the treatment of most types of hypertension and congestive heart failure [3]. However, their lack of specificity is a major reason of exploring alternative therapy. Some of the adverse effects of ACE inhibitors such as dry cough and angioedema have been attributed to the multisubstrate action of ACE [4]. AII-receptor antagonists do not have an effect on biologically active peptides of kallikrein–kinin–prostaglandin and it is expected that these agent create fewer clinical problems. Thus, the specific block of the AII actions at the receptor level represents a potentially advantageous approach to modulate the RAS [5]. In humans, two main types of AII receptors subtypes have been characterised and called AT_1 and AT_2 [6]. The AT_1 receptor subtype mediated virtually all of the known physiological actions of AII in cardiovascular, neuronal, endocrine, hepatic and other cells [7]. The discovery of losartan (2-butyl-4-chloro-5-hydroxymethyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl]-4-yl]methyl-1H-imidazole), a potent and orally active non-peptide AII antagonist, has stimulated the design of a large number of congeners, which share the presence of the biphenyl fragment bearing an acidic moiety (tetrazole ring, –COOH, –SO_2–NH–CO–) and differ about the nature of the pendent heterocyclic system connected to the *para* position of the distal phenyl by means of a methylene group. So, within a wide programme aimed at developing new non-peptidic AII antagonists, we synthesised compound CR 3210 [8] which proved to be a new angiotensin AT_1 antagonist endowed with outstanding in vitro properties.

Several analytical procedures have been described for the determination of benzylimidazole derivatives, i.e. losartan and congeners. Reversed-phase high-performance liquid chromatography (HPLC) has been successfully used to determine losartan and its active metabolite in biological fluids [9,10].

In order to define the pharmacokinetic profile of this new AT_1 antagonist, a high sensitive and selective analytical method was needed, which allowed for the determination of CR 3210 in the nanogram range in plasma samples. Moreover, the need for an efficient samples clean-up and chromatographic separation should not be underestimated.

In the present study, we developed a method for determination of CR 3210 and the internal standard CR 1505 (loxiglumide, I.S.) in plasma of Sprague–Dawley rats. The procedure, based on the use of solid-phase extraction (SPE) and reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection, is simple and rapid and provides accurate and precise results.

Our goal was the determination of the pharmacokinetic profile after intravenous (IV) and intraperitoneal (IP) administration of CR 3210 in Sprague–Dawley rats.

2. Experimental

2.1. Chemicals and standards

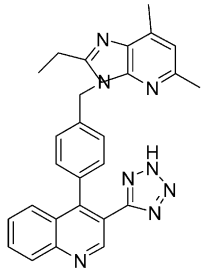
Acetonitrile, methanol and water HPLC grade (Carlo Erba, Milan, Italy) were used; deionized water was utilised for HPLC buffer. All other reagents were of analytical-reagent grade (Carlo Erba, Milan, Italy).

CR 3210 *i.e.* 4-[4-[(2-ethyl-5,7-dimethylimidazo[4,5-*b*]pyridin-3-yl)methyl]phenyl]-3-(2*H*-tetrazol-5-yl)quinoline *w*as synthesized in our laboratories, as previously described [8], while the internal standard CR 1505 i.e. 4-[(3,4-diclorobenzoyl)amino]-5-[(-methoxypropyl)pentylamino]-5-oxopentanoic acid, was a generous gift from Rotta Research Laboratorium (Monza, Italy); the chemical structures are reported in Fig. 1.

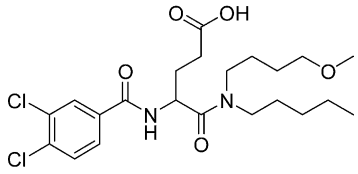
Stock solutions (1 mg/ml) of the studied compound and the I.S. were prepared in methanol. Working solutions of CR 3210 were made by dilution with methanol and used to prepare aqueous standards and spiked plasma samples on a standard curves. The internal standard (I.S.) working solution was prepared at a concentration of 10 μg/ml.

2.2. Chromatography

The chromatographic system comprised a Jasco PU 980 pump and LG 980-02 ternary unit (Tokyo, Japan) with a 100 μl loop injection valve and a variable-wavelength ultraviolet Jasco 975 detector (Tokyo, Japan), set at 238 nm. A Tracer Extrasil ODS1 (25 × 0.46 cm, 5 μm) reversed-phase column (Tecnokroma,



CR3210



CR1505

Fig. 1. Structures of CR 3210 and the internal standard CR 1505.

Barcelona, Spain) was used, with an ODS guard (4.5 × 0.46 cm). The analytical column was heated at 30 °C with a block heater Gastorr GF 103 (Jones Chromatography, CO) The column was eluted with 10 mM potassium dihydrogen orthophosphate/methanol (40:60, v/v, pH 2.85)/acetonitrile, 55:45, v/v. The mobile phase was delivered with a flow rate of 1 ml/min. Data were processed by means of Borwin chromatography software (version 1.21) from Jasco (Tokyo, Japan).

2.3. Animal treatments

Sprague–Dawley rats were housed three per cage in stable conditions of humidity (60 ± 5%) and temperature (22 ± 2 °C), and allowed free access to food and water until the time of the experiments. The animals were maintained on 12-h light, 12-h dark cycle (lights on 07:00–19:00 h, off 19:00–07:00 h).

CR 3210 was dissolved in a solution containing 50% distilled water and 50% sterile saline, and administered intraperitoneally (8 mg/kg body mass) and intravenously (5 mg/kg body mass) to Sprague–Dawley rats. Blood samples were withdrawn from the animals by indwelling catheter ($n = 3$ each time analysed for the two different administration), at 5, 15, 30, 60, 120, 180, 240, 360, 480 and 720 min after IP and IV administrations of the drug. Blood cells were removed by centrifugation and separated plasma was stored at −20 °C until assay.

2.4. Extraction

In the present method, acidified biological samples were purified by solid-phase extraction. A 500-μl aliquot of rat plasma were mixed with 500-μl of 0.2 M citrate buffer (pH 3.0), internal standard (40 μl, 100 μg/ml), and methanol or appropriate calibration standard (50 μl). CR 3210 and I.S. were isolated by means of conditioned Oasis SPE cartridges HLB (3 ml, 30 mg) from Waters (Milford, MA), provided with a LiChrolut extraction unit (Merck). After cartridges have been washed with 2 ml of water containing 5% methanol, the analyte and the I.S. were eluted with 2 ml of methanol, twice. The eluate were dried under a nitrogen stream at 45 °C and the residue were dissolved in 300 μl of H_2O/MeOH (1:3 v/v). Aliquots of 100 μl were injected onto the chromatographic apparatus.

2.5. Calibration curve and method validation

Compounds CR 3210 (50 μl, 10 μg/ml) and the internal standard CR 1505 (40 μl, 100 μg/ml) were injected into the column and identified by their relative retention time. Calibration curves were obtained by plotting the peak area ratio of the CR 3210 to the I.S., versus the theoretical concentration of the analyte added to drug-free rat plasma. The curves were constructed from six replicate measurements of five concentrations of CR 3210, over a range of 0.02–2 μg/ml. The data will be subjected to least-squares regression analysis; the back-calculated calibration standard concentrations were also considered.

Plasma and samples containing a known amount of CR 3210 and I.S. were prepared and stored frozen at −20 °C until use. These samples were utilised as quality control specimens, to compare the measured to the theoretical concentrations of CR 3210.

The precision of the bioanalytical method was evaluated on the basis of the coefficients of variation occurring within (intra-) and between (inter-) batch analyses. The accuracy was expressed in terms of relative error of measurement.

The intra-batch precision and accuracy were calculated by six replicate analysis at each chosen concentration (0.2, 0.5, 1 and 2 μg/ml) and repeated twice (batches 1 and 2). The inter-batch precision and accuracy were determined by considering the coefficients of variation and the mean relative errors of the measurements over a period of six weeks ($n = 6$ at each concentration).

The specificity of the analytical method was assessed by comparison of chromatograms for the presence of interfering peaks and changes in retention times. Chromatograms of extracted plasma samples, derived from non treated animals, and spiked with internal standard at low and high concentration of CR 3210 and internal standard were examined.

2.6. Extraction efficiency

The mean recovery of CR 3210 and I.S. from spiked rat plasma was evaluated to test the efficiency and reproducibility of the extraction procedure. The determination of the extraction efficiency was performed by adding amounts of 1 and 2 μg/ml of both compounds in replicate ($n = 3$), in all samples. The extraction was conducted as described above and 40 μl of internal standard working solution were added prior to the extraction. The responses of these standards taken by means of the extraction procedures have been compared with those of standard solution at the same concentration injected directly into the liquid chromatographic apparatus. The peak-area ratios were compared to the ratio of the standard aqueous samples without extraction.

3. Results and discussion

3.1. Detection and sensitivity

Fig. 2 shows the retention time of the tested compounds. The drugs and the internal standard were detected at 238 nm and the retention times were 4.39

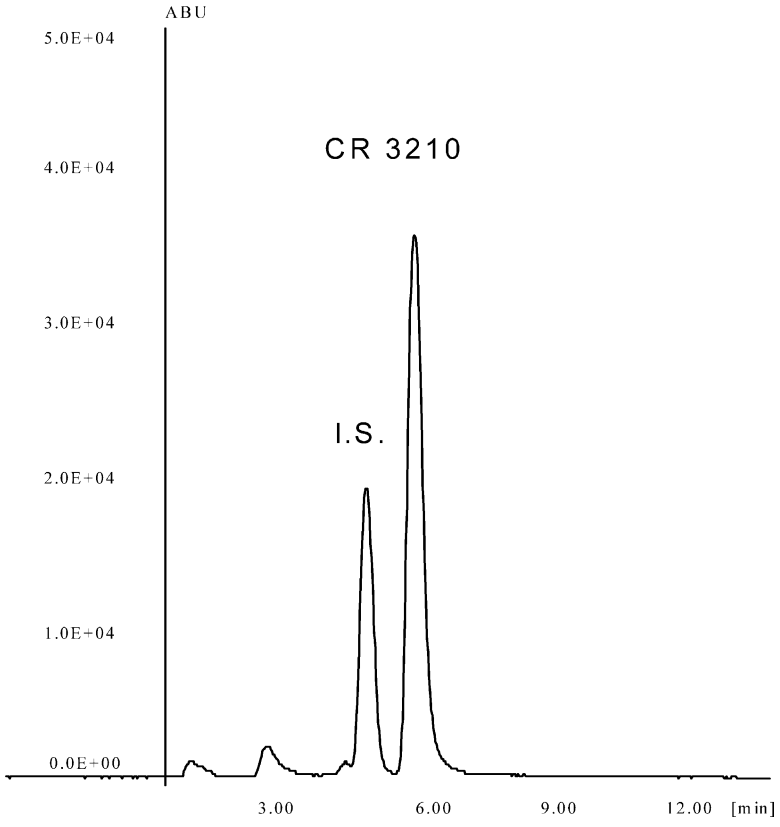


Fig. 2. Representative chromatogram of CR 3210 and the internal standard CR 1505 used in the analytical procedures; the relative retention times are 6.19 and 4.39 min, respectively.

min (±0.14) for CR 1505 and 6.19 min (±0.09) for CR 3210.

The lower limit of detection (LOD), with a signal-to-noise ratio of 3, was taken as 0.014 μg/ml in plasma samples. The lower limit of quantification (LOQ) was taken as 0.02 μg/ml, the lowest calibration standard using 500 μg of rat plasma.

3.2. Linearity

A linear response was observed over the examined concentration range (0.02–2 μg/ml). The mean regression coefficient was 0.99007 ($n = 6$) in plasma. The back-calculated values for each calibration standard within the calibration range, expressed as mean relative error, ranged from 1.4 to +8.6% in plasma. Calibration measurements and back-calculated values are summarised in Table 1.

3.3. Precision, accuracy and extraction efficiency

Table 2 reports the CR 3210 precision and accuracy measurements of intra-batch and inter-batch assays in plasma.

In intra-batch assay the precision, indicated by the coefficient of variation of the measured concentration of replicate control samples and determined in batches one and two, ranged between 1.65 at 0.2 μg/ml, as determined in batches one and 9.45 at 0.5 μg/ml, as determined in batches two. The accuracy of measurement, expressed in terms of mean relative errror in batches one and two, ranged between 4.5 and −4% at a concentration of 0.2 μg/ml; between −2.2 and 1.1% at a concentration of 0.5 μg/ml; between 5.3 and 5.5% at concentration of 1 μg/ml and between 2.2 and 2.3% at concentration of 2 μg/ml, respectively.

In the inter-batch precision measurements of the assay over two occasions ($n = 6$ for each concentration), the accuracy of measurement was −7% at 0.2 μg/ml and 5.1% at 1 μg/ml.

Table 3 reports the extraction efficiency, expressed as mean recovery of CR 3210 from rat plasma. The recovery in plasma ranged from 80.3 to 79.9%, the calculated extraction efficiency was independent from the concentration.

In the extracts of three separate batches of blank rat plasma, there were no interfering peaks present in chromatograms corresponding to the retention times of CR 3210 or I.S., which affected the precision and accuracy of measurements at the low limit of quantification (lowest calibration standard).

Table 1
Calibration measurements of CR 3210 in drug-free rat plasma ($n = 6$)

Calibration no.	Concentration (μg/ml)				
	0.02	0.2	0.5	1	2
Relationship between CR3210 and IS areas					
1	0.0367	0.0892	0.2931	0.7865	1.2982
2	0.0491	0.0990	0.2923	0.6493	1.0563
3	0.0421	0.0930	0.2997	0.6659	1.0646
4	0.0481	0.1133	0.2931	0.5865	0.9902
5	0.0357	0.0976	0.2715	0.6695	1.1985
6	0.0443	0.1095	0.2895	0.7375	1.1940
Mean	0.0427	0.1003	0.2899	0.6825	1.1336
SD [a]	0.0056	0.0094	0.0096	0.0701	0.1151
CV % [b]	13.17	9.35	3.31	10.27	10.15
Retro calculation of concentration values ($n = 6$)					
1	0.018	0.179	0.473	1.094	2.072
2	0.024	0.285	0.523	0.987	1.980
3	0.019	0.197	0.487	1.147	2.077
4	0.024	0.215	0.518	0.979	1.976
5	0.026	0.209	0.507	1.098	1.945
6	0.020	0.176	0.565	1.114	2.119
Mean	0.022	0.210	0.512	1.070	2.028
Sd [a]	0.004	0.040	0.032	0.070	0.07
CV % [b]	16.332	18.957	6.258	6.529	3.452
RE % [c]	8.667	5.083	2.433	6.983	1.408

[a] SD, standard deviation.
[b] CV %, coefficient of variation.
[c] RE %, relative error.

3.4. Application to a pharmacokinetic study

The present method has been applied to the pharmacokinetic study of CR 3210 after IV and IP administration to Sprague–Dawley rats.

Fig. 3 shows the chromatogram from drug-free rat plasma, where is evident that no interfering peaks are present at the retention times of CR 3210 and CR 1505.

Fig. 4 shows the chromatogram from rat plasma sample obtained 5 min after IV administration of CR

Table 2
Precision and accuracy measurements of CR 3210 in plasma ($n = 6$)

Nominal concentration (μg/ml)	Measured concentration (μg/ml) [a]	CV % [b]	RE % [c]
Batch 1			
0.2	0.209 ± 0.003	1.65	4.5
0.5	0.489 ± 0.030	6.05	−2.2
1	1.053 ± 0.058	5.54	5.3
2	2.044 ± 0.053	2.61	2.2
Batch 2			
0.2	0.192 ± 0.007	3.41	−4.0
0.5	0.506 ± 0.039	7.79	1.1
1	1.055 ± 0.028	2.60	5.5
2	2.045 ± 0.077	3.78	2.3
Inter-batch measurements ($n = 6$)			
0.2	0.186 ± 0.010	5.32	−7.0
0.5	0.521 ± 0.015	2.85	4.2
1	1.051 ± 0.036	3.43	5.1
2	2.045 ± 0.032	1.55	2.3

[a] Mean ± standard deviation.
[b] CV %, coefficient of variation.
[c] RE %, relative error.

Table 3
Recovery measurement of CR 3210 from plasma ($n = 3$)

Added concentration (μg/ml)	Measured concentration (μg/ml) [a]	Recovery %	RE % [b]
1	0.803	80.3	19.7
2	1.58	79.9	21.0

[a] Mean ± standard deviation.
[b] RE %, relative error.

3210 spiked with CR 1505. The peak referred to the studied compound and the I.S. are the only two peaks present.

Fig. 5 shows chromatogram from rat plasma sample obtained 120 min after IP administration of CR 3210 spiked with CR 1505. Also in this chromatogram, there is a presence of a peak due to the studied compound and only another peak corresponding to the I.S. This feature suggested that CR 3210 is not subjected to extensive biotransformation in vivo.

Fig. 6 shows the time profiles of plasma concentration of Sprague–Dawley rats treated intravenously and intra-peritoneally with CR 3210. We observe two curves representing the variation of the concentrations over the time, related to the different type of administration of the drug.

The IV administration of CR 3210 determined a peak plasma concentration of 3.404 μg/ml (C_{max}) that was achieved after 5 min (T_{max}); the plasma levels decreased significantly after 30 min.

The IP administration of CR 3210 determined a peak plasma concentration of 0.642 μg/ml (C_{max}) that was achieved after 120 min (T_{max}). This curve showed a quite flat profile, indicating a not significant absorption of the drug.

4. Conclusions

The method here described resulted sensitive and specific for the determination of this new AT_1-antagonist in rat plasma.

The extraction procedures demonstrated a good efficiency. Solid-phase extraction allowed to isolate CR 3210 from rat plasma with a good recovery. The method has been fully validated, the inter-assay accu-

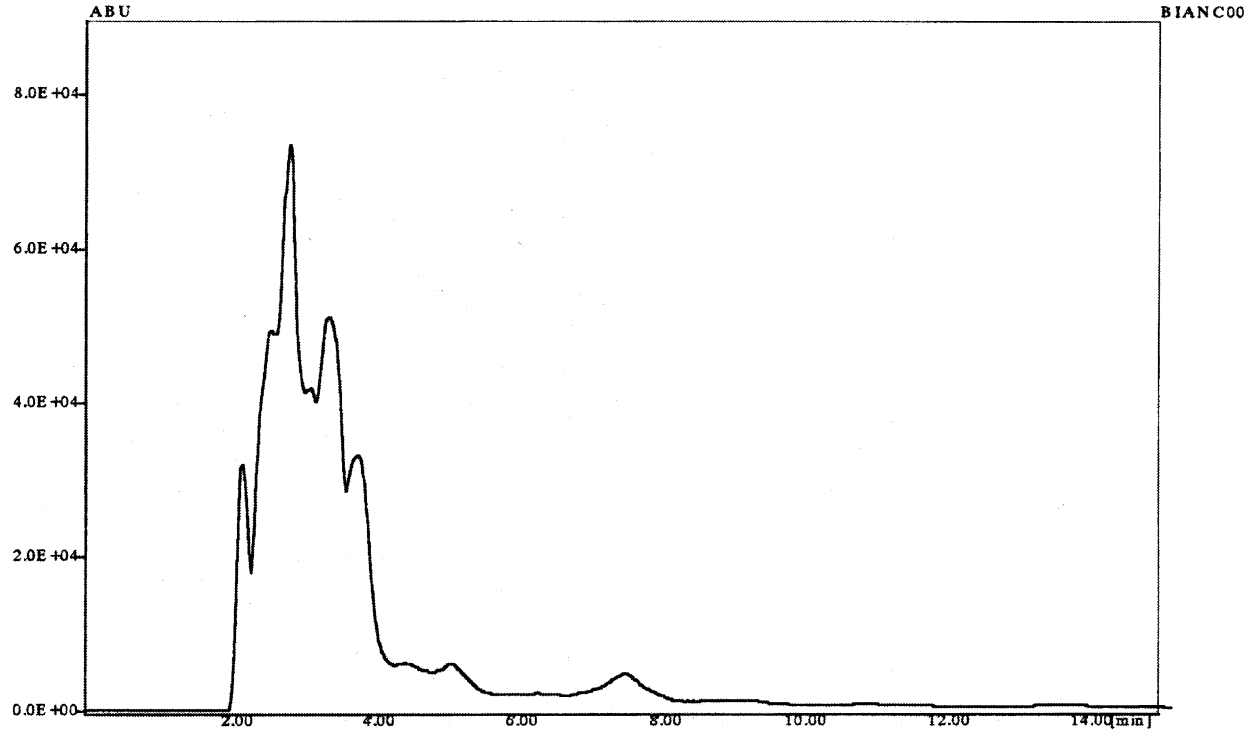


Fig. 3. Chromatogram of drug-free rat plasma.

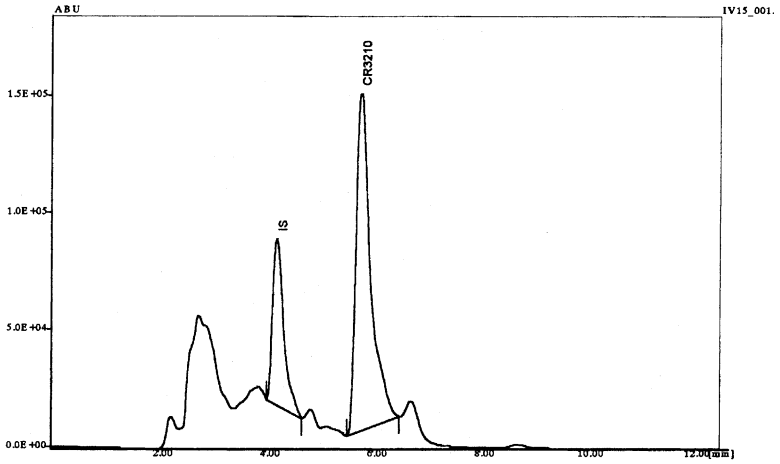


Fig. 4. Chromatogram of rat plasma obtained 5 min after IV administration of CR 3210 spiked with I.S. (40 μl, 10 μg/ml).

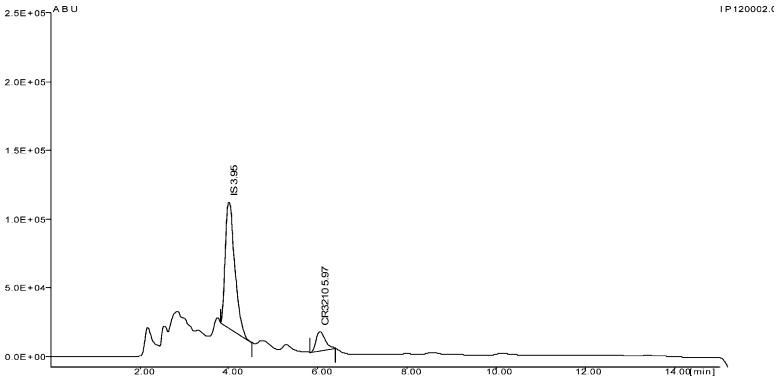


Fig. 5. Chromatogram of rat plasma obtained 120 min after IP administration of CR 3210 spiked with I.S. (40 μl, 10 μg/ml).

racy and recovery showed to be adequate for the analysis of biological samples. Furthermore, the sensitivity of the method allowed the pharmacokinetic study of the CR 3210 in Sprague–Dawley rats.

The plasmatic time course indicated that the intraperitoneal administration of this drug was not useful to reach an adequate plasma concentration. Conversely, the intravenous administration gave an appreciable plasma concentration, even if the CR 3210 plasma level rapidly decreased after 15 min.

Moreover, our study suggested that the CR 3210 is an AT_1 receptor antagonist which is not subjected to extensive biotransformation in vivo.

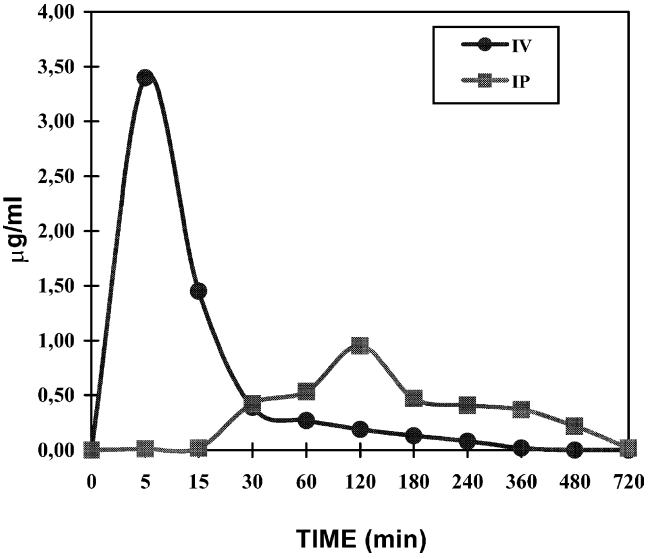


Fig. 6. Time profiles of plasma concentrations after IV and IP administration of CR 3210. The ordinate shows the plasma level, abscissa shows the time after the administrations of the drug ($n = 3$; error bars = SD).

Acknowledgements

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