Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 321–329
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Sensitive SPE–HPLC method to determine a novel angiotensin-$AT_1$ antagonist in biological samples

M. Rizzo [a,*], D. Ventrice [a], F. Monforte [a], S. Procopio [a], G. De Sarro [b], M. Anzini [c], A. Cappelli [c], F. Makovec [d]

[a] *Dipartimento di Scienze Farmacobiologiche, Università degli Studi "Magna Græcia" di Catanzaro, Complesso Ninì Barbieri, Roccelletta di Borgia (CZ) 88021, Italy*
[b] *Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi "Magna Græcia" di Catanzaro, Via T. Campanella, Catanzaro 88100, Italy*
[c] *Dipartimento Farmaco Chimico Tecnologico, Via A. Moro and "European Research Center for Drug Discovery and Development", Università di Siena, Siena 53100, Italy*
[d] *Rotta Research Laboratorium S.p.A., Via Valosa di Sopra, Monza (Mi) 7-20052, Italy*

Received 29 July 2003; received in revised form 4 December 2003; accepted 5 December 2003

**Abstract**

A high-performance liquid chromatography (HPLC)-method after solid-phase extraction (SPE) has been developed in order to determine a new angiotensin-$AT_1$ antagonist, i.e. CR 3210 ($C_{27}H_{24}N_8$; MW = 460.54), 4-[4-[(2-ethyl-5,7-dimethylimidazo[4,5-*b*]pyridin-3-yl)methyl]phenyl]-3-(2*H*-tetrazol-5-yl)quinoline in rat plasma and urine after oral administration to Sprague–Dawley rats. CR 3210 and the internal standard (IS) CR 1505 (loxiglumide), i.e. 4-[(3,4-dichlorobenzoyl)amino]-5-[(3-methoxypropyl) pentylamino]-5-oxopentanoic acid, were isolated from rat urine and plasma by solid-phase extraction. The procedure was optimized regarding the sorbent extraction material, the pH in the conditioning solution, the washing step, the dry time and the type of elution solvent. The separation was 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 capacity factor of 1.87 for CR 3210 and 1.10 for the internal standard. The selectivity of the method was satisfactory. The mean recovery of CR 3210 from spiked rat plasma was 68.5 at 75 ng/ml and 80.9 at 3000 ng/ml; the mean recovery of CR 3210 from spiked rat urine was 69.9 at 75 ng/ml and 78.6 at 3000 ng/ml. The lower limit of detection (LOD) was 14 ng/ml in plasma and 22 ng/ml in urine samples. The lower limit of quantification (LOQ) was taken as 30 ng/ml, the lowest calibration standard using 500 μl rat plasma and urine. The procedures were validated according to international standards with a good reproducibility and linear response from 30 to 3000 ng/ml, for either plasma or urine. The sensitivity of the method allowed for its application to pharmacokinetic studies.


*Keywords:* Angiotensin-$AT_1$ antagonist; SPE–HPLC; Pharmacokinetic

* Corresponding author. Tel.: +39-096-139-1131/1157; fax: +39-096-139-1490.
*E-mail address:* rizzomilena@unicz.it (M. Rizzo).

0731-7085/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.12.004

## 1. Introduction

The most of the peripheral effects of the renin–angiotensin system (RAS) is due to a biologically active component: Angiotensin II (AII). This is a major regulator of blood pressure, aldosterone secretion, and fluids homeostasis that is also an important etiological factor in hypertension and other cardiovascular disorders [1,2]. In recent years, renin inhibitors with high specificity and affinity for human renin have been reported [3], but they have yet to be marketed.

An important class of anti hypertensive agents are represented by angiotensin converting enzyme (ACE) inhibitors that are effective for the treatment of most types of hypertension and congestive heart failure [4]. Nevertheless they possess some adverse effects, such as dry cough and angioedema [5].

A potentially advantageous approach to modulate the RAS is represented by a specific block of the AII actions at the receptor level [6]. The $AT_1$ receptor subtype mediated virtually all of the known physiological actions of AII in cardiovascular, neuronal and other cells [7–9].

The first potent and orally active non-peptide AII antagonist was losartan (2-butyl-4-chloro-5-hydroxymethyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl]-4-yl]methyl-1H-imidazole).

In order to develop new non-peptidic angiotensin $AT_1$ antagonist, we recently synthesized CR3210 ($C_{27}H_{24}N_8$) 4-[4-[(2-ethyl-5,7-dimethylimidazo[4,5-*b*]pyridin-3-yl)methyl]phenyl]-3-(2*H*-tetrazol-5-yl) quinoline [10]. This structure is closely related to losartan, and proved to act in vitro as an angiotensin II receptor antagonist. These substances have been developed in sequence to the angiotensin converting enzyme (ACE) inhibitors as a further therapeutic action on the renin–angiotensin–aldosterone system.

Reversed-phase high-performance liquid chromatography (HPLC) has been successfully used to determine benzylimidazole derivatives, i.e. sartane as losartan and its active metabolites in biological fluids [11–14].

Our previous study reported the time profile of CR 3210 determined after intravenous and intraperitoneal administration to Sprague–Dawley rats [15]. 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.

In consequence, the aim of this study was to define the pharmacokinetic profile of CR 3210 after oral administration, and to asses the urinary excretion over 36 h.

A high sensitive and selective analytical method was developed to determine this new drug in the nanogram range in plasma and urine samples.

Solid-phase extraction (SPE) was chosen to provide an efficient samples clean-up, that should not be underestimated, in particular for urine samples.

HPLC was used to determine CR 3210 and the internal standard (IS) CR 1505 (loxiglumide), 4-[(3,4-dichlorobenzoyl)amino]-5-[(3-methoxypropyl)pentylamino]-5-oxopentanoic acid, in plasma and urine of Sprague–Dawley rats. The method is simple and rapid and provides accurate and precise results.

## 2. Experimental

### 2.1. Chemicals and standards

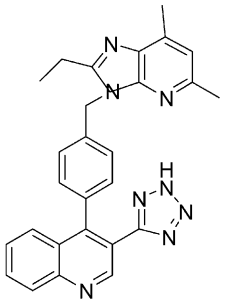
Acetonitrile, methanol and water HPLC grade (Carlo Erba, Milan, Italy) were used; deionized water was utilized to prepare buffers solution. All other reagents were of analytical-reagent grade (Carlo Erba, Milan, Italy).

CR 3210 was synthesized in our laboratories as previously described [8]; the internal standard CR 1505 was loxiglumide. Those compounds were supplied from Rotta Research Laboratorium (Monza, Italy). The structures of CR 3210 and CR 1505 are reported in Fig. 1.

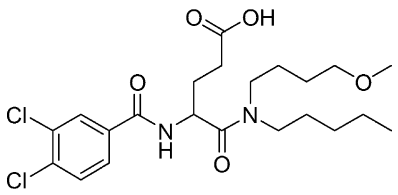
Stock solutions (1 mg/ml) of the studied compound and the IS were prepared in methanol. Working solutions of CR 3210 were made by dilution with methanol and used to prepare spiked plasma and urine samples on a standard curves. The working solution was prepared at a concentration of 10 μg/ml.

### 2.2. Chromatography

A Jasco PU 980 pump and LG 980-02 ternary unit (Tokyo, Japan) with a 100 μl loop injection valve was used. The chromatographic system was



**CR 3210**



**CR 150**

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

associated to a variable-wavelength ultraviolet Jasco 975 detector (Tokyo, Japan), set at 238 nm. The separation was performed on a Tracer Extrasil ODS1 (25 cm × 0.46 cm, 5 μm) reversed-phase column (Tecnokroma, Barcelona, Spain), with a ODS guard (4.5 cm × 0.46 cm). A block heater Gastorr GF 103 (Jones Chromatography, Colorado, USA) was utilized to maintain the analytical column at 30 °C. The mobile phase was formed by solvent A (10 mM potassium dihydrogen orthophosphate–methanol; 40:60 (v/v), pH = 2.85) and solvent B (acetonitrile) in ratio of 55:45 (v/v). It was delivered with a flow rate of 1 ml/min through the columns. Data were processed using 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 7.00 a.m. to 7.00 p.m., off 7.00 p.m. to 7.00 a.m.).

CR 3210 was dissolved in a solution containing 50% distilled water and 50% sterile saline, and administered orally (30 mg/kg body mass) to Sprague–Dawley rats. Blood samples were withdrawn from the animals by indwelling catheter ($n = 3$ each time analyzed), at 0.25–0.5–1–2–4–6–8–12–24 and 36 h after oral (OS) administrations of the drug. Blood cells were removed by centrifugation and separated plasma was stored at −20 °C until assay.

Urine samples were collected in metabolic cages contemporary to plasma collection and stored at 4 °C, before the assay. In detail, eight different aliquots of urine were collected after the drug administration, in sequence from 0 to 1 h, from 1 to 2 h, from 2 to 4 h, from 4 to 6 h, from 6 to 8 h, from 8 to 12 h, from 12 to 24 h and from 24 to 36 h.

### 2.4. Extration

The solid-phase extraction procedure was applied to acidified plasma and urine samples. A 500 μl aliquot of rat plasma and the collected aliquots of urine were mixed with correspondent volumes of 0.2 M citrate buffer (pH = 3.0). The internal standard (40 μl, 10 μg/ml) and methanol, or appropriate calibration standard (50 μl), were also added to each plasma and urine sample. Conditioned Oasis SPE cartridges HLB (3 ml, 30 mg) from Waters (Milford, MA, USA), provided with a LiChrolut extraction unit (Merck), were chosen to isolate CR 3210 and I.S. from biological samples. After cartridges washing with 2 ml of water containing 5% methanol, the analyte and the I.S. were eluted with 2 ml of methanol, twice. For all the dry steps a pressure was maintained at 11 in. Hg for 3 min. The eluate was dried under a nitrogen stream at 45 °C and the residue was dissolved in 300 μl of $H_2O$/MeOH (1:3, v/v). A third part of this extract underwent to the chromatographic separation.

### 2.5. Calibration curve and method validation

The chromatographic identification of the compounds CR 3210 (50 μl, 10 μg/ml) and the internal standard CR1505 (40 μl, 10 μg/ml) were realized by their relative capacity factors. Calibration curves were obtained by plotting the peak-area ratio of the CR 3210 to the IS, versus the theoretical concentration of the analyte added to drug-free rat plasma and urine. The

curves were constructed from replicate measurements of seven concentrations of CR 3210, over a range of 30–3000 ng/ml. The data were subjected to least squares regression analysis (weighted as appropriate), to provided information on correlation coefficients and the back-calculated standard concentrations.

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

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

The intra-batch precision and accuracy were calculated by six replicate analysis at each chosen concentration (30, 60, 350 and 2700 ng/ml) and repeated twice (batch 1 and 2 for plasma and batch 3 and 4 for urine samples).

The inter-batch precision and accuracy were determined by considering the coefficients of variation and the mean relative errors of the combined measurements ($n = 12$ at each concentration).

Chromatograms from blank extracted samples, samples spiked with internal standard alone, and samples spiked with low and high concentration of CR 3210 and internal standard were examined.

Moreover, the presence of interfering peaks and changes in retention times were assessed in chromatograms obtained from plasma and urine analysis to verify the specificity of the analytical method.

### 2.6. Extration efficiency and stability

The efficiency and reproducibility of the extraction procedure was evaluated by measuring the mean recovery of CR 3210 and I.S. from spiked rat plasma and urine.

The determination of the extraction efficiency in all samples was made by adding amounts of 75, 1500 and 3000 ng/ml in replicate ($n = 6$). 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 were compared with those of standard solutions 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. The recovery of test substance was performed at low, medium and high concentrations on the calibration range.

The stability of CR 3210 was assessed in heparinized rat plasma by analyzing 200 μl stability samples at concentrations of 75 and 3000 ng/ml. Samples were analyzed immediately after preparation and after storage at room temperature (ca. +22 °C) for 2 h, frozen (ca. −20 °C) after 14, 28 and 70 days of storage.

The effect of three freeze-thawing cycles on the samples was evaluated at low and high concentrations. Standards were stored at ca. −20 °C for 1 day, thawed at ca. +22 °C for two 2 h; therefore refrozen for 1 day and re-thawed for ca. 2 h twice. Three replicate determinations were made in each case and at each time. The concentrations of CR 3210 determined in the stability samples were compared to the theoretical spiked concentrations.

## 3. Results and discussion

The HPLC method proposed provides a simple procedure for the determination of a novel angiotensin-$AT_1$ antagonist in biological samples.

The ultraviolet spectrum of CR 3210 shows two maxima (238 and 280 nm) and the internal standard CR 1505 has maximum at about 240 nm. A detection of 238 nm was chosen to detect both substances with a good sensitivity.

CR 3210 is a lipophilic molecule, where is present a quinoline bearing an acidic tetrazole moiety. In the structure of loxiglumide is present, as acidic moiety, a carboxylic group. Therefore a solid phase extraction, based on a polymeric matrix with a high hydrophilic–lipophilic balance was chosen. It gives high and reproducible recoveries for acidic and neutral compounds, even if the cartridge runs dry.

In preliminary studies, the removal of interfering plasma ad urine components was attempt by SPE washing the column with eluent at different pH values. Washing with acid acetic 5% or $NH_4OH$ 5% in methanol–water (70:30, v/v) resulted in low recoveries and/or a lot of interferences in the chromatograms. The largest recoveries and the cleanest extraction

procedure were achieved washing the column with water containing 5% methanol, with a dry step of 3 min.

Chromatographic condition were based on the isocratic separation on a reverse phase column. Preliminary studies with different mobile phase combination of acetonitrile and phoshate buffer were considered. The polarity of the drugs made difficult the obtainment of a complete resolution of CR 3210 and CR 1505 in a reasonable time. High percentage of organic solvent needed, therefore methanol was used as second organic modifier. The optimum pH of the hydro–alcoholic mixture was found to be 2.85.

The method was validated with regard to sensitivity, linearity, limits of detection and quantitation, precision, accuracy, extraction efficiency and stability.

### 3.1. Detection and sensitivity

The drugs and the internal standard were detected at 238 nm and the capacity factors were 1.87 for CR 3210 and 1.10 for the internal standard. The lower limit of detection (LOD), with a signal-to-noise ratio of 3, was taken as 14 ng/ml in plasma and 22 ng/ml in urine samples. The lower limit of quantification (LOQ) was taken as 30 ng/ml, the lowest calibration standard using 500 μg rat plasma and urine.

### 3.2. Linearity

A linear response was observed over the examined concentration range (30–3000 ng/ml). The mean regression coefficient was 0.99011 ($n = 6$) in plasma and 0.98970 in urine ($n = 3$). The variation coefficient of calibration measurements ranged between 0.27 and 4.26% in plasma, and between 0.32 and 5.93% in urine. The back-calculated values, for each calibration standard within the calibration range, were determined in plasma and urine samples and are summarized in Table 1.

Table 1
Calibration measurements of CR 3210

| Calibration number | Concentration (ng/ml)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | 30 | 75 | 150 | 300 | 750 | 1500 | 3000 |
| 1 | 30.4 | 71.3 | 146.2 | 295.6 | 749.2 | 1498.0 | 3004.0 |
| 2 | 31.7 | 73.5 | 158.9 | 294.7 | 755.9 | 1495.0 | 3001.5 |
| 3 | 33.1 | 72.8 | 152.9 | 307.7 | 749.8 | 1488.5 | 2989.0 |
| 4 | 32.8 | 71.4 | 154.8 | 305.8 | 759.2 | 1522.5 | 3002.0 |
| 5 | 31.9 | 75.9 | 148.6 | 304.6 | 777.6 | 1511.5 | 3004.5 |
| 6 | 31.3 | 77.4 | 151.2 | 302.7 | 765.2 | 1497.5 | 2998.5 |
| Mean | 31.7 | 72.5 | 152.7 | 299.3 | 751.6 | 1493.8 | 2998.2 |
| CV (%) | 4.26 | 1.55 | 4.16 | 2.43 | 1.24 | 0.33 | 0.27 |
| RE | 5.78 | −3.29 | 1.78 | −0.22 | 0.22 | −0.41 | −0.06 |
| | Concentration (ng/ml)[b] | | | | | | |
| | 30 | 75 | 150 | 300 | 750 | 1500 | 3000 |
| 1 | 29.5 | 72.7 | 147.9 | 298.4 | 751.9 | 1492.0 | 2986.5 |
| 2 | 26.2 | 76.9 | 146.1 | 276.9 | 749.1 | 1496.5 | 3005.7 |
| 3 | 27.8 | 71.2 | 153.9 | 285.2 | 759.5 | 1515.0 | 2993.9 |
| Mean | 27.8 | 73.6 | 149.3 | 286.8 | 753.5 | 1501.2 | 2995.4 |
| CV (%) | 5.93 | 4.01 | 2.74 | 3.78 | 1.88 | 0.81 | 0.32 |
| RE | −7.22 | −1.87 | −0.47 | −4.39 | 0.47 | 0.08 | −0.15 |

CV: coefficient of variation; RE: mean relative error.
[a] Back-calculated values of concentration in drug-free rat plasma.
[b] Back-calculated values of concentration in drug-free rat urine.

Table 2
Determination of precision and accuracy in rat plasma

| Concentration spiked (ng/ml) | Mean concentration found (ng/ml) | S.D. | CV (%) | RE (%) |
|---|---|---|---|---|
| Batch 1 ($n = 6$)[a] | | | | |
| 30 | 33.18 | 1.78 | 5.4 | 10.6 |
| 60 | 57.60 | 4.35 | 7.5 | −4.0 |
| 350 | 345.34 | 4.14 | 1.2 | −1.3 |
| 2700 | 2814.29 | 72.68 | 2.6 | 4.2 |
| Batch 2 ($n = 6$)[a] | | | | |
| 30 | 34.43 | 1.92 | 5.6 | 14.8 |
| 60 | 58.59 | 1.59 | 2.7 | −2.3 |
| 350 | 357.91 | 17.15 | 4.8 | 2.3 |
| 2700 | 2814.29 | 33.12 | 1.2 | 1.3 |
| Summary of combined measurements in batches 1 and 2[b] | | | | |
| 30 | 33.81 | 0.88 | 2.6 | 12.7 |
| 60 | 57.61 | 1.37 | 2.4 | −4.0 |
| 350 | 351.62 | 8.89 | 9.2 | 0.5 |
| 2700 | 2754.36 | 56.67 | 2.1 | 2.0 |

[a] Intra-batch precision and accuracy measurements of CR 3210.
[b] Inter-batch precision and accuracy measurements of CR 3210.

### 3.3. Precision, accuracy and extration efficiency

The CR 3210 precision and accuracy measurements of intra-batch and inter-batch assay in plasma and in urine are reported in Tables 2 and 3, respectively.

Table 3
Determination of precision and accuracy in rat urine

| Concentration spiked (ng/ml) | Mean concentration found (ng/ml) | S.D. | CV (%) | RE (%) |
|---|---|---|---|---|
| Batch 3 ($n = 6$)[a] | | | | |
| 30 | 34.66 | 3.05 | 8.8 | 15.6 |
| 60 | 59.33 | 1.52 | 2.5 | −1.1 |
| 350 | 346.66 | 3.21 | 0.9 | −1.0 |
| 2700 | 2744.33 | 25.89 | 0.9 | 1.6 |
| Batch 4 ($n = 6$)[a] | | | | |
| 30 | 35.33 | 2.51 | 7.1 | 17.8 |
| 60 | 58.50 | 3.12 | 5.3 | −2.5 |
| 350 | 347.66 | 7.50 | 2.1 | −0.7 |
| 2700 | 2739.66 | 33.85 | 1.2 | 1.5 |
| Summary of combined measurements in batches 3 and 4[b] | | | | |
| 30 | 35.00 | 0.47 | 1.3 | 16.7 |
| 60 | 58.91 | 0.58 | 1.0 | −1.8 |
| 350 | 347.16 | 0.70 | 0.2 | −0.8 |
| 2700 | 2742.00 | 3.29 | 0.1 | 1.6 |

[a] Intra-batch precision and accuracy measurements of CR 3210.
[b] Inter-batch precision and accuracy measurements of CR 3210.

In intra-batch assay, the precision was determined in two different occasions. It was indicated by the coefficient of variation of the measured concentrations of replicate control samples, for the chosen concentrations (30, 60, 350 and 2700 ng/ml). It ranged between 1.2 and 7.5% in plasma and between 0.9 and 8.8% in urine.

The inter-batch precision measurement of the assay was determined combining the measurements of batch 1 and batch 2 in plasma and batch 3 and batch 4 in urine. Over the two occasion ($n = 12$ for each concentration), the coefficient of variation ranged between 2.1 (at 2700 ng/ml) and 9.2% (at 350 ng/ml) in plasma, and between 0.1 (at 2700 ng/ml) and 1.3% (at 30 ng/ml) in urine.

The accuracy of measurements was expressed in terms of mean relative error. In plasma it was −4% in batch one and −2.3% in batch two, whereas in urine it was −1.1% in batch 3 and −2.5% in batch 4, when the spiked concentration was 60 ng/ml.

The extraction efficiency, expressed as mean recovery of CR 3210 from rat plasma and urine, was determined. The percentage of CR 3210 recovered from plasma and urine samples was determined at low, medium and high concentration (75, 1500 and 3000 ng/ml); that was 68.5 (±3.8), 79.4 (±5.7) and 80.9 (±8.9), respectively. Similarly, the recovery in urine was 69.9 (±2.9), 75.3 (±4.3) and 78.6 (±10.7).

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

### 3.4. Stability

The results of stability tests of CR 3210 in biological samples are reported in Table 4. The chosen concentrations of CR 3210 added to drug-free plasma were 75 and 3000 ng/ml. The studied compound was shown to be stable in rat plasma at ambient temperature (ca. 22 °C) for up to 2 h, stored frozen at ca. −22 °C for 14, 28 and 70 days and following three freeze/thaw cycles. The percentage of spiked concentrations found in plasma samples ranged from 93 to 104%.

The CR 3210 was also stable in extracted samples at ca. 22 °C up to 24 h.

Table 4
Stability measurements of CR 3210 in rat plasma during storage

| Time of storage (temperature) | Concentration spiked (ng/ml) | Mean concentration found (ng/ml) | S.D. | CV (%) | |
|---|---|---|---|---|---|
| Day 0 | 75 | 72.6 | 4.4 | −3.2 | 3 |
| | 3.000 | 2915.0 | 12.8 | −2.8 | 3 |
| 2 h | 75 | 70.8 | 0.9 | −5.6 | 3 |
| (ca. +22 °C) | 3.000 | 3115.7 | 18.3 | 3.9 | 3 |
| Day 14 | 75 | 73.8 | 5.8 | −1.6 | 3 |
| (ca. −22 °C) | 3.000 | 2798.0 | 14.7 | −6.7 | 3 |
| Day 28 | 75 | 76.0 | 3.8 | 1.3 | 3 |
| (ca. −22 °C) | 3.000 | 2789.0 | 11.9 | −7.0 | 3 |
| Day 70 | 75 | 73.6 | 3.2 | −1.9 | 3 |
| (ca. −22 °C) | 3.000 | 3119.0 | 9.4 | 4.0 | 3 |
| 3 × freeze/thaw | 75 | 77.0 | 2.1 | 2.7 | 3 |
| (ca. −22 °C/ca. +20 °C) | 3.000 | 2876.0 | 6.8 | −4.1 | 3 |

### 3.5. Application to a pharmacokinetic study

The method here described was applied to the pharmacokinetic study of CR 3210 after oral (OS) administration to Sprague–Dawley rats.

Chromatograms obtained from drug-free rat plasma and urine are showed in Fig. 2A and Fig. 3A, respectively, no interfering peaks are present at the retention times of CR 3210 and CR 1505, i.e. 5.61 and 4.10 min, respectively.

Chromatogram from rat plasma sample, obtained 0.5 h after oral administration of CR 3210 and spiked with CR 1505, is showed in Fig. 2B. The peak referred to the studied compound and the I.S. are the only two peaks present. This feature suggested that CR 3210 is not subjected to extensive bio-transformation in vivo.

Chromatogram from rat urine sample, collected from 6 to 8 h after oral administration of CR 3210 and spiked with CR 1505, is showed in Fig. 3B. 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 was excreted unchanged in urine.

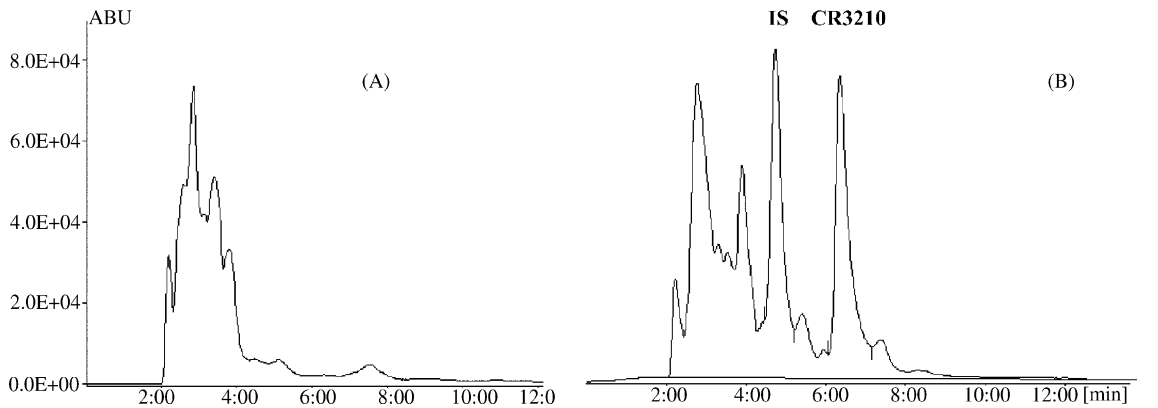



Fig. 2. Chromatograms showing: (A) drug-free rat plasma; (B) rat plasma obtained 0.5 h after oral administration of CR 3210, spiked with I.S. (40 μl, 10 μg/ml). Column: Tracer Extrasil ODS1 (25 cm × 0.46 cm, 5 μm); mobilephase: solvent A (10 mM potassium dihydrogen orthophosphate–methanol; 40:60 (v/v), pH = 2.85) and solvent B (acetonitrile) in ratio of 55:45 (v/v); isocratic flow: 1 ml/min.

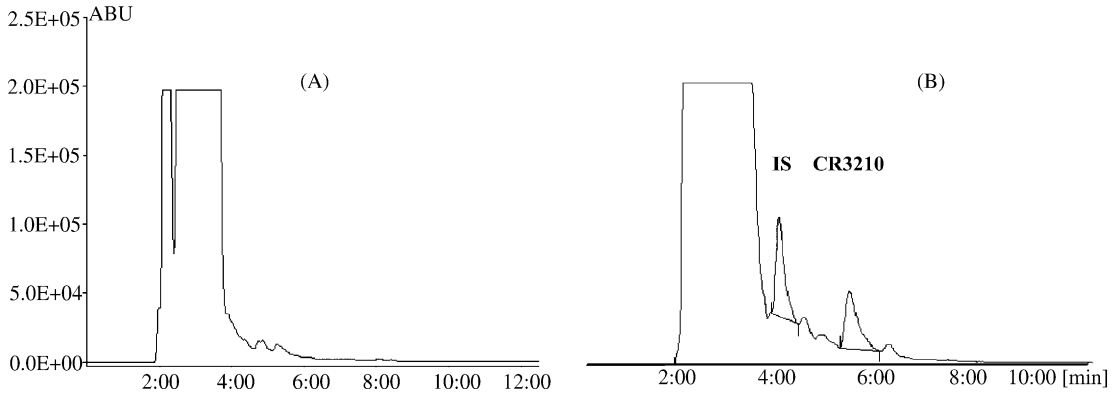



Fig. 3. Chromatograms showing: (A) drug-free rat urine; (B) rat urine collected from 6 to 8 h after oral administration of CR 3210, spiked with I.S. (40 μl, 10 μg/ml). Column: Tracer Extrasil ODS1 (25 cm × 0.46 cm, 5 μm); mobile phase: solvent A (10 mM potassium dihydrogen orthophosphate–methanol; 40:60 (v/v), pH = 2.85) and solvent B (acetonitrile) in ratio of 55:45 (v/v); isocratic flow: 1 ml/min.

The time profiles of plasma concentration of Sprague–Dawley rats ($n = 3$), treated orally with CR 3210, is showed in Fig. 4. The curve represents the variation of the concentrations over the time of 36 h, after the administration of the drug.

The oral administration of CR3210 determined a peak plasma concentration of 4.190 μg/ml ($C_{max}$) that was achieved after 0.5 h ($T_{max}$); the plasma levels decreased significantly after 4 h and the tested compound totally disappeared 6 h after the administration.

The time profile of urine concentration of Sprague–Dawley rats treated orally with CR 3210 is showed in Table 5. The variation of the concentration was determined over the time, and related to the aliquots of urine collected in the metabolic cage. Moreover the total concentration of the investigated drug, detected over the 36 h, was reported.

The oral administration of CR3210 gave a peak urine concentration of 1.607 ng ($C_{max}$), determined in the urine aliquot (3.3 ml) achieved from 8 to 12 h after the drug intake.

The substance was excreted unmodified in urine and it was detected in urine sample 1 h after the oral administration. Nevertheless the concentration of the tested substance remained very low from 1 to 4 h after the oral administration, showing an increased concentration from 6 to 8 h after the drug intake. The excretion drastically decreased after 12 h and CR 3210 disappeared from urine 24 h after the oral administration.

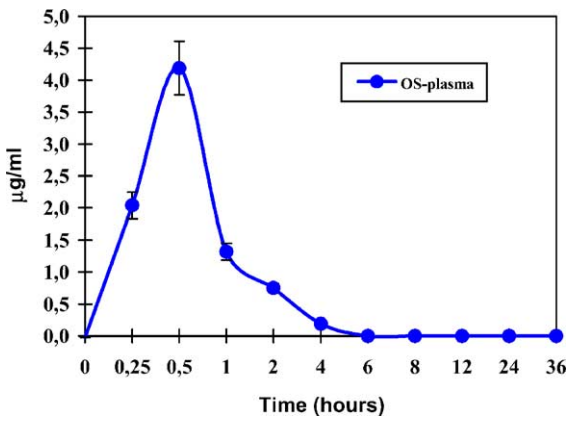



Fig. 4. Time profiles of plasma concentrations after oral (OS) administration of CR 3210. The ordinate shows the plasma level, abscissa shows the time after the administrations of the drug ($n = 3$; errorbars = S.D.).

Table 5
Urinary excretion of CR 3210 in Sprague–Dawley rats ($n = 3$)

| Time of collection (h) | Mean concentration found (ng) | Mean volume collected (ml) |
|---|---|---|
| 0–1 | 132 | 1.1 |
| 1–2 | 152 | 1.5 |
| 2–4 | 159 | 2.1 |
| 4–6 | 694 | 1.5 |
| 6–8 | 914 | 1.2 |
| 8–12 | 1607 | 2.6 |
| 12–24 | 141 | 6.5 |
| 24–36 | 0 | 6.1 |
| 0–36 | 3799 | 22.6 |

## 4. Conclusions

The specific objective of this study was the determination of the new $AT_1$-antagonist CR 3210 in rat plasma and urine.

The assay procedure based on a high performance liquid chromatography was fully validated. The method here described resulted sensitive and specific for the analysis of the test substance in biological samples.

The extraction procedures demonstrated a good efficiency. Solid-phase extraction allowed to isolate CR 3210 from rat plasma and urine with a good recovery. The accuracy determined in the inter-assay both in plasma, and in urine, was adequate for biological samples. The sensitivity of the method allowed the pharmacokinetic study of the CR 3210 in Sprague–Dawley rats.

On the basis of the pharmacokinetic data, plasma profile of CR 3210 seems to be characterized by a bi-compartmental behavior.

The plasmatic time course showed that after oral administration, this drug was able to reach the maximum concentration in 0.5 h, indicating an adequate absorption. Nevertheless the plasma level decreased rapidly and CR 3210 disappeared from plasma 6 h after administration. No other metabolites were detected in the plasma samples analyzed, indicating that the substance did not undergo to bio-transformation. The chromatograms obtained from the analysis of urine samples indicated that CR 3210 was excreted unchanged in urine. Moreover, considering the total amount of the urinary excretion over the 36 h, we observed that the concentration of CR 3210 detected in urine was low respect to intake dosage, suggesting the possibility of further kind of excretion of the tested substance.

In conclusion, the high performance liquid chromatographic method here described resulted sensitive and specific for the determination of CR 3210, an $AT_1$ receptor antagonist. The assay, fully validated, allowed the pharmacokinetic study of CR 3210 in Sprague–Dawley rats.

## Acknowledgements

The authors are deeply grateful to Rotta Research Laboratorium (Monza, Italy) for the financial support. Furthermore the authors would thank Dr. N. Costa, supplier of plasma and urine samples for analyses.

## References

[1] C.M. Ferrario, J.M. Flack, J. Cardiovasc. Drug Ther. 10 (1996) 511–518.
[2] J.P. Tsikouris, C.D. Cox, Pharmacotherapy 23 (2003) 1141–1152.
[3] A. Stanton, J. Renin Angiotensin Aldosterone Syst. 4 (2003) 6–10.
[4] M.J. Wyvratt, A.A. Patchett, Med. Res. Rev. 5 (1985) 483–531.
[5] M.D. Coulter, I.R. Edwards, Br. Med. J. 294 (1987) 1521–1523.
[6] E.G. Erdos, R.A. Skidgel, Hypertension 8 (1986) 1.
[7] L. Quadri, M. Gobbini, L. Monti, Curr. Pharm. Des. 4 (1998) 489–512.
[8] M. De Gasparo, K.J. Catt, T. Inagami, J.W. Wright, T. Unger, Pharmacol. Rev. 52 (2000) 415–472.
[9] T. Unger, J. Hypertens. Suppl. 21 (2003) 3–7.
[10] A. Cappelli, G. Pericot Mohr, A. Gallelli, M. Rizzo, M. Anzini, S. Vomero, L. Mennuni, F. Ferrari, F. Makovec, M.C. Menziani, P.G. De Benedetti, G. Giorgi, J. Med. Chem., submitted for publication.
[11] R.C. Williams, M.S. Alasandro, V.L. Fasone, et al., J. Pharm. Biomed. Anal. 14 (1996) 1539–1546.
[12] P.K. Yeung, A. Jamieson, G.J. Smith, D. Fice, P.T. Pollak, Int. J. Pharm. 204 (2000) 17–22.
[13] G. Carlucci, G. Palumbo, P. Mazzeo, M.G. Quaglia, J. Pharm. Biomed. Anal. 23 (2000) 185–189.
[14] L. Gonzales, J.A. Lopez, R.M. Alonzo, R.M. Jimenez, J. Chromatogr. A 949 (2002) 49–60.
[15] M. Rizzo, M. Anzini, A. Cappelli, S. Vomero, D. Ventrice, G. De Sarro, S. Procopio, N. Costa, F. Makovec, Farmaco 58 (2003) 837–844.