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Determination of XR510, a balanced angiotensin II receptor antagonist, in dog and rat plasma by combined liquid–liquid/solid-phase extraction and high-performance liquid chromatography

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Abstract

A sensitive and selective high-performance liquid chromatographic method for the determination of XR510 (I), a new non-peptide angiotensin II (AII) receptor antagonist with balanced affinity for AT_1 and AT_2 receptor subtypes is described. I and the internal standard, XR513, were extracted from acidified plasma by combined liquid – liquid/solid-phase extraction and chromatographed on a phenyl column with ultraviolet absorbance detection at a wavelength of 272 nm. The mobile phase consisted of a mixture of acetonitrile and sodium phosphate buffer. For both rat and dog plasma, the limit of quantitation was 5 ng/ml. This method has been validated and successfully utilized to investigate the disposition of I.

Keywords: Liquid–liquid extraction; Solid-phase extraction; XR510; Angiotensin II receptor antagonist

1. Introduction

Losartan, the prototype non-peptide angiotensin II (AII) receptor antagonist, has recently been approved for the treatment of hypertension. The antihypertensive effects of losartan and its active metabolite, EXP3174, are attributed to the selective antagonism of the AII receptor [1]. With the discovery of the existence of two subtypes of the AII receptor (AT_1 and AT_2), it has been determined that losartan binds only to the AT_1 receptor [2]. It has been proposed that this selectivity to the AT_1 receptor could result in increased circulating AII. This excess AII may be available to bind to AT_2 receptors. Although the physiological function of the AT_2 receptor is not clearly understood [3], concern that excess circulating AII could result in undesirable side effects has led to the search for an AII antagonist with a balanced affinity for AT_1 and AT_2. The potassium salt of 1-((2′-((i-amyl carbonyl-amino)sulfonyl) -3-fluoro-(1,1′-biphenyl)-4-yl)methyl)-5-[2-(N-butyryl-N-3-pyridinoamino)ethyl carbonyl] -4-ethyl-2-propyl-1H-imidazole, XR510 (I), is a non-peptide AII receptor antagonist with balanced affinity for AT_1 and AT_2 receptor subtypes as determined by radioligand receptor assays [4]. In order to investigate the bioavailability and pharmacokinetics of I in rats and dogs, a high-performance liquid chromatographic (HPLC) assay was developed and validated for the determination of I in plasma. The structures of I and the internal standard, XR513, (I.S.) are shown in Fig. 1.

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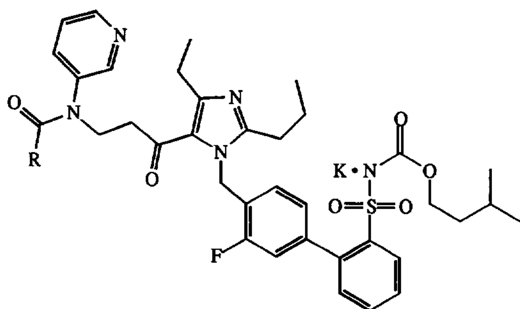

Fig. 1. Structures of I (R=*n*-propyl) and I.S. (R=ethyl).

2. Experimental

2.1. Chemicals and reagents

Compounds I and I.S. were synthesized by The DuPont Merck Pharmaceutical Company (Wilmington, DE, USA). Hydrochloric acid, phosphoric acid (HPLC), glacial acetic acid (HPLC), sodium hydroxide, acetonitrile (HPLC), methanol (HPLC), methylene chloride, and *n*-hexane (spectroscopic) were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade water was obtained from a Barnstead Ultrapure purification system. Sprague–Dawley rat plasma, with EDTA as the anticoagulant, was purchased from Cocalico (Reamstown, PA, USA), and used to prepare standards. Unless specified otherwise, all chemicals were of reagent grade.

2.2. Preparation of solutions

Stock solutions of I and I.S. (1.0 mg/ml free acid equivalent) were prepared by dissolving accurately weighed quantities of I and I.S. in methanol. Plasma standards of I were prepared by diluting an aliquot (100 μl) of the stock solution directly into plasma (10 ml) to produce a concentrated plasma standard. This concentrated plasma standard was subsequently diluted to produce plasma calibration standards at concentrations ranging from 5 ng/ml to 3 μg/ml. Working internal standard solution was prepared by diluting I.S. to a concentration of 1 μg/ml in water.

HPLC mobile phase was prepared by combining 420 ml of acetonitrile and 580 ml of 0.05 *M* phosphoric acid. The mobile phase pH was adjusted to 6.5 for the analysis of rat plasma samples with 5 *M* sodium hydroxide solution. For the analysis of dog plasma samples, the pH of the mobile phase was adjusted to 6.3.

Extraction solvent was prepared by combining 300 ml of methylene chloride and 700 ml of *n*-hexane.

2.3. Extraction apparatus

The liquid–liquid extraction procedure for I and I.S. was carried out using borosilicate glassware and Teflon® lined screw caps from Kimble (Vineland, NJ, USA). Standard solutions and reagents were prepared using class A volumetric glassware, where appropriate. For the extraction procedure, plasma samples and extraction reagents were transferred using Eppendorf pipettors (Brinkmann, Westbury, NY, USA). A reciprocating shaker (Eberbach Ann Arbor, MI, USA) was utilized to provide mixing for extraction. Other equipment used in the extraction procedure included a Sorvall RT6000D centrifuge (DuPont Wilmington, DE, USA), and an N-EVAP evaporator (Organomation, Berlin, MA, USA). Solid-phase extraction (SPE) columns, 1 ml Bondelut NH2 (aminopropyl, P/N 1210–2014) and a VacElut processing station were purchased from Bodman (Aston, PA, USA).

2.4. Extraction procedure

Aliquots of either plasma standards or unknown plasma samples (500 μl) were pipetted into 16×100 mm screw-cap culture tubes, containing 250 μl of working internal standard solution. The plasma I.S. mixture was acidified with 0.1 *M* HCl (500 μl) and briefly mixed by vortexing. The mixture was extracted with 6 ml of extraction solvent by shaking for 10 min. After centrifugation for 15 min at 1600 *g*, the extraction solvent layer was transferred to a clean 13×100 mm culture tube. The solvent was evaporated at 40°C under nitrogen and the residues were reconstituted with methylene chloride (1.0 ml) in preparation for SPE. Aminopropyl SPE columns were activated with hexane (1.0 ml). After the methylene chloride residues were extracted onto the columns, endogenous interferences were washed from the columns with methanol (1 ml). The columns were air dried under vacuum for 2 to 3 min

and I and I.S. were eluted with an aliquot of 2% acetic acid in methanol (1.0 ml). Again, the solvent was evaporated at 40°C under nitrogen. The residues were reconstituted with 50% mobile phase in water (250 μl), and transferred to polypropylene autosampler vials for HPLC analysis.

2.5. HPLC apparatus

The HPLC system consisted of a LC10AD Pump, a SIL10A autosampler (Shimadzu, Columbia, MD, USA), with a 3 μm (100×4.6 mm I.D.) YMC-Pack C_8 analytical column (YMC, Wilmington, NC, USA), and a Model 486 Tunable Absorbance Detector (Waters, Milford, MA, USA). Integration of the absorbance signal at 272 nm was accomplished using Waters 860 Data Acquisition software.

2.6. Chromatography and data acquisition parameters

Plasma extracts (150 μl) were injected onto the 3-μm YMC-Pack C_8 column, where I and I.S. were separated from endogenous interferences. For the analysis of rat plasma samples, mobile phase was pumped at a flow-rate of 1.5 ml/min, which resulted in retention times of approximately 8.5 and 6.1 min, for I and I.S., respectively. Mobile phase, pumped at a flow-rate of 1.7 ml/min for the analysis of dog plasma extracts resulted in I and I.S. retention times of approximately 9.5 and 6.7 min, respectively. The UV absorbance of the analytes was monitored at 272 nm, at a rate of 2 samples per second using the Waters 860 Data Acquisition software. The total run time for each injection was ≤12 min.

2.7. Data analyses

The peak height ratios (I/I.S.) of extracted plasma standards, calculated by the Waters Data Acquisition software, were used to perform a weighted ($1/C^2$) least squares linear regression. The 'best fit' slope and intercept were subsequently used to calculate the concentration of I in plasma standards and unknown samples.

3. Results and discussion

3.1. Specificity

Fig. 2A and Fig. 3A show typical chromatograms of extracts of blank rat and dog plasma, respectively. The mobile phase that was used for quantification of I in rat plasma was adjusted to pH 6.5 and pumped at a flow-rate of 1.5 ml/min. This resulted in chromatograms free of endogenous plasma interferences. However, when this mobile phase was used for the analysis of dog plasma extracts, a small endogenous peak not found in rat plasma interfered with the internal standard peak. Adequate separation of this peak was achieved by adjusting the mobile phase to pH 6.3. The resulting longer retention times were compensated for by increasing the flow-rate to 1.7 ml/min.

For this class of compounds, selective plasma

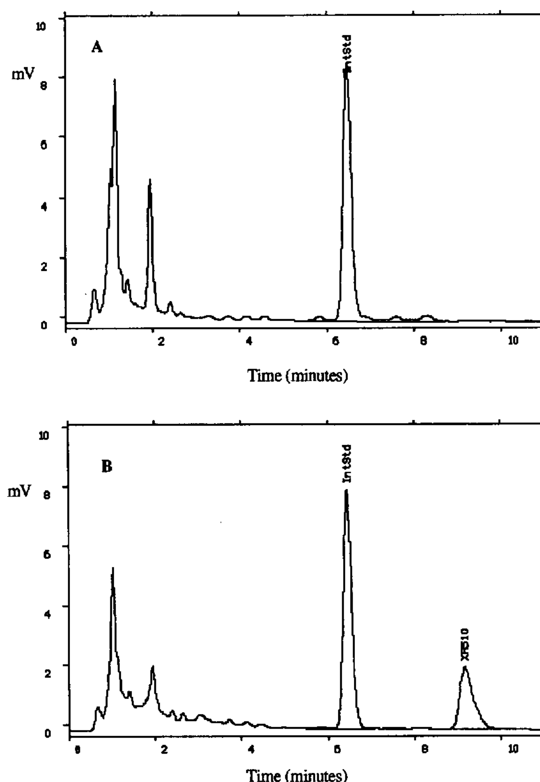

Fig. 2. Representative chromatograms of rat plasma extracts. (A) Predose and (B) 1 h after 5 mg/kg oral dose of I (plasma concentration 0.252 μg/ml).

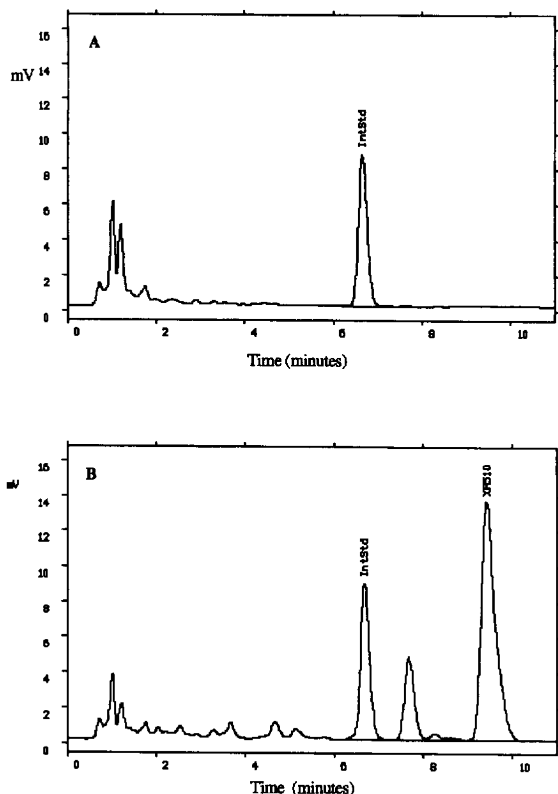

Fig. 3. Representative chromatograms of dog plasma extracts. (A) Predose and (B) 1 h after 10 mg/kg oral dose of I (plasma concentration 1.31 μg/ml).

extraction, free of endogenous interferences, has been challenging. For the analysis of losartan, methyl-*t*-butyl ether was chosen as the extraction solvent because it provided cleaner extracts, although more polar extraction solvents gave somewhat better extraction recovery [5]. Typically, cleaner chromatograms for these compounds have been obtained by back extracting the analytes into dilute sodium hydroxide followed by a hexane wash [5–7]. For DMP 811, chromatographic run times were lengthened to accommodate these late-eluting peaks [7].

The current procedure combines a polar SPE with solvent extraction to provide chromatograms free of endogenous interferences and late-eluting peaks. The aminopropyl SPE column was chosen because it is a very polar sorbent with strong hydrogen bonding capability. The acidic nature of I and the ability of the aminopropyl SPE column to function as an anion exchanger were also considered when selecting this sorbent. Potential wash solvents were evaluated according to increasing polarity to determine the most polar wash solvent which did not elute I or I.S.. Both compounds were retained on the SPE column after washes with hexane, methylene chloride, ethyl acetate, acetonitrile, and even methanol. Acidification of the methanol with acetic acid resulted in complete elution of both compounds. The combination of a methanol wash and elution with 2% acetic acid in methanol resulted in chromatograms free of interferences and late-eluting peaks.

3.2. Recovery

The recoveries of I and I.S. were determined by comparing peak heights of plasma extract to peak heights observed in spiked solutions prepared in 50% mobile phase in water. Although compounds I and I.S. could be extracted at acidic and neutral pH (Fig. 4), improved recovery was observed after acidification. The percentage of methylene chloride in the extraction solvent was optimized based on the recovery of I from acidified plasma. For SPE, recovery of I and I.S. was essentially quantitative. In extracts of plasma, the overall recovery of I was determined at 10, 100 and 1000 ng/ml ($n=5$) and found to be 92±13%, 86±2%, and 83±3%, respectively.

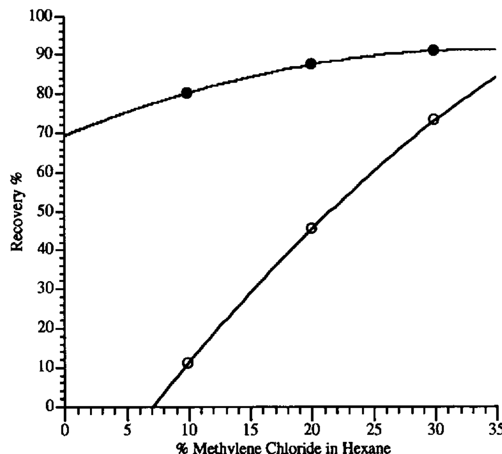

Fig. 4. Solvent extraction recovery of I at neutral pH (○) and acidic pH (●).

3.3. Linearity

Peak height ratios of plasma standards, over a range of 5 ng/ml to 3 μg/ml, were proportional to concentration. The relatively small percent differences between the nominal and calculated concentrations indicate that the response is linear, and the regression model is appropriate.

3.4. Assay precision and accuracy

The precision of the assay was defined as the coefficient of variation (C.V.%) of the replicate measurements. The accuracy of the assay was defined as the mean of the absolute values of the percent difference of the determined concentrations, from the nominal value. The percent difference for each measurement is determined by:

$$\% \text{ difference} = \frac{\text{determined} - \text{nominal}}{\text{nominal}} \times 100$$

Intra-day assay precision and accuracy were determined in quality control samples, prepared separately from plasma standards, but at the same concentrations as plasma standards. Results of these determinations are shown in Table 1.

Table 1
Intra-day assay precision and accuracy from rat and dog plasma (n=5)

Nominal plasma concentration (ng/ml)	Mean found concentration (ng/ml)	C.V. (%)	Difference (%)
rat			
5	5.3	8.0	9.3
10	9.9	10.9	7.5
30	28.9	1.4	3.7
100	111	1.6	11.0
300	305	0.6	1.7
1000	1088	1.4	8.8
3000	3175	3.1	5.9
dog			
5	5.1	4.1	3.1
10	10.4	7.6	7.3
30	26.7	5.4	10.8
100	99.4	8.2	5.2
300	312	2.6	4.0
1000	994	2.0	1.7
3000	2719	1.9	9.4

Table 2
Inter-day assay precision and accuracy from rat and dog plasma (n=4)

Nominal plasma concentration (ng/ml)	Mean found concentration (ng/ml)	C.V. (%)	Difference (%)
rat			
5	5.1	3.1	2.4
10	9.8	6.7	1.7
30	26.7	0.6	10.9
100	107	2.9	6.5
300	290	1.5	3.4
1000	1039	2.4	3.9
3000	3101	5.8	3.4
dog			
5	5.0	9.7	6.9
10	11.1	7.6	11.0
30	29.2	7.3	5.6
100	99.3	2.4	1.9
300	312	4.0	4.0
1000	1019	5.1	4.0
3000	3020	6.6	4.7

Inter-day assay precision and accuracy were determined on four different days. The results of the inter-day analyses, found in Table 2, are comparable to the intra-day results. For both dog and rat, the precision and accuracy of the method within a single day and between days demonstrate the reproducibility of the extraction procedure.

3.5. Applications

This assay has been used successfully to evaluate the pharmacokinetics of I in rats and dogs [8]. Representative plasma concentration versus time profiles are shown for rat and dog in Fig. 5. In addition, plasma samples collected as part of a cardiovascular safety study in dogs have been assayed to correlate plasma concentrations with cardiovascular effects.

4. Conclusions

An assay for I, a new drug candidate with a balanced affinity for the AT_1/AT_2 receptors, has been developed and validated. The plasma ex-

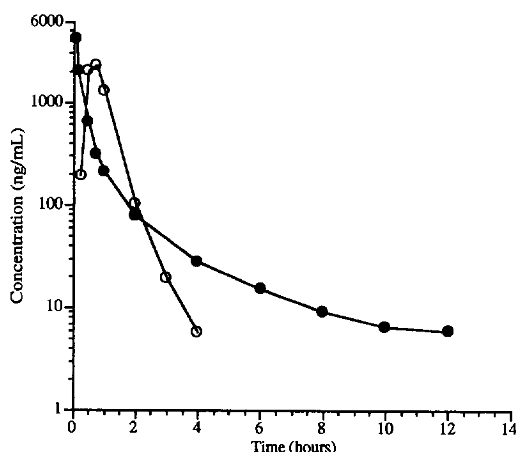

Fig. 5. Representative plasma concentrations versus time profiles after oral administration in dog, (○, 10 mg/kg) and rat (●, 5 mg/kg).

traction combines a solvent extraction with SPE utilizing an aminopropyl column to effectively eliminate endogenous interferences and late-eluting peaks. The assay sensitivity and throughput of the method have allowed the application of the method for the analysis of plasma samples from pharmacokinetic and safety studies.

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