Short communication

Determination of candesartan cilexetil, candesartan and a metabolite in human plasma and urine by liquid chromatography and fluorometric detection

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Abstract

Liquid chromatographic methods are described for the determination of a new effective anti-hypertensive drug candesartan (CV-11974), its prodrug candesartan cilexetil (TCV-116) and a metabolite, CV-15959 in human plasma and urine. The assays comprise liquid–liquid extraction and separation on a phenyl column with fluorometric detection. The methods give absolute recoveries of 70, 83 and 78% for candesartan cilexetil, candesartan and CV-15959, respectively, and the limit of quantification is 5, 1 and 3 nM of plasma (RSD<20%), respectively. The methods were applied to plasma and urine samples from biopharmaceutical and clinical studies in man. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: TCV-116; Candesartan; Angiotensin II receptor antagonist

1. Introduction

Candesartan cilexetil (CC) is a potent, orally active and selective angiotensin II type 1 receptor blocker. Since angiotensin II (A-II) is the primary effector molecule of the renin–angiotensin system (RAS), candesartan (CV-11974) is believed to reduce blood pressure by a minimum of intervention in this endogenous system. CC is a pro-drug that is transformed during absorption to the active compound, candesartan, which is further metabolised to the inactive MII (CV-15959). Analytical methods for angiotensin II antagonists have largely been based on liquid chromatography (LC) and photometric or fluorometric detection for losartan [1–3], valsartan [4] and irbesartan [5]. CC, candesartan and MII have been assayed by column switching LC and fluorometric detection with different sample work-up and separation mode, depending on the compounds measured and the biological matrix [6,7].

Owing to extensive pharmacokinetic and clinical studies we needed a fast and easy method for determination of CC, candesartan and MII in blood plasma and urine, with a more uniform sample work-up procedure as compared to published methods. The present assays comprise liquid–liquid extraction with dichloromethane–diethyl ether (1:4, v/v), evaporation, redissolution and separation on a phenyl column with fluorometric detection. The advantages of our method are that we use an internal standard and that the limit of quantification is lower. So far the

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methods have been used for more than 10 000 biological samples.

2. Experimental

2.1. Chemicals and reagents

CC, candesartan, MII and the internal standard T-57536 (IS), see Fig. 1, were obtained from Takeda (Osaka, Japan). Diethyl ether was of pro analysis grade from Prolabo (Lyon, France). Acetonitrile, methanol, hexane and dichloromethane were of Rathburn HPLC grade (Walkerburn, UK). Citric acid, orthophosphoric acid, sodium dihydrogenphosphate, hydrochloric acid and sodium hydroxide (Titrisol) were of analytical grade from Merck (Darmstadt, Germany). Tetrabutylammonium (TBA) hydrogensulfate was obtained from Fluka (Buchs,

Fig. 1. Chemical structures for candesartan cilexetil (CC), candesartan, MII (CV-15959) and T-57536 (I.S.).

Switzerland). Water was purified using an Elga System (High Wycombe, UK).

2.2. Chromatographic system

The chromatographic system consisted of a gradient (ternary) LC-pump, Chrompack GRAS (Middelburg, The Netherlands) or an isocratic LC-pump, Gynkotek model 480 (Munich, Germany), an autosampler, Spectra-System AS 3000, Thermo Separation Products (Fremont, CA, USA) and a fluorescence detector, Jasco FP-920 (Tokyo, Japan) set at excitation and emission wavelengths of 265 and 395 nm, respectively. Data collection and computation were performed automatically by a chromatography data system VG MULTICHROM, Labsystem (Altrincham, UK). The shaking machine used (SM 25) was from Bühler (Tübingen, Germany). The guard column was a 15×3.2 mm Brownlee Cyano Newguard, 7 μm from Perkin-Elmer (Norwalk, CT, USA) and the analytical column a 100×4.6 mm Spherisorb S3P (phenyl), 3 μm, Hichrom (Reading, UK). The mobile phases are described below, before use they were ultrasonicated and were used at room temperature.

The mobile phase for candesartan in plasma was prepared by mixing 100 ml citrate buffer (pH 3.1, $I = 0.5$ containing 50 mM TBA), 185 ml acetonitrile, 180 ml methanol and diluting to 1000 ml with water. The flow-rate was 0.9 ml/min giving a retention time of about 9 min for candesartan and 11 min for the I.S.

Mobile phase for CC in plasma and urine was prepared by mixing 200 ml phosphate buffer (pH 2.8, $I = 0.1$ containing 12.5 mM TBA), 420 ml acetonitrile and diluting to 1000 ml with water. The flow-rate was 1.0 ml/min giving a retention time of about 20 min for CC and about 4 min for the I.S.

Mobile phases for candesartan and MII in plasma and urine, phase A were prepared by mixing 200 ml phosphate buffer (pH 2.8, $I = 0.1$ containing 12.5 mM TBA), 200 ml acetonitrile and diluting to 1000 ml with water, phase B by mixing 200 ml phosphate buffer (pH 2.8, $I = 0.1$ containing 12.5 mM TBA), 600 ml acetonitrile and diluting to 1000 ml with water. The acetonitrile gradient was as follows: 0–9 min 20% of B, 9–14 min linear increase to 32%, 14–25 min back to 20% and 25–29 min 20%. The
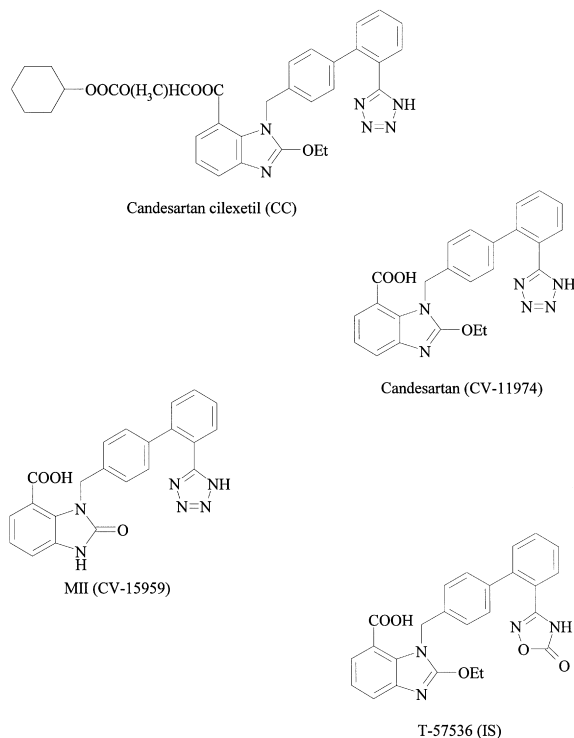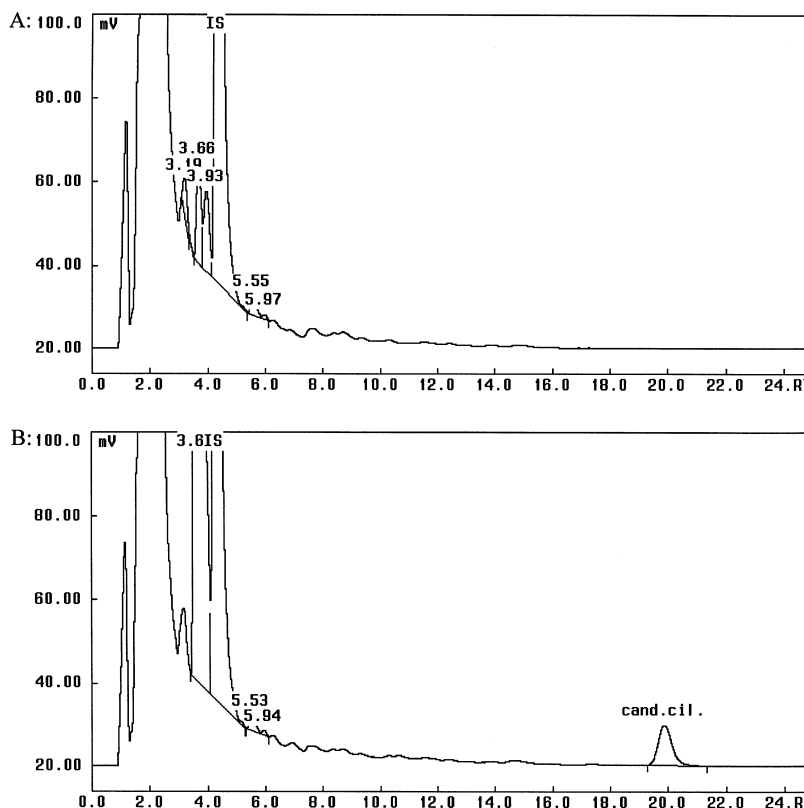
Fig. 2. Chromatogram of a human plasma sample taken before dose (A) and 0.5 h after dose (B), using an isocratic system for determination of CC (8.5 n*M*), I.S. (220 n*M*).

flow-rate was 1.0 ml/min giving a retention time of about 10, 18 and 21 min for MII, candesartan and the I.S., respectively.

2.3. Sample preparation procedure

Venous blood samples were collected into heparin tubes and separated by centrifugation for 5 min at 1500 *g*. The plasma samples were transferred to disposable polypropylene tubes and frozen at −18°C. Urine samples were collected and frozen. The frozen plasma or urine samples were allowed to thaw at room temperature, vortex-mixed and centrifuged. A 500-μl volume of plasma or a 50-μl volume of urine (+450 μl of drug-free plasma) was pipetted into a 10-ml glass tube and mixed with 50 μl I.S. solution (I.S., 2.2 μ*M* in phosphate buffer, pH 7.0, *I*=0.1

Table 1
Absolute recoveries and within-day variation for CC, candesartan and MII in plasma

Substance	Nominal conc. (n*M*)	Absolute recovery (%)	RSD *n*=6–8 (%)
CC	3.6	72.0	3.8
	7.1	70.0	3.1
	170	65.6	3.1
Candesartan	2.4	82.4	2.6
	59	83.0	2.8
	237	81.7	1.0
MII	2.5	73.8	8.8
	62	78.7	1.9
	247	78.4	0.9
T-57536 (I.S.)	230	83.6	

containing 10% methanol) and 500 µl of HCl (0.2 *M* for candesartan and MII, 0.15 *M* for CC). Thereafter 5.0 ml of the extraction solvent (dichloromethane–diethyl ether, 1:4, v/v) was added and the tubes were shaken (300/min) for 20 min. After centrifugation for 5 min, the aqueous phase was frozen in a dry ice–ethanol bath (to aid in phase separation) and the organic phase transferred to glass tubes with conical bottoms and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 400 µl redissolution liquid (20 ml acetonitrile, 10 ml citrate buffer (pH 3.1, I=0.5, 50 m*M* TBA) diluted to 100 ml with water), for candesartan and MII, or 300 µl redissolution liquid (60 ml methanol, 20 ml phosphate buffer (pH 2.8, I=0.1, 12.5 m*M* TBA) diluted to 100 ml with water) for CC. An 80-µl volume was injected onto the LC-column. Two plasma or urine calibration samples were injected

before the unknown samples and then after every 10–15 samples.

The calibration plasma samples were prepared from 500 µl drug-free plasma and 50 µl of a standard solution containing either candesartan and MII in phosphate buffer (pH 7.0, I=0.1 containing 10% methanol) or CC dissolved in 100% methanol. The concentrations in the prepared calibration samples corresponded to a sample concentration of 65, 500 and 130 n*M* for CC, candesartan and MII, respectively. The urine samples for calibration were prepared in the same way except 50 µl drug-free urine and 450 µl drug-free plasma were taken. The urine samples contained ten times as high a concentration of the compound as the plasma samples. These samples were run in parallel with unknown plasma or urine samples according to the described procedure.
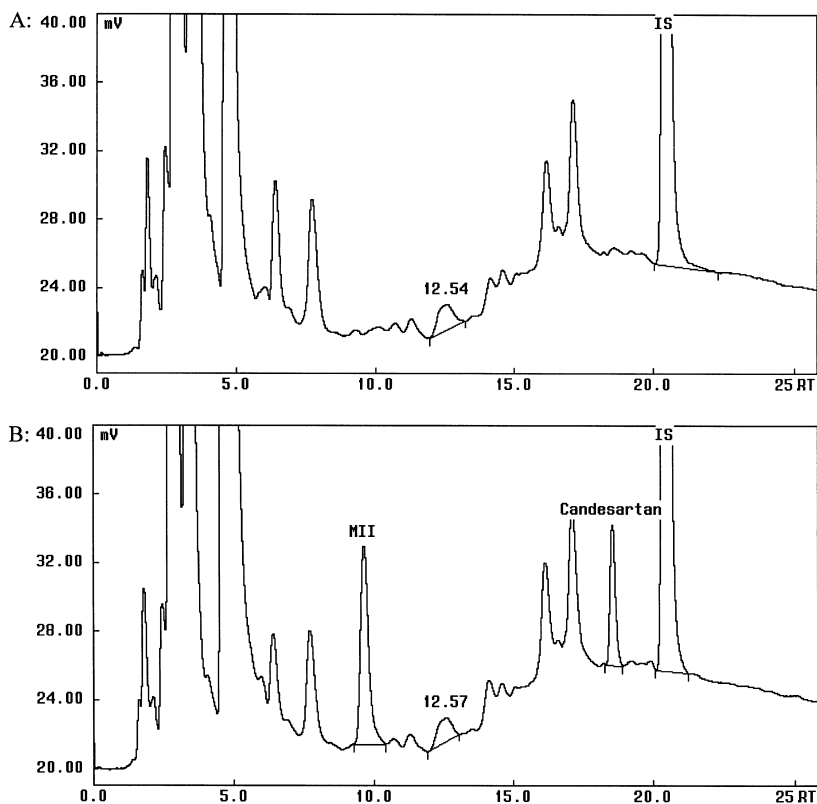

Fig. 3. Chromatogram of a human plasma sample taken before dose (A) and 24 h after dose (B), using a gradient system for determination of candesartan (9.4 n*M*), MII (24.8 n*M*) and the I.S., T-57536 (140 n*M*).

3. Results and discussion

3.1. Extraction

The absolute recoveries from plasma were calculated by comparing the peak heights of CC, candesartan, MII and the I.S. with those obtained for calibration solutions containing the same amounts of respective compound injected directly onto the analytical column. The absolute recoveries were above 80% for candesartan and I.S. and somewhat lower for CC and MII (Table 1). We did not investigate other extraction systems, e.g. more efficient organic solvents or solid-phase extraction which might have increased the extraction recovery but may also have introduced more interfering co-extractants. In the

extraction from urine samples, plasma was added to act as adsorption inhibitor in the evaporation step, which improved recovery.

3.2. Chromatographic conditions

The mobile phase used in most clinical studies for determination of candesartan was straightforward with acetonitrile and methanol in citrate buffer, pH 3.1, with an added ion-pairing agent, TBA, to increase retention and influence selectivity (Fig. 4). For CC, acetonitrile was the only organic modifier in phosphate buffer pH 2.8 with added TBA (Fig. 2) and this was also valid for the gradient system used for simultaneous measurement of candesartan and MII (Figs. 3 and 5). Thanks to the excellent de-
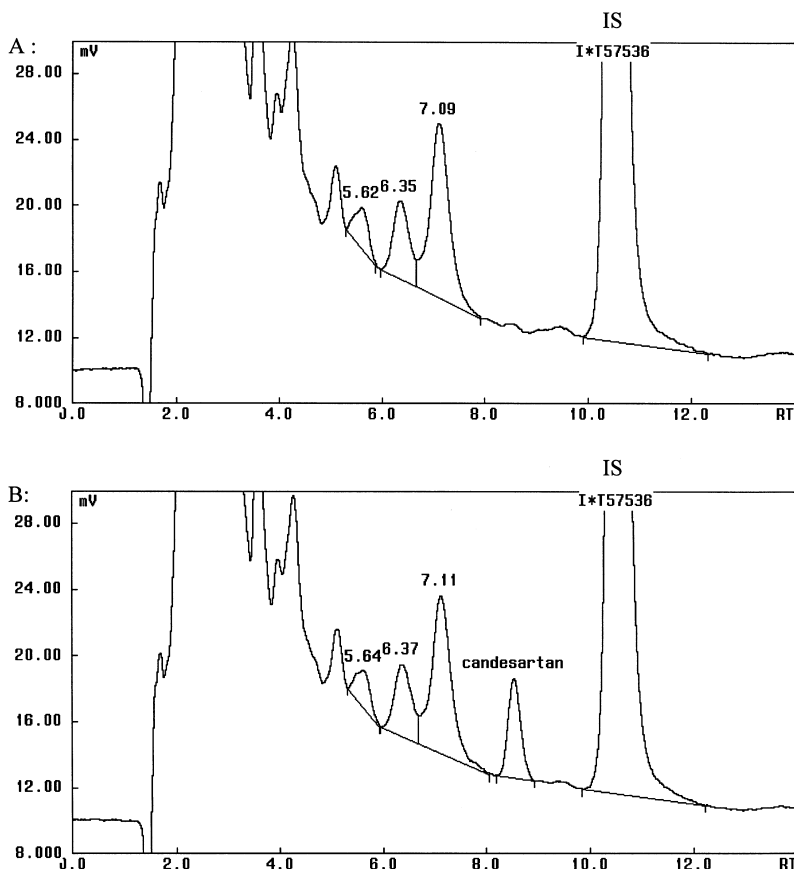

Fig. 4. Chromatogram of a human plasma sample taken before dose (A) and 0.5 h after dose (B), using an isocratic system for determination of candesartan (7.0 n*M*), I.S. (150 n*M*).

tection properties of the compounds through fluorescence, a low limit of quantification could be achieved enabling concentrations in the low nanomolar region to be determined. The chromatographic systems showed no interfering peaks but consistent separation and sensitivity on long-term routine analyses.

3.3. Quantification and accuracy

Quantification was performed using peak height measurement. The mean value of peak height ratio of the respective substance to the internal standard in the plasma/urine calibration samples was used for the calculation of the concentrations in the unknown samples. Within-day precision of the analytical method was estimated using spiked samples at different concentration levels. The relative standard deviations (RSDs) in the determination of absolute recovery

were in the range of 1–9% as seen in Table 1. Between-day variability was 4.4% for CC at 65 nM plasma ($n=7$) and 2.9 and 4.2% for candesartan and MII, respectively, at 120 and 130 nM plasma ($n=25$).

The daily calibration was based on at least six replicate calibration samples at one concentration besides a blank plasma sample. Full standard curves for candesartan in plasma comprising eight different concentrations were run at least once a month during periods of routine analysis. There were three replicates at each concentration, except for the level used in daily calibration and at limit of quantification (LOQ, defined as a concentration with within-day precision of <20%), where eight replicates of each were assayed. The curves were found to be linear over the range of 1 to 1000 nM for candesartan and LOQ was set at 1 nM. CC and MII were dealt with in a corresponding way, with LOQs of 5 and 3 nM,
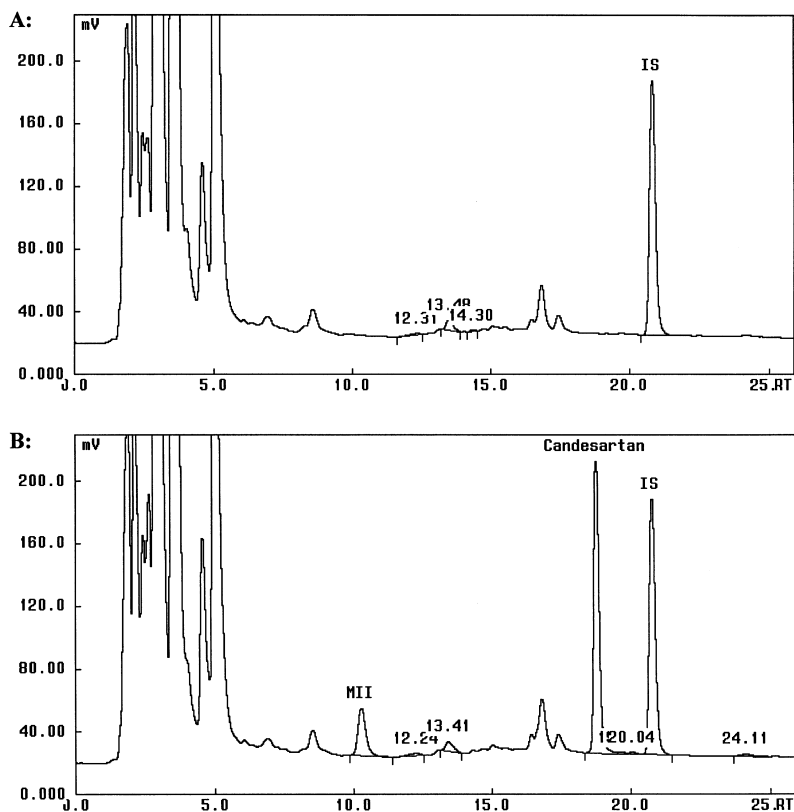

Fig. 5. Chromatogram of a human urine sample taken before dose (A) and 12–24 h after dose (B), using a gradient system for determination of candesartan (1070 nM), MII (337 nM) and the I.S., T-57536 (1390 nM).

respectively. The total capacity of the methods were about 50 authentic samples a day and four such series of analyses a week. For urine samples, LOQ was 10 times higher than in plasma.

3.4. Stability

Candesartan in standard solution was stable for at least 6 months at $-18°C$, MII and CC for at least 3 months at $-18°C$. Candesartan in spiked plasma samples was stable for at least 24 h at $+22°C$ and for at least 10 months at $-18°C$. Candesartan in study plasma samples was stable for at least 5 days in a refrigerator at $+6°C$ and in pooled, study plasma stored at $-18°C$ for at least 11 months. Candesartan in authentic or spiked plasma samples was stable during the process of thawing, repeated freezing and thawing. Candesartan in processed samples from authentic plasma was stable for at least 2 days at $+22°C$ and for at least 7 days at $-18°C$.

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