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Automated quantitative determination of the new renin inhibitor CGP 60536 by high-performance liquid chromatography

Gilbert Lefèvre[a,*], Sonia Gauron[b]

[a]*Novartis Pharma AG, WSJ-27.7.029, 4002 Basle, Switzerland*
[b]*Novartis Pharma SA, Rueil-Malmaison, France*

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Abstract

A fully automated high-performance liquid chromatography method with fluorescence detection for the determination of the renin inhibitor CGP 60536 in animal and human plasma and urine has been developed and validated. After addition of an internal standard, the compounds were automatically extracted from 400 μl of plasma or urine with methyl alcohol–acetic acid (99:1, v/v) on 100-mg Bond-Elut CN cartridges using the Gilson ASPEC system. The on-line chromatographic separation was performed on a LiChrospher 100 RP8 5-μm particle size packed analytical column (25×0.4 cm I.D.). The mobile phase consisted of acetonitrile–0.01 *M* potassium dihydrogenphosphate (65:35, v/v) at a flow-rate of 0.8 ml/min. The analytes were detected using a fluorescence detector at excitation and emission wavelengths of 280 and 330 nm, respectively. The limit of quantitation was established at 4.5 ng/ml in plasma (accuracy 106% and precision 1%), and 9.0 ng/ml in urine (accuracy 101% and precision 13%). The method was applied to the investigation of the pharmacokinetics of CGP 60536.

Keywords: Renin inhibitor; CGP 60536

1. Introduction

The renin angiotensin system (RAS) plays an important role in the short- and long-term control of blood pressure through various direct and indirect effects on the vasoconstrictive hormone angiotensin II, which is generated from the hydrolysis of angiotensin I by angiotensin converting enzyme (ACE). To date, angiotensin converting enzyme inhibitors (ACEIs) are recognized as a good therapy to slow the progression of renal insufficiency and to treat hypertension and congestive heart failure [1–6]. However, they are not effective in all patients – as a monotherapy – and they produce certain adverse effects such as cough and angioneurotic oedema [7,8]. Renin, in contrast to ACE, is highly specific since angiotensinogen (the precursor of angiotensin I) is its only known substrate. Therefore, renin inhibitors which specifically block the RAS at the first and rate limiting step in the formation of the active vasoconstrictive hormone are expected to be free of the side effects associated with the use of ACEIs. Accordingly, the search of renin inhibitors is a very active research field [9]. CGP 60536B (Fig. 1) is the first new, completely non-peptide, low molecu-

*Corresponding author. Tel.: +41-61-324-6114; fax: +41-61-324-6112.
E-mail address: gilbert.lefevre@pharma.novartis.com (G. Lefèvre)

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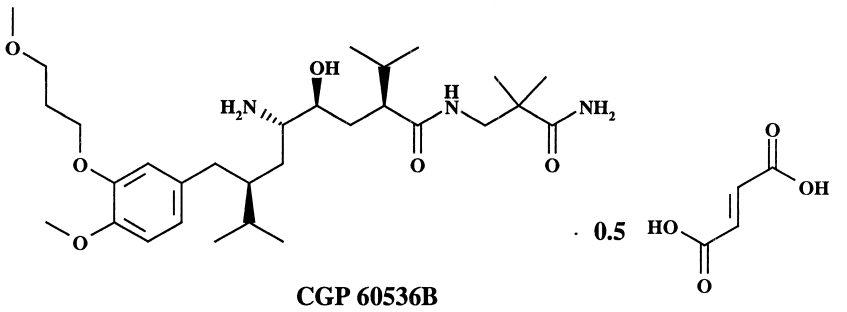
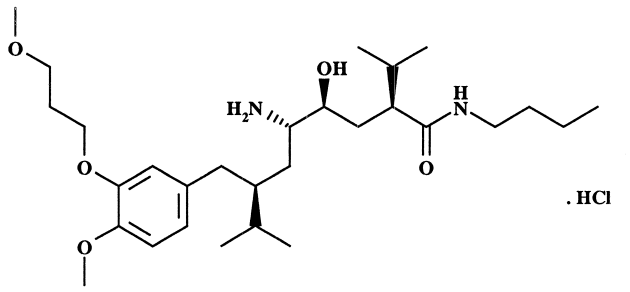


CGP 60536B



CGP 56962A (IS)

Fig. 1. Chemical structures.

lar weight renin inhibitor under investigation [10] in the treatment of hypertension and chronic renal failure.

The objective of the work reported in this paper was to develop and validate a selective and sensitive analytical method for the measurement of CGP 60536 in animal and human plasma and urine. The present paper describes a high-performance liquid chromatography (HPLC) procedure with fluorescence detection involving liquid–solid extraction from 400 μl of plasma or urine and which is fully automated by using the automatic sample preparation with extraction columns (ASPEC) system [11,12].

2. Experimental

2.1. Chemicals and reagents

CGP 60536 refers to the free base ($C_{30}H_{53}N_3O_6$, M_r=551.77) and CGP 60536B to the hemi-fumarate salt ($C_{30}H_{53}N_3O_6+1/2C_4H_4O_4$, M_r=609.8). The following reference compounds (Fig. 1) were supplied by Novartis Pharma AG (Basle, Switzerland): CGP 60536B (hemi-fumarate salt) and CGP 56962A ($C_{29}H_{52}N_2O_5$·HCl, M_r=545.2) was used as the internal standard (I.S.).

All solvents and reagents were of analytical grade and used without further purification: methyl alcohol and acetonitrile from Carlo-Erba (Milan, Italy), acetic acid, anhydrous potassium dihydrogenphosphate, di-potassium hydrogenphosphate, di-sodium hydrogenphosphate dihydrate and sodium azide from Merck (Chelles, France), gelatin from porcine skin from Fluka (St. Quentin Fallavier, France). Water was purified and deionized using a Milli-Q® Plus reagent grade water purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

The chromatographic system consisted of a pump (Model 305, Gilson, Villiers-Le-Bel, France), an ASPEC system (Model XL, Gilson, Villiers-Le-Bel,

France), a model L-7480 fluorescence detector (Hitachi) from Merck (Nogent-sur-Marne, France) set at 280 and 330 nm for excitation and emission wavelengths, respectively, and a model C-R3A integrator-recorder (Shimadzu, Kyoto, Japan).

2.3. Extraction column

Bond-Elut solid-phase extraction cartridges with a 1 ml capacity packed with 100 mg polar phase cyanopropylsilane were used. They were manufactured by Varian and purchased by Prolabo (Fontenay-sous-Bois, France).

2.4. Calibration and validation samples

The stock solutions of CGP 60536B were prepared by dissolving 5 mg of substance (hemi-fumarate salt) in 50 ml of water. Appropriate serial dilutions of the stock solutions with water were then made in order to prepare the spiking solutions at concentrations ranging from 90.5 to 18 100 ng/ml. The spiking solutions were used for the preparation of the calibration samples. Other stock solutions of CGP 60536B in water at the same concentrations were prepared from a second weighing and were appropriately diluted to give spiking solutions to be used for the preparation of validation (accuracy and precision assessments), quality control and stability samples. The I.S. stock solution was prepared by dissolving 5 mg of CGP 56962A in 50 ml of 0.01 *M* KH_2PO_4 solution. The concentration of the I.S. in the working solutions, obtained by further dilution of the stock solution with 0.01 *M* KH_2PO_4 solution, was 2.8 μg/ml for the assay in plasma and 5.6 μg/ml for the assay in urine. All the solutions were prepared in glass flasks and stored in dark at +4°C while not in use. CGP 60536 samples in the concentration range of 4.5–450 ng/ml (plasma) and 9.0–900 ng/ml (urine) were prepared for calibration, accuracy and precision, quality control and stability assessments by adding 20 μl of the appropriate standard solutions to 400 μl of drug-free human plasma or urine aliquots.

2.5. Sample handling

To 400 μl of plasma or urine in disposable tubes 20 μl of the I.S., 20 μl of the appropriate standard solution for standard samples or drug-free water for actual samples and 400 μl of phosphate-buffered saline containing 0.2% of gelatin (and an additional 0.2% bovin serum albumin for urine assay) were added. Gelatin was added to the buffer since it was shown to improve the extraction of the compounds. Each tube was vortex-mixed for 10 s and placed on the rack of the ASPEC system. The automated sequences are described in Table 1. Each sample was prepared separately during the chromatography of the previous sample. After each liquid transfer, the

Table 1
Automated procedure for the extraction of CGP 60536 and the internal standard (IS), CGP 56962, from plasma and injection of the extract

Steps	Fluid dispensed		Dispensing flow-rate (ml/min)	Pressurizing air volume (ml)
	Composition	Volume		
Conditioning the cartridge	methyl alcohol	1 ml	5.0	0.0
	water	1 ml	5.0	0.03
Loading the sample	diluted fluid	840 μl	0.18	0.5
Washing	methyl alcohol–0.01 *M* K_2HPO_4 (1:1, v/v)	200 μl	3.0	2.0
	water	200 μl	3.0	2.0
Elution	methyl alcohol–acetic acid (99:1, v/v)	2×250 μl	0.5	1.0
Dilution of the extract	0.01 *M* KH_2PO_4	500 μl	3.0	–
Mixing of the extract	air	2 ml	48.0	–
Injection via a 200-μl loop	diluted extract	200 μl[a]	12.0[b]	–

[a] 100 μl in the case of urine assays.
[b] 0.5 ml/min in the case of urine assays.

needle was rinsed with 1 ml of water–methyl alcohol (70:30, v/v), with a flow-rate of 80 μl/s.

Plasma samples with concentrations higher than 450 ng/ml had to be diluted with a blank pool plasma prior to the sample handling procedure.

2.6. Chromatography

Chromatographic separations were performed at room temperature on a pre-packed column (stainless-steel tube, 25 cm×4 mm I.D.), filled with LiChrospher 100 RP8, 5-μm particle size (Merck, Chelles, France). The mobile phase consisted of acetonitrile–0.01 *M* KH_2PO_4 (65:35, v/v) and was used at a flow-rate of 0.8 ml/min. A protective pre-column (4×4 mm I.D.) filled with the same material was inserted between the injector and the analytical column. This pre-column was replaced every week.

2.7. Calibration and sample quantification

Calibration (standard) samples at seven different concentrations, in the range of 4.5–450 ng/ml of CGP 60536 for the assay in plasma and 9.0–900 ng/ml for the assay in urine, were prepared and analysed. Calibration curves, represented by the plots of the peak height ratios (y) of CGP 60536 to the I.S. versus the concentrations (x) of the calibration samples, were generated using weighted ($1/x$) linear least-squares regression as the mathematical model [13]. Concentrations in unknown preclinical or clinical, quality control and stability samples were calculated from the resulting height ratios and the regression equation of the calibration curve ($y = mx + b$). The drug concentration in plasma or urine samples is expressed in ng/ml of free base CGP 60536.

3. Results and discussion

3.1. Selectivity

Representative chromatograms of extracts of CGP 60536 and the I.S. from clinical human plasma or urine samples are shown in Fig. 2.

CGP 60536 and the I.S. were eluted from the column with retention times of approximately 6.4 and 10.5 min, respectively. Peak assignment was established from samples spiked individually with each compound and treated as described in the sample preparation procedure. As shown, the compounds of interest were well separated from co-extracted endogenous plasma or urine components. Similar chromatographic profiles were observed for different plasma collected from control healthy volunteers receiving no medication and for animal plasma samples (baboon, rat and rabbit). Two chromatographic columns of the same characteristics but with a different production batch number demonstrated comparable chromatographic profiles.

3.2. Extraction efficiency

The extraction efficiency of CGP 60536 in human plasma and urine was assessed by comparison of the peak heights from extracted samples ($N=9$) to those from non-extracted aqueous standards spiked at the same CGP 60536 concentrations and at a concentration of 600 ng/ml for the I.S.

The mean±SD efficiencies of the extraction in plasma were 83±6% (100 ng/ml) and 87±8% (2500 ng/ml) for CGP 60536 and 86±5% for the I.S.

The mean±SD efficiencies of the extraction in urine were 94±10% (18.1 ng/ml) and 100±9% (450 ng/ml) for CGP 60536 and 76±5% for the I.S.

The extraction efficiencies were also calculated in plasma from animals. The mean ($N=3$) efficiencies of the extraction were 80 and 87% in baboon, 77 and 87% in rabbit and 86 and 94% in rat for CGP 60536 at the two concentrations of 100 and 2500 ng/ml, respectively. They were 89% in baboon, 81% in rabbit and 86% in rat for the I.S. at the concentration of 600 ng/ml.

3.3. Calibration curves

Representative calibration curves of CGP 60536 for plasma and urine (Fig. 3) gave relevant regression lines of $y=0.0108x+0.0345$ and $y=0.0048x+0.0128$, respectively. Correlation coefficients were 0.9998 (plasma, $N=7$) and 0.9993 (urine, $N=7$). Individual fittings of the calibration standards to the curve were assessed from the relative error (RE) in percentage, calculated as 100×[(back-calculated concentration from the regression line equation)-

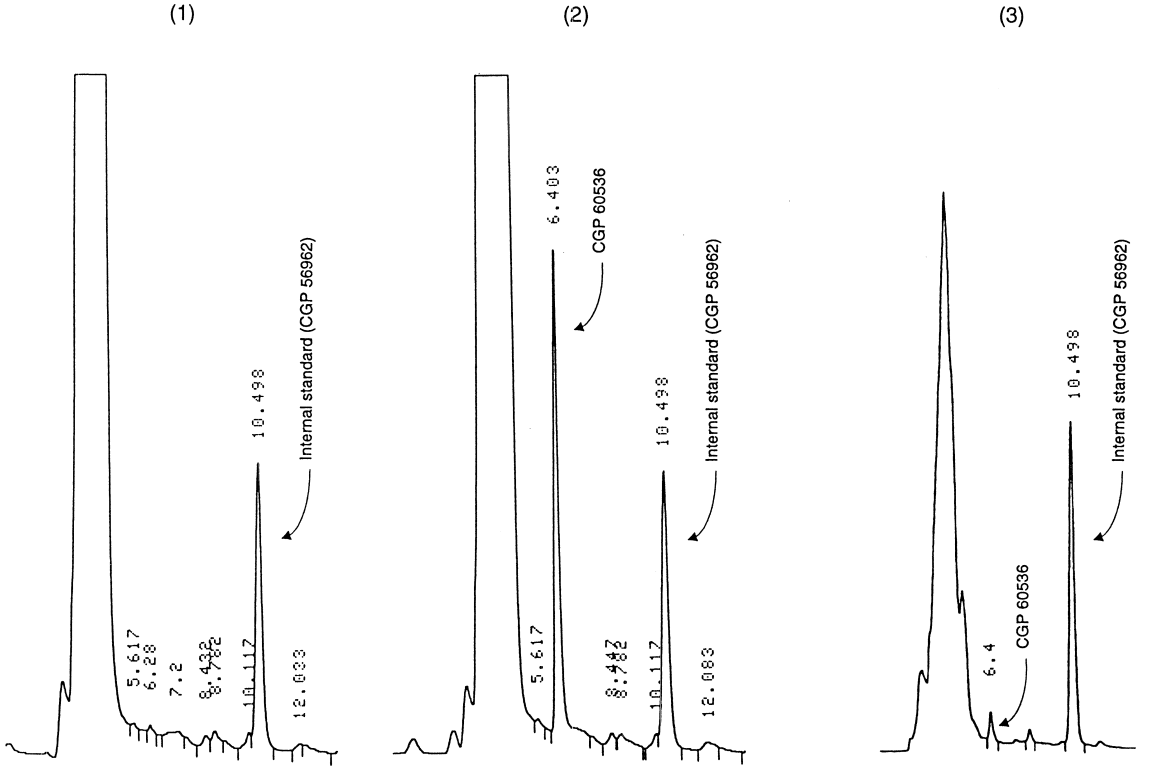


Fig. 2. Examples of chromatograms. Extracts of plasma samples prior to (1) and following (2) drug administration (found concentration 133 ng/ml) and of urine sample (3) (found concentration 18.1 ng/ml) from one human subject given an oral dose of CGP 60536B.

(nominal concentration)]/(nominal concentration). The differences for back-calculated concentrations did not exceed 5% on average from theory in both fluids, indicating a good fit of the weighted regression model over the range of the calibration curves. The reproducibility of the calibration curves, performed over 2 months, was highly satisfactory, as assessed by the low coefficient of variation of the curve slopes (C.V. of 5.5% in plasma and 2.6% in urine, $N=6$).

3.4. Accuracy and precision

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest, near the lowest, near the middle and the highest concentration values of the calibration range [14]. Accuracy was determined by calculating the mean recovery for the found concentrations in percentage of the nominal concentrations in validation samples. Precision was assessed from the coefficient of variation (C.V.) in percentage of the mean recoveries. The following validation criteria for accuracy and precision were used to assess the method suitability: mean recoveries should be within 85–115%, except at the limit of quantitation (LOQ) where it should be within 80–120%; C.V. should not exceed 15%, except at the LOQ where it should not exceed 20% [15].

Human plasma or urine samples were analysed on the same day (intra-day assays) and on successive days (inter-day assays). Recoveries and corresponding coefficients of variation are presented in Table 2.

In plasma, mean intra-day assay recoveries over concentrations from 4.5 to 450 ng/ml ranged from 98 to 106% and the C.V. from 1 to 11%. Mean

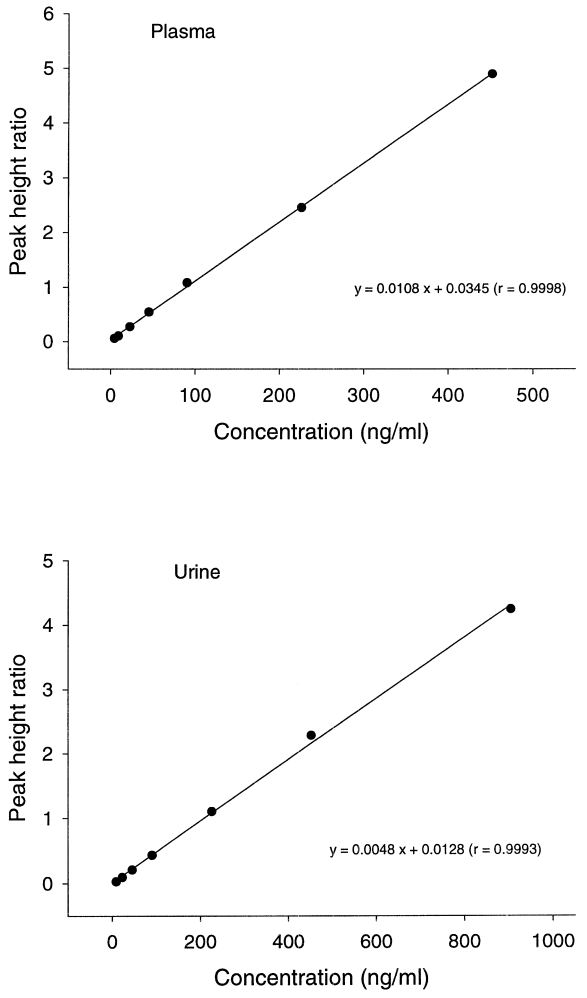


Fig. 3. Representative calibration curves in plasma and urine.

inter-day assay recoveries over concentrations from 4.5 to 450 ng/ml ranged from 99 to 104% and the C.V. from 4 to 8%.

In urine, mean intra-day assay recoveries over concentrations from 9.0 to 900 ng/ml ranged from 97 to 107% and the C.V. from 3 to 13%. Mean inter-day assay recoveries over concentrations from 9.0 to 900 ng/ml ranged from 99 to 107% and the C.V. from 4 to 8%.

Accuracy data obtained in baboon, rat and rabbit plasma (recoveries from 95 to 114%, $N=9$) were comparable to those obtained in humans and demonstrates that the method is also suitable to quantify CGP 60536 in animal kinetic studies.

3.5. Limit of quantitation

The LOQ was defined as the lowest concentration that can be measured with acceptable accuracy, precision and variability. As indicated in Section 3.4, the mean recovery should be within 80–120% of the expected value with a C.V. not exceeding 20%. The lowest concentration values of 4.5 ng/ml (in plasma) and 9.0 ng/ml (in urine) whose accuracy and precision (Table 2) were within the proposed criteria were quoted as the LOQ.

3.6. Stability

The stock solutions of CGP 60536 in water (100 μg/ml) were stable for at least 7 months at +4°C. The stock solutions of the I.S. (100 μg/ml) in 0.01 *M* KH_2PO_4 were stable for at least 2 months at +4°C.

The plasma sample were found to be stable for at least 24 h at room temperature on the auto sampler of the ASPEC system. The concentrations following this storage period were on average 98% of the nominal values of 50, 100, 250, 500, 1000 and 2500 ng/ml.

No loss of CGP 60536 was observed after 7 days of storage at −20°C, but degradation of the compound became apparent after 14 days of storage at −20°C, showing mean recovery of 81% ($N=3$) after 14 days and 76% ($N=3$) after 21 days. CGP 60536 in plasma was stable for at least 12 months at −80°C. No loss of compound was observed after three freeze(−80°C)-thaw cycles of plasma samples at concentrations of 100, 500 and 2500 ng/ml. No stability data was available in urine.

4. Application

This method was applied to the plasma samples from rats, rabbits, marmosets and humans given oral doses of CGP 60536B. Fig. 4 depicts the mean ($N=8$) plasma concentrations measured in rabbits given 10, 50 or 100 mg CGP 60536B/kg body weight as a solution administered orally by gavage. CGP 60536 was rapidly absorbed, reaching peak plasma levels at around 0.25 h post-administration and was then rapidly eliminated from the plasma.

Table 2
CGP 60536 in human plasma and urine: intra- and inter-day accuracy and precision

Nominal concentration (ng/ml)	Intra-day accuracy		Inter-day accuracy	
	Mean±SD recovery [a] (range)	Precision C.V. (%)	Mean±SD recovery (range)	Precision C.V. (%)
Plasma				
4.5	106±1 (106–108)	1	99±4 (91–103)	4
9.0	99±11 (83–110)	11	104±6 (96–110)	6
45.0	98±8 (82–110)	8	102±8 (93–111)	8
450.0	104±3 (100–108)	3	100±6 (94–109)	6
Urine				
9.0	101±13 (87–116)	13	102±6 (96–110)	6
18.1	105±10 (88–116)	10	107±5 (99–113)	5
45.0	97±3 (93–100)	3	101±4 (97–108)	4
450.0	101±5 (97–108)	5	99±8 (86–109)	8
900.0	107±4 (102–111)	4	104±6 (94–108)	6

[a] Recovery = Found concentration expressed in a percentage of the nominal concentration.

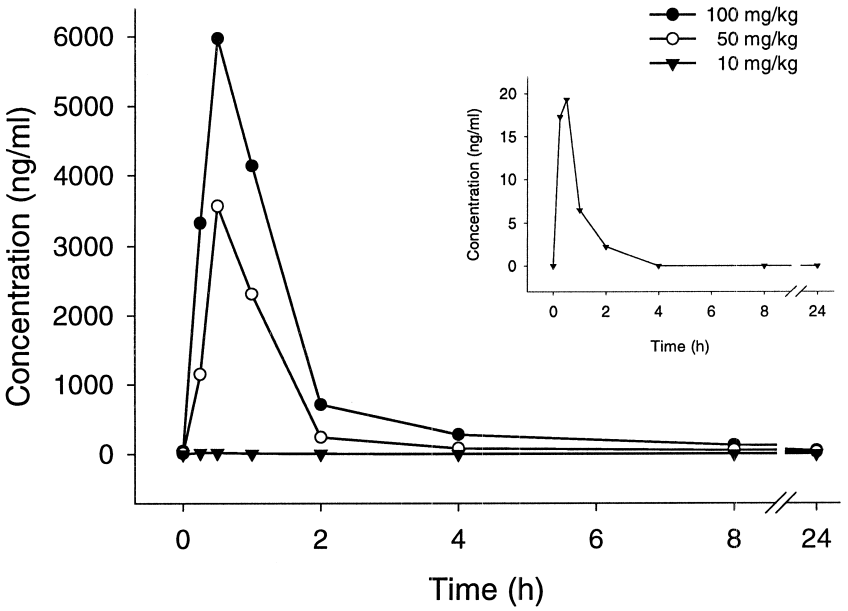


Fig. 4. Mean plasma concentration–time profiles of CGP 60536 in rabbits ($N=8$). Profile after the 10 mg/kg dose is enlarged in insert.

5. Conclusions

A selective and sensitive HPLC method has been developed and validated for quantifying plasma and urine concentrations of the new renin inhibitor CGP 60536 in animals and humans. The working calibration curves range from 4.5 to 450 ng/ml for assay in plasma and from 9.0 to 900 ng/ml for assay in urine. The biological fluid samples must be stored at −80°C immediately after collection. The limit of quantitation is 4.5 ng/ml in plasma and 9.0 ng/ml in urine. In addition, it is fully automated, which greatly increases sample throughput.

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