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Determination of the renin inhibitor Ro 42-5892 in human plasma by automated pre-column derivatization, reversed-phase high-performance liquid chromatographic separation and electrochemical detection after post-column irradiation

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Abstract

The renin inhibitor Ro 42-5892 has been found to be very potent, thereby necessitating a sensitive assay method for the evaluation of its pharmacokinetics in man. We report here the development of a very sensitive and selective HPLC assay for the analysis of this compound in human plasma. Ro 42-5892 was extracted from plasma with dichloromethane, derivatized with 2,4-dinitrofluorobenzene and then chromatographed on a Novapak C_{18} column (150 × 3.9 mm I.D.) with acetic acid buffer (pH 7)–acetonitrile (100:85). Detection was performed by irradiation at 254 nm, followed by electrochemical oxidation at 550 mV. The extraction recovery of Ro 42-5892 from human plasma (mean 102%) was quantitative. With this method a limit of quantitation of 0.3 ng/ml was achieved. The assay was linear up to 5 ng/ml, had acceptable inter-assay precision (12.2%) and accuracy (9.3%) and was successfully tested for selectivity. This assay was successfully applied to over 250 samples from a pharmacokinetic study in hypertensive patients.

1. Introduction

Renin and the angiotensin converting enzyme (ACE) are peptidases of the renin-angiotensin system (RAS). This system is a multi-regulated proteolytic cascade producing potent pressor peptides [1,2]. In accordance with the participation of the RAS in the control of blood pressure homeostasis, treatment with ACE inhibitors reduces blood pressure in hypertensive patients [1,3,4]. Although ACE inhibitors are generally well tolerated, they give rise to adverse effects in some patients [5].

Inhibition of the first enzyme of the RAS, renin, potentially produces fewer clinical side effects, since renin is highly specific, possessing only one known substrate, namely angiotensinogen [1,2]. Accordingly, the search for renin inhibitors is a very active research field [6]. (*S*)-α-[(*S*)-α-[(*tert.*-butylsulfonyl)methyl]hydrocinnamamido]-N-[(1*S*, 2*R*, 3*S*)-1-(cyclohexylmethyl)-3-cyclopropyl-2,3-dihydroxypropyl]imidazole-4-propionamide methanesulfonate (Ro 42-5892/001, I, Fig. 1) is a developmental renin inhibitor currently under investiga-

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tion as an antihypertensive agent [7]. It has been found to be very potent, thereby necessitating a sensitive assay method for the evaluation of its pharmacokinetics in man [8].

Since the renin inhibitor I contains no structural group allowing highly sensitive detection, derivatization was necessary to reach the required quantification limit. Fortunately, renin inhibitor I possesses an amine group in the imidazole ring of its histidine moiety. This amine functionality has already been used as the site of derivatization in the development of an analytical method for toxicokinetic purposes [9]. The method was based on increased UV absorption after derivatization with 4-dimethylaminoazobenzene-4′-sulfonyl chloride. However, the resulting quantification limit of 3–5 ng/ml was not sufficient to establish a pharmacokinetic profile in humans. Consequently, derivatization with the fluorescence label 9-fluorenylmethylchloroformate was developed [10]; however, with a quantification limit of 2 ng/ml this method still proved to be insufficiently sensitive.

Electrochemical detection has long been established as a sensitive and selective technique [11]. Derivatization with 2,4-dinitrofluorobenzene (DNFB, Sangers reagent) has been introduced as one possibility to make molecules which possess no electrochemically active group amenable to this detection mode [12]. Another, independent possibility to introduce amenability to and/or increase sensitivity of electrochemical detection is irradiation of the HPLC-eluent with UV-light before, or in, the electrochemical cell [13–18]. Depending on the type of analyte and the conditions during irradiation, different chemical processes are induced. Amino alcohols and amino acids were analyzed with high sensitivity using a combination of derivatization and irradiation [19], including the determination of serine in beer. The same principle has also been applied to the determination of prostaglandins in a limited number of plasma and urine samples [20], using 2,4-dinitrophenylhydrazine or *p*-nitrobenzyloxy-amine hydrochloride as derivatization agents.

We now report on the first application of the original approach [19] to a routine assay, used for pharmacokinetics. Renin inhibitor I and the internal standard (Ro 42-4661/000, II, Fig. 1) were extracted from plasma and then derivatized with 2,4-dinitrofluorobenzene. The products (dinitrophenylderivative of Ro 42-5892: Ro 42-8809/000, III, Fig. 1) were then chromatographed on a C_{18} stationary phase and detected by electrochemical oxidation after UV irradiation. The usefulness of the assay in bioanalytical routine was established by its successful application to over 200 samples from a pharmacokinetic study.

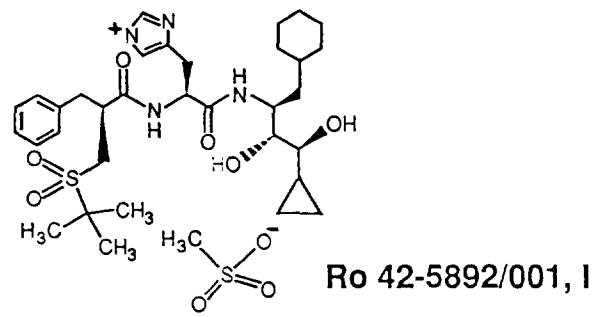
2. Experimental

2.1. Materials, reagents and solvents

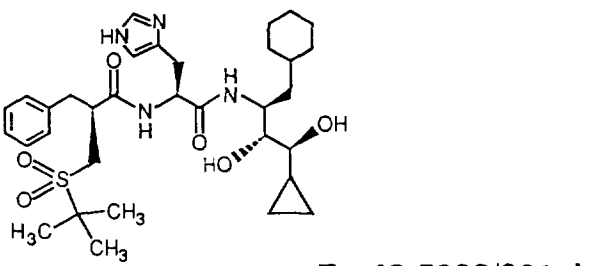
The following materials, reagents and solvents were used: Reacti-Therm heating module and Reacti-Vap evaporation unit (Pierce, Rockford, IL, USA); centrifuges: Hettich Rotixa KS (for glass tubes) and Mikrorapid K (for polypropylene vials; Hettich, Tuttlingen, Germany); degassing unit: Shodex Degas (Henggeler Analytik, Riehen, Switzerland); HPLC-pump: L-6000 (E. Merck, Darmstadt, Germany); pulse damper: Portmann Instruments type PM-PD (Innovativ-Labor AG, Adliswil, Switzerland); Gilson 232-401 sample processor (Synmedic AG, Zürich, Switzerland); Haake thermostat F3CH (Digitana AG Horgen, Switzerland); precolumn: LiChrospher 100 RP-18, 5 μm, 4 × 4 mm (E. Merck, Darmstadt, Germany); analytical column: Novapak C_{18}, 4 μm, 150 × 3.9 mm I.D. (Millipore-Waters, Volketswil, Switzerland); Beam Boost UV-irradiation unit (ICT, Basel, Switzerland); electrochemical detector: ESA Coulochem II with 5011 measuring cell and 5020 guard cell (Stagroma AG, Wallisellen, Switzerland); integrator: SP4200 (Spectra-Physics AG, Basel, Switzerland).

Ro 42-5892/001 (analyte, I, Fig. 1), Ro 42-4661/000 (internal standard, II, Fig. 1) and Ro 42-8809/000 (dinitrophenylderivative of Ro 42-5892, III, Fig. 1) from certified batches.

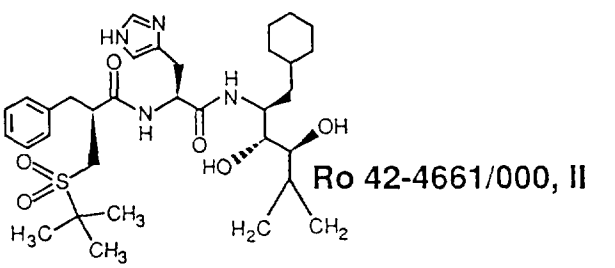
2,4-Dinitrofluorobenzene (Catalogue No.



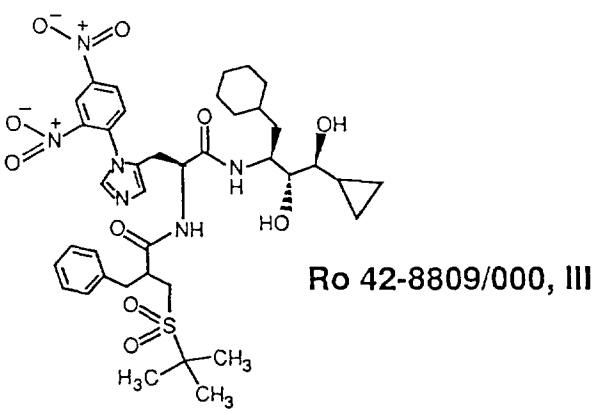
Ro 42-5892/001, I



Ro 42-5892/000, Ia



Ro 42-4661/000, II



Ro 42-8809/000, III

Fig. 1. Structures of renin inhibitor Ro 42-5892/001 (I), Ro 42-5892/000 (Ia), internal standard Ro 42-4661/000 (II) and the dinitrofluorobenzene-derivative of I, Ro 42-8809/000 (III).

D19,680-0, Aldrich, Steinheim, Germany); acetonitrile HPLC grade S (Rathburn, Walkerburn, UK); water: Baker Analyzed HPLC Reagent (J.T. Baker BV, Deventer, Netherlands); dichloromethane LiChrosolv, methanol p.a. (E. Merck, Darmstadt, Germany); Helium 46 for degassing (99.996% pure, Carbagas, Liebefeld-Bern, Switzerland).

2.2. Preparation of standards

Stock solutions of renin inhibitor I and internal standard II were prepared in methanol. Water as solvent should be avoided because of insufficient solubility (internal standard) and possible adhesion to the container (renin inhibitor). The concentrations of the stock solutions were 0.575 mg/ml for I (corresponding to 0.5 mg/ml Ro 42-5892/000, Ia, Fig. 1) and 1.0 mg/ml for II, respectively.

For the internal standard, a working solution in acetonitrile (7 ng/0.8 ml = 8.75 ng/ml) was prepared.

For the preparation of the calibration standards, a stock solution of 0.5 mg/ml of renin inhibitor was diluted in several steps with plasma to obtain calibration concentrations of 5.0, 3.0, 2.0, 1.0, 0.75, 0.5, 0.3 or 0.2 ng/ml. The concentrations of the independent quality control samples were 0.75 or 2.0 ng/ml.

Calibration standards and quality control samples (1-ml portions) were stored at −20°C (see sample preparation procedure).

2.3. Sample preparation procedure

Plasma samples were obtained using EDTA as anticoagulant and stored at −20°C until analysis. Samples containing concentrations above 5 ng/ml were diluted with tested blank plasma. All processes were carried out at room temperature unless stated otherwise. In a polypropylene vial, plasma (1 ml) was mixed with NaOH (1 *M*, 85 μl) and internal standard (800 μl working solution). After 10 min, the mixture was centrifuged (10 min, 1500 *g*) and the liquid phase was added to dichloromethane (2 ml) in a glass tube before head-over-head extraction (10 min, 20 rpm). After centrifugation (20 min, 1700 *g*), the lower, organic phase was evaporated to dryness at 70°C under helium.

The residue from the extraction was dissolved in acetonitrile–water (1/1, v/v, 70 μl). After centrifugation (10 min, 4600 *g*), an aliquot (65 μl) was transferred into a crimp-capped vial. The derivatization was performed as an automated, sequential process with a Gilson 232-401 sample processor, which added sodium borate buffer (0.2 *M*, pH 8.0, 15 μl) and 2,4-dinitrofluorobenzene (2.0 μmol in 15 μl of acetonitrile, kept at 10°C), and transferred the mixture from a rack at 10°C to one at 80°C. After 25 min an aliquot (50 μl) was injected, while still hot, onto the column.

2.4. Chromatographic system and conditions

A schematic representation of the HPLC-system is given in Fig. 2. The mobile phase consisted of 1000 ml of solution A [92.5 mg disodium-EDTA in 0.1 *M* acetic acid, pH 7.0 (1 *M* NaOH)] plus 850 ml of acetonitrile, flow-rate 1.2 ml/min. Degassing was performed with an online membrane degasser; the precolumn (4 × 4 mm) was filled with LiChrospher 100 RP-18, 5 μm, the analytical column (150 × 3.9 mm I.D.) with Novapak C_{18}, 4 μm. Irradiation at 254 nm was performed with a Beam Boost using a teflon coil of 1 m × 0.3 mm. The ESA Coulochem II was used as electrochemical detector, with the preconditioning cell set at 800 mV. The potential settings on the 5011 dual electrode were 300 mV and 550 mV for the first and the second electrode, respectively.

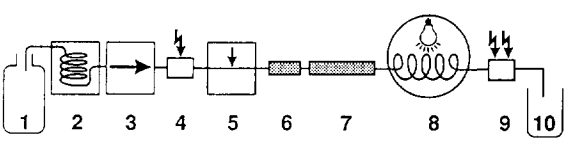


Fig. 2. Schematic representation of the HPLC-system. (1) Mobile phase, (2) degassing unit, (3) pump, (4) preconditioning cell, (5) sample processor, (6) pre-column, (7) analytical column, (8) irradiation unit, (9) electrochemical detector, (10) waste.

2.5. Calibration and calculations

The concentrations of unknown samples were calculated from a linear calibration curve. This calibration curve was obtained by computing a weighted least-squares regression (weighting factor $1/y^2$) of the peak-height ratios Y (derivatized renin inhibitor/derivatized internal standard) versus the renin inhibitor concentrations X from 7 standard samples. The calculations were performed using an improved version of a special BASIC programm described for the Spectra-Physics SP4100 integrator [21]. Since duplicate analyses are always performed on different days, only inter-assay precision was investigated.

2.6. Recovery

For the determination of recovery, quadruplicate analyses of two renin inhibitor concentrations, 0.5 and 3.0 ng/ml, were performed. The mean response for the worked up samples (8 ng of II were added before derivatization) was compared to that obtained by analysis of blank plasma aliquots (1 ml) to which I (0.5 or 3.0 ng) and II (8 ng) had been added at the end of the extraction procedure.

3. Results and discussion

3.1. Sample preparation procedure

The extraction procedure described for the analytical method used for toxicokinetics [9] was applied without modification.

Due to the low reactivity of renin inhibitor I, the derivatization process had to be performed at 80°C to obtain reasonably short reaction times (Fig. 3), even though the reagent DNFB was of high reactivity, and had to be prepared fresh for each series and kept at 10°C until use. The pH, on the other hand, was of no great influence in the range 7.1–8.4 (Fig. 4). The decline in peak height at higher pH values is most likely due to the increase in the reaction rate of hydrolysis of DNFB [22]. The salt concentration was set at the highest value not giving precipitation (0.2 M), to ensure a constant pH during the reaction. The compound was stable at 4°C in the extract before (tested up to 5 days), and after derivatization (tested up to 7 days). It was found that the reaction yield for such small concentrations (0.5–5 ng/ml) did not need a constant excess, but a constant amount of reagent for optimal results. This is most likely due to the fact that the matrix coextracted from the plasma is the main consumer of the reagent, irrespective of the concentration of analyte. A concentration of 2 μM seemed to give the best compromise between the maximally achievable peak height and the front peak generated by the excess of reagent. The use

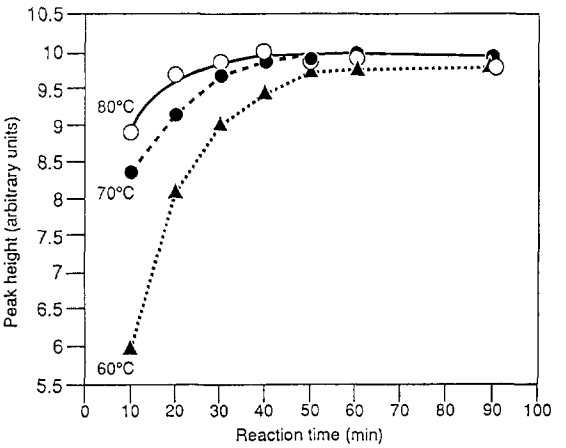


Fig. 3. Dependence of peak height of renin inihibitor I on reaction time and temperature during pre-column derivatization.

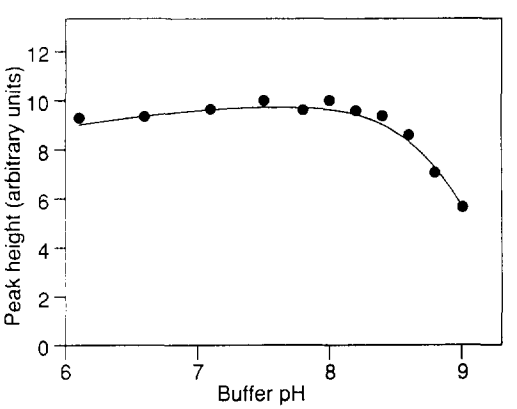


Fig. 4. Influence of buffer pH during pre-column derivatization on peak height for renin inihibitor I.

of a versatile sample processor allowed the automation of the derivatization. This had the combined advantages of minimizing manual sample handling and allowing freshly derivatized, hot sample to be injected onto the column. This last point is of importance due to the sensitivity of the detection method to dissolved gas (see below), since hot solutions contain less dissolved gases than cold solutions. Additionally, the time per sample could be reduced by derivatizing the next sample during the chromatographic analysis of the preceding sample. The automation of the derivatization process also sped up the development process, since several different conditions could be tested during the same, unattended, overnight series.

3.2. Analytical system and chromatography

The use of DNFB allows the application of electrochemical redox detection, which involves reduction at high potential at the first electrode (−0.8 V) and reoxidation at low potential (+0.1 V) at the second electrode. However, although this detection scheme was very sensitive (10 pg of III, the DNFB-derivative of I, could easily be detected), a rapid and dramatic fouling of the electrodes prevented its further use. This fouling could not be avoided and was irreversible, even when the electrochemical cell was cleaned off-line.

We therefore tested the approach of irradiation prior to the electrochemical oxidation [19] for III. The irradiation step was a necessary prerequisite to obtain any oxidative signal. Fig. 5 shows the relative dependence of signal and background current on the voltage applied on the second detection electrode of the dual-electrode cell. As a compromise between maximal signal intensity and acceptable baseline noise and drift, a standard setting of 550 mV was chosen. The first electrode was set at 300 mV, since at this potential no signal was lost, but easily oxidized interferences were eliminated. The preconditioning cell was set at 800 mV to prevent interferences and to decrease background current caused by constituents of the mobile phase. In addition, 0.1 *M* sodium acetate was a compromise with respect to increased signal at higher molarity and an even stronger increase in background current. The pH was set to 7, since higher values are not acceptable for silica-based LC columns. Another compromise had to be found for the irradiation time. Fig. 6 shows that a 13-s irradiation gave the highest signal, but the baseline drift was much smaller after an irradiation of only 3.5 s; this duration was therefore chosen.

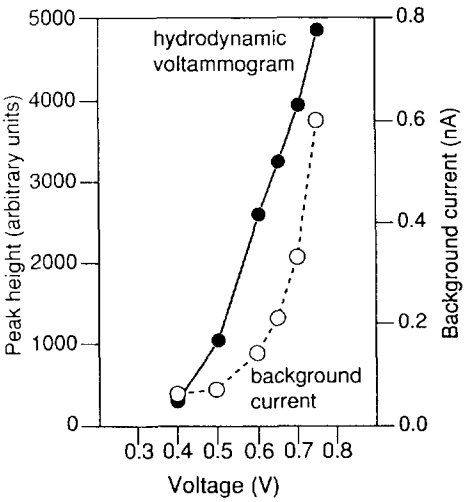


Fig. 5. Hydrodynamic voltammogram of III, detection by irradiation followed by electrochemical oxidation, compared to the dependence of the background current on applied potential.

Effective degassing of the mobile phase was important. An on-line degasser was necessary.

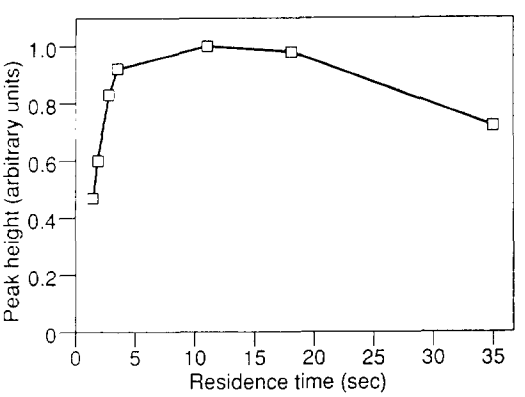


Fig. 6. Dependence of peak height for III on residence time in the post-column irradiation reactor.

Without degassing, addition of new mobile phase induced a baseline drift lasting for several hours.

Since the mobile phase had to be optimized with regard to detection, adjusting the selectivity of the chromatographic system was restricted to the selection of the appropriate stationary phase. Accordingly, 15 different silica gel-based phases (3 to 10 μm particle size, phenyl-, C_6-, C_8- or C_{18}-bonded) and 3 other phases (organic polymer, porous graphitic carbon, aluminium oxide) were tested in 24 different column formats (lengths 70–250 mm, I.D. 3.9–4.7 mm). Only Novapak C_{18}, 150 × 3.9 mm I.D., 4 μm particle size, gave the required selectivity between interfering peaks and the analyte (Fig. 7a,b). This phase also showed a high column-to-column reproducibility of selectivity and retention times (t_R = 11.26 min ± 2.2% for first injection of III on 8 different columns).

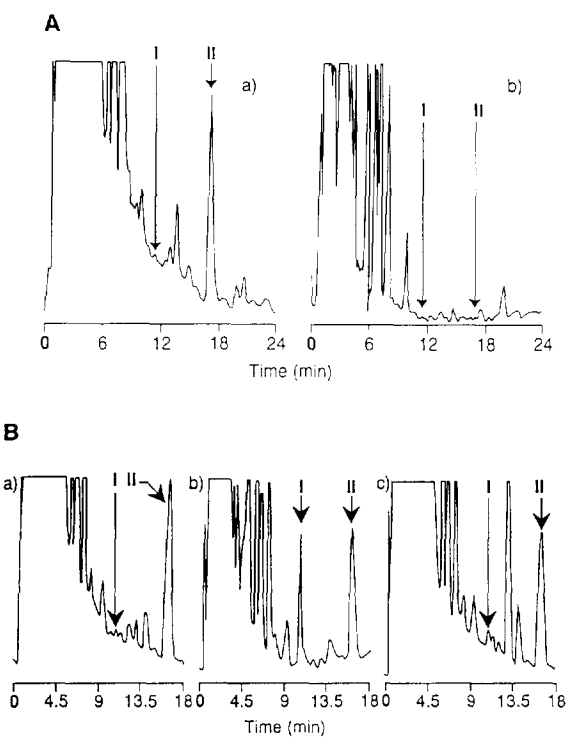


Fig. 7. (A) Chromatograms (a) of human blank plasma spiked with 7 ng/ml II, (b) reagent blank; the retention times of analyte (I) and internal standard (II) are indicated. (B) Chromatograms of human plasma containing I (t_R = 11.4 min) and spiked with II (internal standard, 7 ng/ml, t_R = 17.0 min); (a) standard spiked with 0.2 ng/ml I (lowest calibrator); (b) standard spiked with 5.0 ng/ml I (highest calibrator); (c) plasma sample collected 3 h after dosing of 200 mg I p.o. (concentration found 0.56 ng/ml).

3.3. Recovery

The two concentrations chosen for the test of recovery (0.5 and 3 ng/ml) were selected to cover the calibration range of 0.2 to 5 ng/ml. Practically quantitative recoveries of 97.9 and 107% were found for the 0.5 and 3 ng/ml concentrations, respectively.

3.4. Selectivity

The chromatograms presented in Fig. 7 show that the method was sufficiently selective. All ten different blank plasmas tested during the validation were free from interferences.

3.5. Linearity

The calibration curve data presented in Table 1 show that the assay was linear over the range tested (0.2–5 ng/ml). Most of the analyses of the lowest calibration concentration (0.2 ng/ml) were excluded from the regression calculation because of chromatographic problems.

Table 1
Linearity of the calibration curve, as expressed by the regression parameters and the mean deviation of the calibration samples from the regression line[a]

Day	r	a	b	Mean deviation[b]
1	0.995	465.7	1.492	5.0
2	0.993	658.6	2.845	5.9
3	0.998	674.7	1.447	2.9
4	0.988	559.2	1.429	8.6
5	0.988	−24.3	1.322	4.7
6	0.987	215.0	1.205	6.1
7	0.996	49.8	1.378	3.6

[a] Weighted least-squares regression (weighting factor $1/y^2$); $Y = a + bX$.

[b] Mean deviation = Mean deviation of back-calculated concentrations from added concentrations, in percent.

Table 2
Inter-assay precision and accuracy of the determination of Ro 42-5892 in human plasma determined from quality control samples, quantification limit (QL) test samples, and study samples

Concentration added (pg/ml)	Mean concentration found (pg/ml)	n[a]	R.S.D.[b] (%)	IAC[c] (%)
300 (QL)	315	4	19.6	5.1
500	551	6	10.7	10.1
750	884	9	11.2	17.8
2000	2085	10	7.4	4.3
Study samples	300–26800	126	13.0	n.a.

[a] n: For the study samples, n refers to the number of duplicates of different samples; for the other samples, n represents the number of single determinations; all replicates were performed on different days.
[b] R.S.D. (%): Inter-assay precision, expressed as relative standard deviation in percent.
[c] IAC (%): Inaccuracy, expressed as relative deviation of concentration found from concentration added in percent.

3.6. Precision and accuracy

The data on precision and accuracy are presented in Table 2. As a consequence of the low concentrations analyzed, the mean inter-assay precision (12.2%) and the mean inaccuracy (9.3%) are relatively high, but still acceptable. The mean inter-assay precision of the duplicate determinations of study samples (13.0%) is in very good agreement with the result for the quality control samples.

3.7. Limit of quantification

The lack of chromatographic reliability at the lowest calibration concentration (0.2 ng/ml) was one of the reasons to set the limit of quantification at 0.3 ng/ml. At the latter concentration, our criterion for precision (<20%) was just fulfilled.

3.8. Stability

In a previous study, Ro 42-5892 was shown to be stable in human plasma for up to 5 days at 25°C and for up to 6 months at −20°C [23].

3.9. Application to biological samples

The method was successfully applied to over 250 samples from a pharmacokinetic study in hypertensive patients [24]. The overall precision of all 126 double determinations (mean R.S.D. 13.0%; Table 2) was acceptable. The higher concentrations were determined with better precision (mean R.S.D. 7.1%, $n = 39$, conc. found >2000 ng/ml).

4. Conclusions

The potent renin inhibitor Ro 42-5892 requires a sensitive assay method for the evaluation of its pharmacokinetics in man. We report here the development of a very sensitive and selective HPLC assay for the analysis of this compound in human plasma.

Ro 42-5892 was extracted from plasma with dichloromethane, derivatized with 2,4-dinitrofluorobenzene and then chromatographed on a reversed-phase HPLC column with acetic acid buffer–acetonitrile. Detection was performed by irradiation, followed by electrochemical oxidation.

With this method, a limit of quantification of 0.3 ng/ml was achieved. The assay was linear up to 5 ng/ml and had acceptable inter-assay precision and accuracy. It was successfully tested for selectivity. The extraction recovery of Ro 42-5892 from human plasma was quantitative. This assay was successfully applied to over 250 samples from a pharmacokinetic study in hypertensive patients. The stability of Ro 42-5892 was satisfactory.

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