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Oral platelet aggregation inhibitor Ro 48-3657: Determination of the active metabolite and its prodrug in plasma and urine by high-performance liquid chromatography using automated column switching

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Abstract

A sensitive and highly automated high-performance liquid chromatography (HPLC) column-switching method has been developed for the simultaneous determination of the active metabolite III and its prodrug II, both derivatives of the oral platelet inhibitor Ro 48-3657 (I), in plasma and urine of man and dog. Plasma samples were deproteinated with perchloric acid (0.5 *M*), while urine samples could be processed directly after dilution with phosphate buffer. The prepared samples were injected onto a pre-column of a HPLC column switching system. Polar plasma or urine components were removed by flushing the precolumn with phosphate buffer (0.1 *M*, pH 3.5). Retained compounds (including II and III) were backflushed onto the analytical column, separated by gradient elution and detected by means of UV detection at 240 nm. The limit of quantification for both compounds was 1 ng/ml (500 μl of plasma) and 25 ng/ml (50 μl of urine) for plasma and urine, respectively. The practicability of the new method was demonstrated by the analysis of about 6000 plasma and 1300 urine samples from various toxicokinetic studies in dogs and phase 1 studies in man.

Keywords: Column switching; Platelet inhibitor; GP IIb–IIIa receptor; Ro 48-3657

1. Introduction

[Z] - (*S*) - [[1-[2-[[4 - (aminohydroxyiminomethyl)-benzoyl]amino] - 1 - oxopropyl] - 4 - piperidinyl]oxy]-acetic acid ethyl ester, I (Ro 48-3657; F. Hoffmann-La Roche, Basle, Switzerland) (see Fig. 1) is being developed for secondary prevention of arterial thrombosis, i.e., following unstable angina, acute myocardial infarction, percutaneous transluminal coronary angioplasty, transient ischemic attacks and stroke. In contrast to aspirin, which predominantly inhibits the generation of the platelet agonist, thromboxane A2, direct GP IIb–IIIa inhibitors like III act on the final common pathway of platelet aggregation, the fibrinogen receptor. It is hoped that the higher

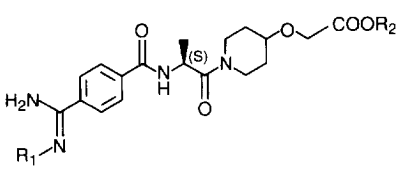

Fig. 1. Chemical structures for the compounds referred to in the text: I, R_1=OH, R_2=C_2H_5, double protected prodrug; II, R_1=OH, R_2=H, inactive prodrug; III, R_1=H, R_2=H, active metabolite.

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intrinsic potency of GP IIb–IIIa receptor antagonists will translate into a better clinical benefit–risk ratio compared to aspirin [1].

After oral administration, the double protected prodrug, I (amidoxime ethyl ester), is moderately absorbed and metabolized into the inactive prodrug, II (amidoxime free acid), and into the active metabolite, III (amidine free acid), both of which are found in plasma and urine [2].

For pharmacokinetic studies performed in dog and man, a sensitive and precise method was required for the determination of II and III in plasma and urine. Since III binds to blood platelets [1], the new method should be able to separate between free and total (i.e., free plus bound) concentrations of III in plasma.

2. Experimental

2.1. Reagents and solvents

Ethanol (absolute, p.a.), methanol (HPLC grade) and perchloric acid (70%, p.a.) were obtained from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade S) was purchased from Rathburn (Walkerburn, UK). Phosphate buffer solutions of different molarities were prepared by diluting *ortho*-phosphoric acid (85%, p.a.; Merck) with bidistilled water and adding sodium hydroxide (p.a.; Merck) until the desired pH values of 3.5 and 5.5 were obtained.

2.2. Solutions

Stock solutions were obtained by dissolving the pure compounds II and III in bidistilled water. Aliquots of the stock solutions were diluted with water to provide working solutions. Stock solutions could be stored at 5°C for about three months. Working solutions were prepared prior to use.

2.3. Plasma standards

Standards and QC samples were obtained by spiking dog and human blank plasma (10 ml) with 100 μl of the corresponding working solution, providing concentrations of between 1 and 10 000 ng/ml (dog studies) and between 1 and 250 ng/ml (human studies). The samples were divided into 1.2 ml volumes and stored in a deep-freeze (−20°C) until required for analysis.

2.4. Urine standards

Standards and QC samples were obtained by spiking drug-free human urine (10 ml) with 500 μl of the corresponding working solution, providing concentrations of between 25 and 10 000 ng/ml. The samples were divided into 1.2 ml volumes and stored in a deep-freeze (−20°C) until required for analysis.

2.5. Sample preparation

Plasma (0.5 ml) was mixed with 0.5 ml of perchloric acid (0.5 *M*). After centrifugation (ca. 20 000 *g*, 6 min), a 0.8 ml volume of the deproteinated sample was diluted with 0.75 ml of phosphate buffer (1 *M*, pH 5.5). Finally, 1.4 ml of the resulting solution was injected into the chromatographic system.

Urine (0.05 ml) was mixed with 1.5 ml of phosphate buffer (0.1 *M*, pH 3.5). Finally, 1.4 ml of the resulting solution was injected into the chromatographic system.

2.6. Instrumentation

A schematic representation of the column-switching system is given in Fig. 2. A LC pump P1 (Model L-6000A; Merck-Hitachi, Darmstadt, Germany) delivered the solvents M1A, M1B and M1C via the solvent selector, SS (LabSource, Reinach, Switzerland), for purging the precolumn, PC, at flow-rates of between 0.05 and 3 ml/min. Samples (1.4 ml) were injected by the autosampler, AS (Model AS-4000; Merck-Hitachi), onto one of the pre-columns, PC. In order to inject large sample volumes, the autosampler was used with a 5-ml syringe, a 3-ml loop, and the "slow needle-down-speed"-mode. The gradient pump, P2 (Model L-6200A; Merck-Hitachi), in connection with the mixing chamber, MC (Merck-Hitachi), delivered the gradient mobile phases M2A–M2B–M2C at flow-rates of between 0.05 and 2.5 ml/min, for chromatography of the retained components on the analytical column, AC. A column oven (Model Pelcooler; Labsource) allowed the

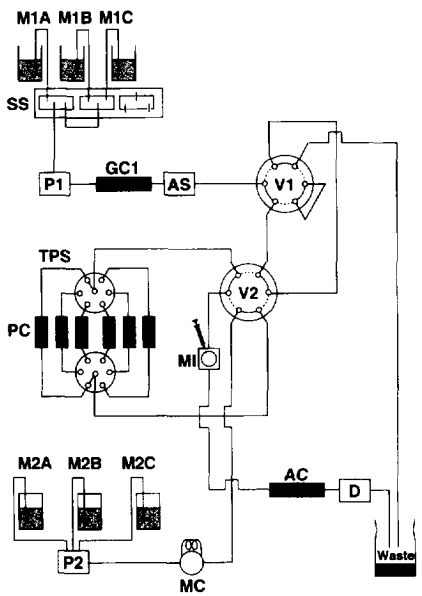

Fig. 2. Schematic representation of the column-switching system (see text for details).

analytical separation to be performed at constant temperature (25°C). Detection of the eluted compounds was carried out at 240 nm with a UV detector, D (Model SPD-10A; Shimadzu, Zürich, Switzerland). A manual injector, MI (Model 7125 with a 100-μl loop; Rheodyne, Cotati, CA, USA), was used for direct injections onto the analytical column (e.g., for recovery experiments).

The two high-pressure switching valves, V1 and V2 (Model High Speed Valve 7000E; LabSource), operated electrically and were controlled by the external time events of the gradient pump, P2. The back-pressure of the precolumns, PC, was monitored by a pressure controller, which was part of a tandem precolumn selector, TPS (Model E.A. 6 Port Valve 7066 CPR; LabSource). When a certain pressure limit was reached (250 bar), the TPS switched automatically to a fresh precolumn, PC (selection of new precolumns could also be triggered manually).

2.7. Columns and mobile phases

Cartridges (4×4 mm I.D.) filled with LiChrospher 100 RP-18 (5 μm; Merck) were used as the guard column, GC1. The tandem precolumn selector, TPS, was equipped with six cartridges (20×4.6 mm I.D.) filled with Supelcosil LC-ABZ (5 μm) (Supelco, Bellefonte, PA, USA) acting as precolumns PC. The analytical column, AC, (150×4.6 mm) filled with Supelcosil LC-ABZ (5 μm) was commercially available (Supelco).

Three mobile phases were used during the enrichment process; M1A, phosphate buffer (0.1 *M*, pH 3.5); M1B, ethanol–water (1:1, v/v) and M1C, ethanol.

The gradient mobile phase prepared by pump P2 consisted of three components; M2A, phosphate buffer (0.11 *M*, pH 3.5)–acetonitrile–methanol (92:4:4, v/v); M2B, phosphate buffer (0.12 *M*, pH 3.5)–acetonitrile–methanol (84:8:8, v/v); M2C, phosphate buffer (0.2 *M*, pH 3.5)–acetonitrile–methanol (50:25:25, v/v). The mobile phases were degassed on-line by a commercially available solvent degasser (Model SDU 2003; Labsource).

2.8. Analytical procedure

A complete automated sample analysis lasted 15 min, and included four column-switching steps, which are illustrated in Table 1. The corresponding flow diagram for both pumps is shown in Fig. 3.

2.9. Calibration and calculations

Along with the unknown samples, at least six plasma (or urine) standards covering the expected concentration range were processed. The calibration curve for the prodrug was established by weighted linear least-squares regression (weighting factor=$1/Y^2$) of the measured peak height (y) of II versus the concentration (x) of II added to the plasma (or urine). The calibration curve for III was established in the same way. These calibration curves were then used to interpolate the concentrations of II and III in plasma (or urine) samples from measured peak heights.

3. Results and discussion

3.1. Sample collection

The measurement of plasma concentrations of GP IIb–IIIa antagonists can result in a substantial unde-

Table 1
Scheme of the automated column-switching process

Step	Time (min)	Valve Position	Precolumn	Analytical column
A	0–4.5	V1=0 V2=0	Deproteinated sample is injected by means of the AS onto the PC. Hydrophilic compounds are flushed with M1A to waste, while lipophilic components remain on top of the PC	AC is conditioned with 100% M2A
B	4.5–5.0	V1=1 V2=0	PC is backflushed with M1A in order to remove trapped solid material without washing out enriched analytes	
C	5.0–7.5	V1=1 V2=1	Enriched material is desorbed and transferred from PC to AC in the backflush mode by means of the analytical mobile phase (gradient M2A–M2B). All capillaries between P1–AS–V1–waste are purged with 100% M1B	
	7.5–11	V1=1 V2=0	Removal of strongly retained material by backflushing the PC with 100% M1C	Gradient elution of transferred material on AC. Detection of separated components in Detector D
D	11–13	V1=0 V2=0	Reconditioning of the PC with M1B	Removal of remaining material from AC with M2C
	13–15	V1=0 V2=0	Reconditioning of PC with M1A	Reconditioning of AC with M2A

restimation of the real drug level in whole blood. Compound III is a potent inhibitor of platelet aggregation [1] and, therefore, binds with high affinity to the platelet receptor GP IIb–IIIa. Due to the rather high concentration of GP IIb–IIIa in blood (about 30 n*M*), the circulating blood platelets have the capacity to bind approximately 11 ng of III/ml of blood.

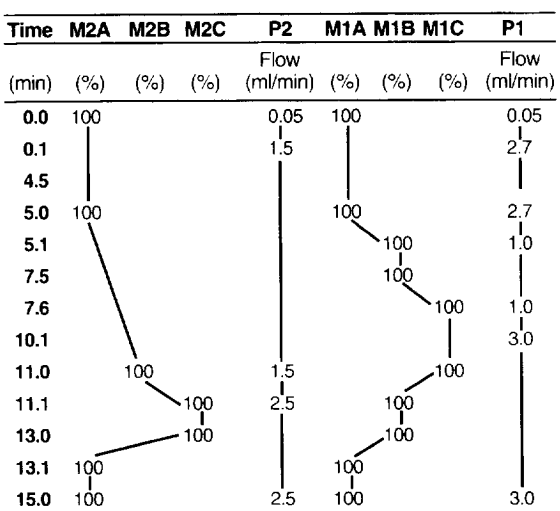

Fig. 3. Flow diagram of the pumps P1 and P2.

In a series of experiments, the effect of the anticoagulant on the recovery of II and III in plasma samples was investigated. Fresh human blood was drawn from the antecubital vein of normal volunteers, directly into trisodium citrate (0.38% final concentration) or into ethylene diamine tetraacetic acid (EDTA; 5 m*M* final concentration). The anticoagulated blood was then spiked either with 5 ng/ml II and III or with 20 ng/ml II and III and plasma was prepared.

When 5 ng/ml of either compound was added to whole blood, the concentration of III measured in citrated plasma was only 39% of the plasma concentration calculated under the assumption that III did not bind to any blood cells (Table 2). Thus, 61% of the compound was missing in the citrated plasma due to its binding to platelets. In contrast, 95% of II was recovered in citrated plasma, indicating that II (at 5 ng/ml) does not interact with the platelet receptor. This result is in good agreement with data determined in solid-phase receptor assays, where II was about 300 times less potent than III in inhibiting

Table 2
Citrate versus EDTA anticoagulation

Compound	Blood concentration (ng/ml)	Plasma concentration calculated[a] (ng/ml)	Citrated plasma		EDTA-treated plasma	
			(ng/ml)	(%)[b]	(ng/ml)	(%)[b]
II	5.0	8.77	8.29	95	8.83	101
	20.0	35.09	30.49	87	33.04	94
III	5.0	8.77	3.39	39	8.00	91
	20.0	35.09	20.95	60	32.03	91

[a] These concentrations were calculated using a hematocrit of 43% (i.e., cell volume, 43%; plasma volume, 57%).
[b] Percentage of recovery (relative to 8.77 and 35.09, respectively).

fibrinogen binding to GP IIb–IIIa [1]. When 20 ng/ml of either compound was added to the citrated blood, 60% of III was recovered in the plasma, representing the non-bound compound. The recovery of III was higher in this case because the binding capacity of platelets is limited.

When the blood was anticoagulated with EDTA, the recovery of both compounds, II and III, in the plasma was more than 90%, independent of the initially added concentration (Table 2). This result indicated that, in the presence of EDTA, III is unable to bind to GP IIb–IIIa on the platelet surface and can therefore be detected in the plasma sample. In addition, EDTA was found to efficiently displace already bound III from the platelets (data not shown). The recovery of III was slightly lower when the active drug III had to be displaced from the platelets by EDTA, compared to the experiments where EDTA prevented the binding of III to the platelets (83±6 and 91±3%, respectively). However, no difference in recovery was detected between the samples containing low and high concentrations of III.

Thus, EDTA was used as an anticoagulant in order to determine the total (bound plus free) plasma concentration of III, whereas citrate was used to allow an estimation of the free plasma concentration of III.

3.2. Sample clean-up

Only minimal sample pre-treatment was required: Following protein precipitation and centrifugation, plasma samples were buffered and then injected. Urine samples were just buffered before injection. Further clean-up of the samples was done on-line as part of the automated column-switching process.

Modern column-switching techniques offer a number of benefits compared to the rather simple systems available in 1981 [3]. The solvent selector, SS, allowed the precolumn and all capillaries between the autosampler, AS, and the switching valves to be purged with solvents of different polarity, preventing memory effects caused by non-polar material adsorbed in the precolumn and the steel capillaries [4]. Valve V1 allowed forward- and backward-flushing of the precolumn. In this way, solid material (particulate matter from seal abrasion, precipitated proteins, etc.), which had settled during the purge process on the inlet of the precolumn, could be backflushed to waste, instead of being transferred onto the top of the analytical column [5]. The tandem precolumn selector, TPS, and the pressure monitor enabled routine overnight injections without possible interruption by precolumn clogging [4].

3.3. Chromatography

In order to prevent any further band-broadening during column transfer, the fraction retained on the PC during enrichment was eluted in the backflush mode onto the top of the analytical column, AC. II and III were separated from endogenous components by carefully optimizing the composition, slope and flow-rate of the gradient eluent system. To suppress any ionization of the acidic compounds, chromatography was carried out under slightly acidic conditions (pH 3.5). The buffer strength during the gradient had to be carefully adjusted in order to obtain a stable baseline, which turned out to be crucial in maintaining a quantification limit of 1

ng/ml. To remove any strongly retained components, the analytical column was purged at the end of each run with different solvents and was then re-conditioned.

3.4. Recovery

Recovery was measured using blank plasma and urine samples spiked with II and III at different concentrations. These spiked samples were analyzed in replicate as described and compared with spiked eluent mixtures, directly injected onto the analytical column, AC, via the manual injector, MI, providing the 100% values.

Acceptable recoveries were obtained for both compounds (Table 3). No relevant matrix dependence could be detected when recoveries from dog plasma, human plasma and human urine were compared. In some cases, a certain variability of the individual recoveries at different concentrations was observed, probably reflecting the difficulties in determining recoveries in a column-switching system, rather than demonstrating a concentration dependence of the recovery.

3.5. Selectivity

Simple column-switching methods with UV detection often suffer from decreased selectivity compared to extraction methods. Using common packing materials such as C_8 or C_{18}, only proteins, salts and other highly polar plasma and urine components are flushed to waste, while the majority of endogenous compounds are retained on the PC and transferred to the AC, together with the substances of interest. In contrast, the sophisticated column-switching systems used these days in bioanalysis are highly optimized and offer improved selectivity, due to the use of multiple valves, different purge fluids during enrichment and sophisticated gradient mobile phase elution during separation on the analytical column.

In the hundreds of blank plasma samples (predose, placebo) from various dogs and human volunteers analysed so far, only in very rare cases were interfering compounds observed that coeluted with either II or III. In contrast, the selectivity of the urine method was somewhat critical. For this reason, the practical limit of quantification was limited to 25 ng/ml, although both compounds could also be

Table 3
Recovery of II and III from plasma and urine

Compound	Matrix	Concentration added (ng/ml)	Concentration found (ng/ml)	Recovery (%)	Precision (%)	n
II	Dog plasma (EDTA)	5	4.0	79.5	6.2	4
		50	37.2	74.3	2.4	4
		500	407	81.3	3.9	4
	Human plasma (Citrate)	5	3.8	75.6	25.9	4
		50	44.0	87.9	2.7	4
		500	422	84.4	1.0	4
	Human urine	50	40.0	80.0	3.7	5
		500	386	77.1	1.3	5
		5000	3770	75.4	0.7	5
III	Dog plasma (EDTA)	5	4.7	94.0	5.2	4
		50	34.5	69.0	2.0	4
		500	376	75.1	2.0	4
	Human plasma (Citrate)	5	4.2	83.7	14.8	4
		50	40.0	79.1	2.2	4
		500	384	76.8	1.2	4
	Human urine	50	35.1	70.2	3.7	5
		500	389	77.8	0.9	5
		5000	3715	74.3	0.9	5

quantified around levels of 10 ng/ml and less, with acceptable precision and accuracy. Figs. 4a, 5a, 6a and 7a show representative chromatograms of dog plasma, human plasma (citrate), human plasma (EDTA) and human urine collected before administration.

3.6. Precision and accuracy

The precision (relative standard deviation of replicate analyses) and the inaccuracy (percentage deviation between found and added concentration) of the method were evaluated for toxicokinetic and clinical concentrations in dog plasma, human plasma and human urine. For each level (1 to 10 000 ng/ml, dog plasma; 1 to 250 ng/ml, human plasma and 10 to 10 000 ng/ml, human urine), a spiked sample was prepared and analysed on different days (using a separate calibration line on each day). The data in Table 4 Table 5 demonstrate the good precision and accuracy for II and III in plasma and urine over all of the concentration ranges investigated.

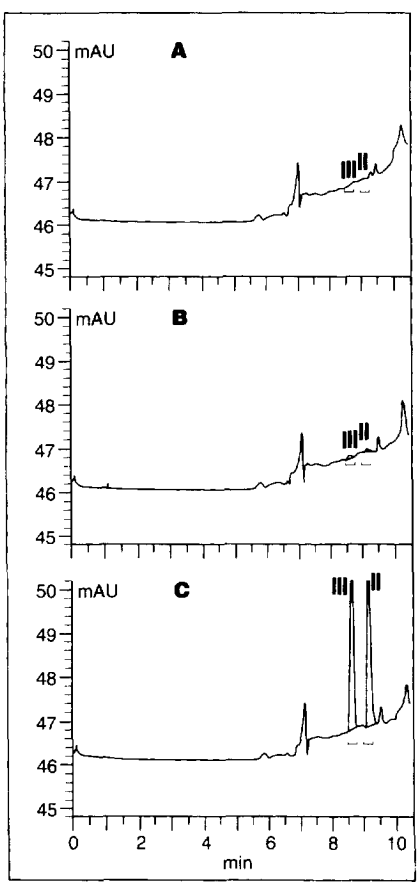

Fig. 4. Chromatograms of dog plasma samples (EDTA): (a) Before application; (b) blank plasma spiked with 1 ng/ml of II and III (limit of quantification); (c) collected 8 h after a multiple oral dose of 80 mg/kg I. Measured concentrations, 43 ng/ml II and 71 ng/ml III.

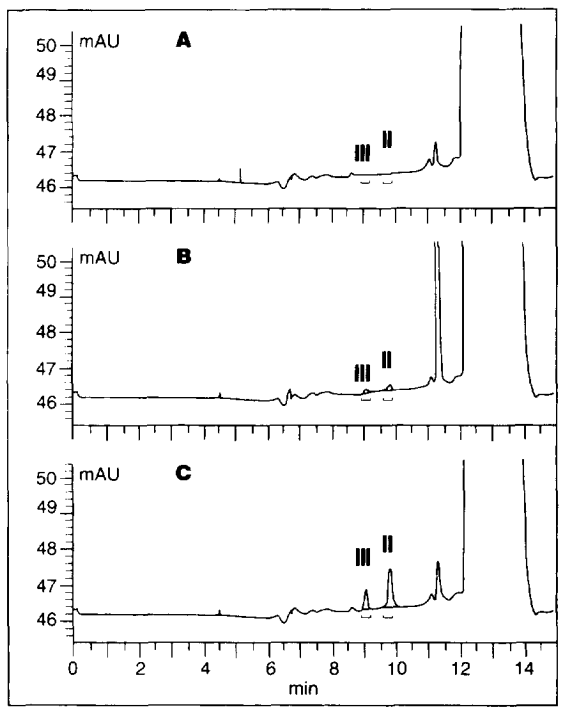

Fig. 5. Chromatograms of human plasma samples (citrate): (a) Before application; (b) blank plasma spiked with 1 ng/ml of II and III (limit of quantification); (c) collected 3 h after a single oral dose of 5 mg of I. Measured concentrations, 16.1 ng/ml II and 6.9 ng/ml III.

3.7. Linearity

Experience with the first toxicokinetic studies showed that the analytical method had to deal with high plasma levels of II, exceeding 60 000 ng/ml, when repeated oral doses of 400 mg/kg were administered to dogs. On the other hand, a high sensitivity of around 1 ng/ml was desirable to acquire meaningful information on the excretion phase of the two compounds after administration of low doses. The data presented in Tables 4 and 5

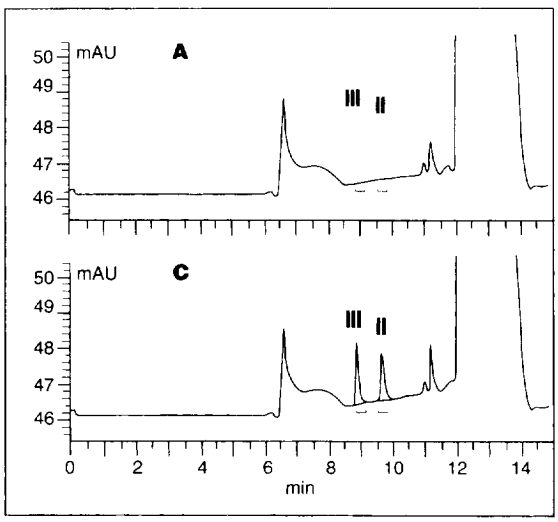

Fig. 6. Chromatograms of human plasma samples (EDTA): (a) Before application; (c) collected 3 h after a single oral dose of 5 mg of I. Measured concentrations, 18.5 ng/ml II and 19.3 ng/ml III.

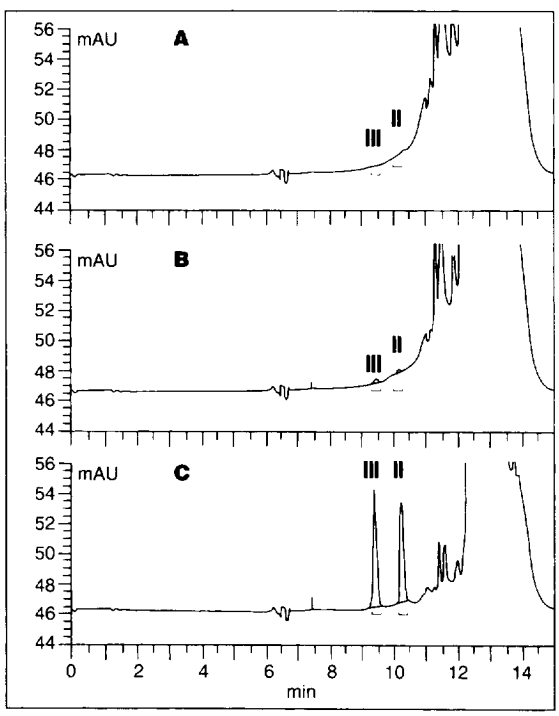

Fig. 7. Chromatograms of human urine samples: (a) Before application; (b) blank urine spiked with 25 ng/ml of II and III (limit of quantification); (c) collected 4–8 h after a single oral dose of 5 mg of I. Measured concentrations, 900 ng/ml II and 1350 ng/ml III.

show that the precision was virtually constant over a wide concentration range (indicating variable variances), which meant that a "conventional" regression procedure could not be applied to the calibration data. Instead of dividing the concentration graph into smaller sub-ranges (and thereby complicating the analytical procedure), a weighted linear least-squares regression procedure was applied, using $1/Y^2$ as the weighting factor [6]. Under these conditions, excellent linearity and a negligible y-intercept were found routinely between 1 and 10 000 ng/ml (plasma) and 25 and 10 000 ng/ml (urine) for both compounds.

3.8. Limit of quantification

The limit of quantification for both compounds, defined here as the minimum concentration that could be measured routinely with acceptable precision (<20%) and inaccuracy (<20%), was 1 ng/ml (plasma) and 25 ng/ml (urine) (see Tables 4 and 5). Figs. 4b, 5b and 7b show chromatograms of dog plasma, human plasma and human urine spiked with II and III at the limits of quantification.

3.9. Stability in body fluids

Compounds II and III were added to two matrices (plasma and urine) from two species (dog and human) at two concentrations (10 and 1000 ng/ml, plasma, and 250 (or 100) and 1000 ng/ml, urine) and then stored for different time intervals at different storage temperatures (6 and 24 h at 25°C, plasma and urine; three and six (or seven) months at −20°C, plasma; six months at −20°C, urine). After storage, the samples were analysed together with an equal number of freshly prepared samples providing the 100% values. The statistical interpretation [7] of the data indicated that the two compounds were stable in dog plasma, human plasma and human urine under the storage conditions investigated. Only the stability of II in urine for six months at −20°C was somewhat critical, indicating that urine samples should not be stored for more than three months after collection.

3.10. Application to biological samples

The new method has been applied successfully to all studies performed to date in dogs (1660 plasma

Table 4
Inter-assay precision of II and III in plasma

Compound	Matrix	Concentration added (ng/ml)	Concentration found (ng/ml)	Precision (%)	Inaccuracy (%)	*n*
II	Dog plasma (EDTA)	1.00[a]	0.98	12.3	−2.5	3
		2.50	2.38	10.4	−4.4	6
		25.0	24.5	4.7	−2.0	6
		250	248	3.9	−0.8	6
		2500	2460	9.0	−1.5	6
		4.9 [b]	4.86	4.7	−1.3	19
		52.9 [b]	53.0	3.7	+0.3	20
		523 [b]	528	4.2	+0.8	20
		5260 [b]	5260	5.7	−1.7	20
	Human plasma (Citrate)	1.00[a]	0.97	1.9	+3.4	4
		2.00	1.98	9.4	−0.9	4
		5.00	5.28	6.2	+5.6	4
		25.0	24.5	1.2	−2.0	4
		250	255	8.4	+2.0	4
		5.39[b]	5.11	5.9	−5.2	20
		25.3 [b]	25.4	2.5	+0.4	20
		97.2 [b]	99.4	2.8	+2.3	20
		480 [b]	488	2.6	+1.7	20
	Human plasma (EDTA)	5.15[b]	5.04	9.6	−2.1	19
		24.4 [b]	25.3	3.5	+3.7	19
		95.8 [b]	98.8	3.0	+3.1	20
		471 [b]	489	3.1	+3.8	20
III	Dog plasma (EDTA)	1.00[a]	1.07	11.6	+6.7	4
		2.50	2.54	8.5	+1.7	6
		25.0	24.1	5.3	−3.6	6
		250	248	3.0	−0.6	6
		2500	2490	7.7	−0.2	6
		4.63[b]	4.59	5.3	−0.9	20
		51.5 [b]	50.8	4.8	−1.4	20
		532 [b]	509	4.8	−4.3	20
		5290 [b]	4990	5.0	−5.7	20
	Human plasma (Citrate)	1.00[a]	1.06	7.8	+6.0	4
		2.00	1.84	7.8	−8.3	4
		5.00	5.17	6.1	+3.3	4
		25.0	25.3	0.8	+1.2	4
		250	259	6.3	+3.4	4
		4.87[b]	4.81	2.7	−1.2	20
		25.4 [b]	25.1	2.3	−1.2	20
		100 [b]	99.7	2.0	−0.3	20
		500 [b]	495	2.1	−1.0	20
	Human plasma (EDTA)	5.09[b]	5.11	5.6	+0.4	19
		26.1 [b]	25.9	3.6	−1.0	19
		100 [b]	100	3.9	+0.0	20
		490 [b]	491	3.3	+0.2	20

[a]Limit of quantification.
[b]Quality control sample.

Table 5
Inter-assay precision of II and III in urine

Compound	Matrix	Concentration added (ng/ml)	Concentration found (ng/ml)	Precision (%)	Inaccuracy (%)	n
II	Human urine	10.0[c]	10.4	5.1	+4.3	7
		25.0[a]	25.3	8.4	+1.1	6
		50.0	46.9	8.8	−6.2	7
		100	99.5	3.2	−0.5	7
		250	247	2.2	−1.1	7
		500	512	2.5	+2.4	7
		1 000	1 015	2.0	+1.5	7
		2 500	2 819	2.1	+12.8	7
		5 000	5 133	2.5	+2.7	7
		10 000	10 051	2.4	+0.5	7
		192[b]	192	3.8	+0.0	26
		1 980[b]	1 980	2.9	+0.0	26
III	Human urine	10.0[c]	10.4	5.4	+3.7	7
		25.0[a]	24.2	7.1	−3.1	7
		50.0	49.5	6.7	−0.9	7
		100	98.7	3.4	−1.2	7
		250	246	2.8	−1.8	7
		500	504	3.1	+0.8	7
		1 000	1 002	2.4	+0.2	7
		2 500	2 862	2.5	+14.5	7
		5 000	5 064	2.5	+1.3	7
		10 000	9 886	2.9	−1.1	7
		202[b]	203	3.7	+0.7	26
		2 081[b]	2 054	3.3	−1.3	26

[a] Limit of quantification.
[b] Quality control sample.
[c] Below the limit of quantification.

samples) and man (4400 plasma and 1300 urine samples). Figs. 4c, 5c, 6c and 7c show representative chromatograms from these studies. The method was sensitive enough to measure precisely the low plasma concentrations for up to 12 h (II) or 48 h (III) after a single oral dose of 5 mg of I to human volunteers (Fig. 8).

The productivity of the highly automated column-switching method was very good, due to minimal sample pre-treatment, short analysis time (15 min), optimal data acquisition (modern PC-based systems) and quick handling, reporting and archiving of data by a pharmacokinetic Laboratory Information Management System (LIMS) [8,9]. 70 unknown samples, together with eight calibration standards and eight quality control samples, could be analysed every 24 h by a single person with a single instrument.

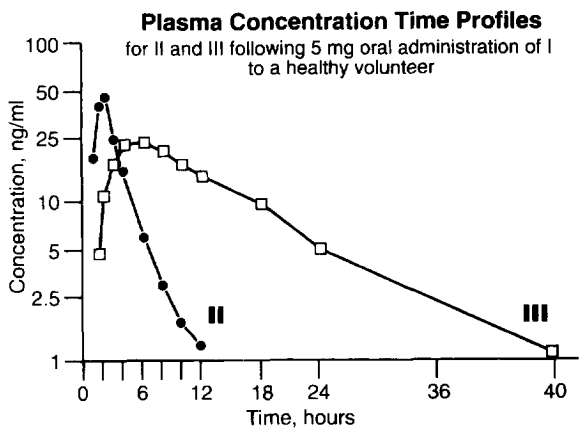

Fig. 8. Plasma concentration time profiles following the administration of a 5-mg oral dose of I to a human volunteer. EDTA was used as an anticoagulant.

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