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Determination of the oral platelet aggregation inhibitor Sibrafiban® in rat, dog, and human plasma utilising HPLC-column switching combined with turbo ion spray single quadrupole mass spectrometry☆

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Abstract

A sensitive and selective HPLC-column switching method with single quadrupole mass spectrometric detection was developed for the simultaneous determination of the oral platelet aggregation inhibitor Sibrafiban® (double protected prodrug), its prodrug and the active metabolite in rat, dog, and human plasma. The three analytes together with their tri-deuterated internal standards were isolated from plasma by protein precipitation (0.5 M perchloric acid). The de-proteinated samples were injected onto a standard-bore trapping column (4.0 mm i.d., LC-ABZ) of an HPLC-column switching system. Polar plasma components were removed by flushing the trapping column with ammonium formate (pH 3.6; 5 mM). Enriched compounds (including the analytes of interest) were backflushed onto a narrow-bore analytical column (2.1 mm i.d., Inertsil ODS-2) and separated by gradient elution (formic acid/methanol). The whole effluent (200 μl/min) from the analytical column was passed to the turbo ion spray interface without splitting. Selected ion monitoring (SIM) was used for mass spectrometric detection. The limit of quantification for all three analytes was 1 ng/ml, using a 250-μl specimen of plasma. The mean precision and inaccuracy for the three analytes in all species were < 6 and $< 5\%$, respectively. The practicability of the new analytical method was demonstrated by the analysis of about 500 rat and dog plasma and about 14 000 human plasma samples. The new method represents a successful example for the application of LC single MS with ionspray ionisation to the analysis of small molecule drugs in biological matrices from toxicokinetic studies and large clinical trials.

Keywords: Sibrafiban; Platelet aggregation inhibitor; HPLC-column switching; LC–MS; Single quadrupole mass spectrometric detection; Cross-validation

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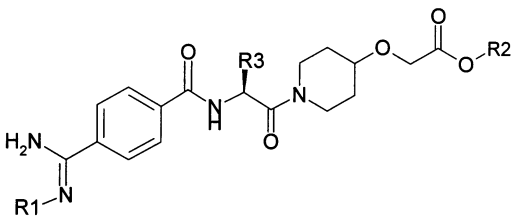

Fig. 1. Chemical structures for the compounds referred to in the text: (I) R1, OH; R2, C_2H_5; R3, CH_3; Sibrafiban®; double protected prodrug. (II) R1, OH; R2, H; R3, CH_3; prodrug. (III) R1, H; R2, H; R3, CH_3; active metabolite. (IV) R1, OH; R2, C_2H_5; R3, CD_3; deuterated internal standard for the double protected prodrug. (V) R1, OH; R2, H; R3, CD_3; deuterated internal standard for the prodrug. (VI) R1, H; R2, H; R3, CD_3; deuterated internal standard for the active metabolite.

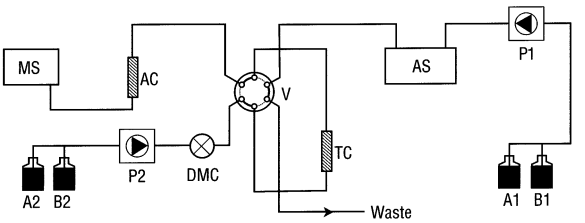

Fig. 2. Schematic representation of the HPLC-column switching system. P1, first gradient pump; P2, second gradient pump; DMC, dynamic mixing chamber; A1, B1, mobile phases for gradient pump 1; A2, B2, mobile phases for gradient pump 2; TC, trapping column; AC, analytical column; V, HPLC-switching valve; MS, mass spectrometer.

1. Introduction

[Z]-(S)-[[[1-[2-[[4-(aminohydroxyiminomethyl)-benzoyl]amino]-1-oxopropyl]-4-piperidinyl] oxy]-acetic acid ethyl ester, I (Sibrafiban®; F. Hoffmann–La Roche Ltd., 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 [1]. After oral administration, the double protected prodrug I (amidoxime ester) is moderately absorbed and metabolised to the prodrug II (amidoxime free acid) and the active metabolite III (amidine free acid).

To support the pharmacokinetic program in human volunteers and patients, the analytical method can be restricted to the determination of the active metabolite and its prodrug in plasma and urine. In order to follow therapeutic doses during the late elimination phase, a limit of quantification (LOQ) of at least 1 ng/ml is required for both compounds in plasma and urine. Concentration maxima of both compunds appear at ca. 100 (plasma) and 3000 ng/ml (urine). In the case of large phase III studies with thousands of collected samples, the costs of sample analysis play a very important role. For preclinical safety studies the inactive double protected prodrug I also has to be measured, together with II and III in mouse, rat, and dog plasma. However, I is degraded by plasma esterases to II [2], which has to be considered during method development. A limit of quantification of 1–5 ng/ml is desirable for all three components. Experience with early toxicology studies showed that peak concentrations of up to 10 000 ng/ml or even more can be expected for II and III in plasma of test animals. In cases where a distinction between total and free concentrations of III is required, two anticoagulants (EDTA and sodium citrate) are used for plasma preparation [3], which has also an impact on the method development.

Table 1
Scheme of the automated column switching process for II and III in human plasma

Step	Time (min)	Valve position	Trapping column	Analytical column
A	0.0–1.5	0	Loading the deproteinated sample onto TC. Flushing of TC (100% A1)	Start of gradient (100% A2 to 15% A2/85% B2)
B	1.5–3.5	1	Transfer of trapped material from TC to AC in backflush mode.	Chromatography 2.5 min: end of gradient
			Purging of all capillaries between P1–AS–V–Waste (100% B1)	2.5–3.5 min: isocratic phase (15% A2/85% B2)
C	3.5–7.0	0	Reconditioning of TC (100% A1)	Reconditioning of AC (100% A2)

Table 2
Scheme of the automated column switching process for I, II, and III in dog and rat plasma

Step	Time (min)	Valve position	Trapping column	Analytical column
A	0.0–2.0	0	Loading the deproteinated sample onto TC. Flushing of TC (100% A1)	Start of gradient (100% A2 to 10% A2/90% B2)
B	2.0–6.0	1	Transfer of trapped material from TC to AC in backflush mode. Purging of all capillaries between P1–AS–V–waste (100% B1)	Chromatography 2.5 min: end of gradient 2.5–6.0 min: isocratic phase (10% A2/90% B2)
C	6.0–10	0	Reconditioning of TC (100% A1)	Reconditioning of AC (100% A2)

Table 3
Release of I, II, and III from plasma proteins during sample clean-up

Compound	Matrix	Concentration added (ng/ml)	Recovery[a] (%)	Repl. (n)
I	Human plasma (EDTA)	5	n.d.	–
		100	n.d.	–
	Human plasma (Citrate)	5	n.d.	–
		100	n.d.	–
	Dog plasma (EDTA)	5	92.4	9
		100	102	5
	Dog plasma (Citrate)	5	93.6	5
		100	95.3	5
	Rat plasma (EDTA)	5	79.2	9
		100	85.7	9
	Control (water)	5	100[b]	5
		100	100[b]	5
II	Human plasma (EDTA)	5	101	5
		100	97.6	5
	Human plasma (Citrate)	5	98.3	5
		100	100	5
	Dog plasma (EDTA)	5	84.4	9
		100	97.5	5
	Dog plasma (Citrate)	5	92.9	5
		100	98.8	5
	Rat plasma (EDTA)	5	79.2	9
		100	85.7	9
	Control (water)	5	100[b]	5
		100	100[b]	5
III	Human plasma (EDTA)	5	95.6	5
		100	99.5	5
	Human plasma (Citrate)	5	100	5
		100	94.4	5
	Dog plasma (EDTA)	5	94.4	9
		100	101	5
	Dog plasma (Citrate)	5	103	5
		100	99.9	5
	Rat plasma (EDTA)	5	74.6	9
		100	76.3	9
	Control (water)	5	100[b]	5
		100	100[b]	5

[a] n.d. not determined.
[b] Reference, arbitrarily set to 100%.

A number of assays have been developed during the last 3 years, which met the immediate needs of their respective studies but lacking somewhat toward the ideal goals described above: a triple quadrupole mass spectrometric method (LC–MS/MS) for the determination of all three compounds in mouse, rat, dog, and human plasma with a LOQ of 0.2 ng/ml [2]; a less expensive HPLC-column switching method with UV detection (LC–UV) with sufficient sensitivity (1 ng/ml) for dog and human plasma but restricted to the determination of II and III [3] and an HPLC method restricted to II in rat plasma with an LOQ around 40 ng/ml [4].

It was felt, that, for routine applications, a new liquid-mass spectrometric method would be desirable with sufficient sensitivity for all three compounds but at a lower cost than the existing LC–MS/MS procedure. Most of the quantitative liquid mass spectrometric assays reported are based on complex and expensive triple quadrupole mass spectrometers. More recently, low cost single quadrupole instruments (LC–MS) became available using the same design as the larger instruments. The analytical method described here represents one of the first bioanalytical applications of this new type of instrumentation.

2. Experimental

2.1. Reagents and solvents

Water (for chromatography), methanol (HPLC grade), ethanol (absolute, p.a.) and perchloric acid (70%, p.a.) were obtained from Merck (Darmstadt, Germany). Formic acid (ca. 98%, puriss p.a.) and ammonium formate (purum p.a.) were purchased from Fluka (Buchs, Switzerland). Acetonitrile (HPLC grade S) was obtained from Rathburn (Walkerburn, UK). Dichlorvos was purchased from Riedel de Haen (Seelze, Germany).

2.2. Solutions

Stock solutions were obtained by dissolving the pure test compounds and internal standards in methanol (I, II, IV, V) or water (III, VI). Stock solutions could be stored at 5°C for about 3 months. Three sets of working solutions were prepared by diluting aliquots of the stock solutions with water: two solutions containing all three analytes at appropriate concentrations to prepare rat and dog plasma standards in two calibration ranges and one containing just II and

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

Compound	Matrix	Concentration added (ng/ml)	Precision (%)	Inaccuracy (%)	Repl. (*n*)
II	Human plasma (EDTA)	1[a,b]	4.4	1.4	5
		10[b]	3.8	1.5	4
		100[b]	4.0	−1.7	4
		5[c]	6.7	−0.7	70
		50[c]	5.8	−0.1	70
III	Human plasma (EDTA)	1[a,b]	7.0	−2.4	6
		10[b]	5.2	4.1	4
		100[b]	4.8	2.8	4
		5[c]	6.7	3.6	70
		50[c]	5.8	−1.1	70

[a] Limit of quantification.
[b] Evaluated during method development.
[c] Evaluated during sample analysis, using quality control samples.

Table 5
Inter-assay precision of I, II, and III in dog and rat plasma

Compound	Matrix	Concentration added (ng/ml)	Precision (%)	Inaccuracy (%)	Repl. (n)
I	Dog plasma (EDTA)	1[a,b]	3.9	5.9	6
		100[b]	2.0	7.9	6
		10000[b]	4.0	−8.7	5
	Dog plasma (citrate)	5[c]	4.7	2.2	5
		500[c]	5.0	0.9	8
		5000[c]	5.9	−1.7	7
	Rat plasma (EDTA)	1[a,b]	7.2	1.4	5
		100[b]	8.1	−2.8	5
		10000[b]	7.2	−8.3	5
		5[d]	4.6	−4.6	8
		500[d]	6.1	−8.6	8
		5000[d]	5.0	4.2	8
II	Dog plasma (EDTA)	1[a,b]	7.7	3.2	6
		100[b]	3.0	7.8	6
		10000[b]	6.0	−4.0	6
	Dog plasma (citrate)	5[c]	1.6	3.3	5
		500[c]	6.1	5.3	8
		5000[c]	3.4	4.4	7
	Rat plasma (EDTA)	1[a,b]	2.6	1.1	5
		100[b]	5.4	−0.5	5
		10000[b]	1.3	−7.8	5
		5[d]	3.6	−2.6	8
		500[d]	5.5	−7.1	8
		5000[d]	4.5	−1.0	8
III	Dog plasma (EDTA)	1[a,b]	3.7	−2.5	6
		100[b]	2.1	9.1	6
		10000[b]	3.8	−5.8	6
	Dog plasma (citrate)	5[c]	4.1	5.2	5
		500[c]	5.5	4.5	8
		5000[c]	4.6	−2.2	7
	Rat plasma (EDTA)	1[a,b]	6.7	−1.6	5
		100[b]	2.1	−0.9	5
		10000[b]	3.3	−8.0	5
		5[d]	3.8	−5.0	8
		500[d]	3.4	4.9	8
		5000[d]	3.3	−0.3	8

[a] Limit of quantification.
[b] Evaluated during method development.
[c] Interpolated from EDTA calibration curves.
[d] Evaluated during sample analysis, using quality control samples.

III for setting up human plasma standards. Working solutions were always freshly prepared. Two dichlorvos solutions were prepared to stabilise I in plasma; one for rat plasma by dissolving 200 mg dichlorvos in 2 ml of acetonitrile and the other for dog plasma by dissolving 25 mg dichlorvos in 10 ml of tetrahydrofuran: water (1:4, v/v). Both dichlorvos solutions could be stored at 5°C for about 4 weeks. Two aqueous internal standard solutions were prepared for the simultaneous determination of I, II, and III in rat and dog plasma; one containing the tri-deuterated compounds IV, V, and VI at concentrations of 1 ng/μl (low calibration range), the other at concentrations of 10 ng/μl (high calibration range). A third internal standard solution for the assay of II and

III in human plasma contained the tri-deuterated compounds V and VI at concentrations of 0.4 ng/μl water. All three solutions could be stored at 5°C for 3 months.

2.3. Plasma standards

The blank plasma required for preparation of the standards was prepared from rat, dog, and human blood by centrifugation (1000 × *g*, 20 min), using EDTA/NaF or sodium citrate as anticoagulant. Rat and dog plasma were then mixed with the corresponding dichlorvos solution yielding final dichlorvos concentrations of 1 mg/ml (rat plasma) and 10 μg/ml (dog plasma). Human blank plasma was used without stabilisation.

Dog and rat plasma standards were prepared by spiking dichlorvos treated blank plasma (20 ml) with 200 μl of the corresponding working solution (containing I, II and III) providing concentrations between 1 and 100 ng/ml (low calibration range) and between 100 and 10 000 ng/ml (high calibration range). Human plasma standards were obtained by spiking blank plasma (20 ml) with 200 μl of working solution (containing II and III) providing concentrations between 1 and 100 ng/ml. Plasma standards were divided into aliquots and stored deep-frozen at −20°C (dog and human) or −80°C (rat) until required for analysis.

Table 6
Concentrations of II and III in cross-validation samples obtained by LC–MS/MS and LC–MS

Sample ID	II			III		
	LC–MS/MS Reference (ng/ml)	LC–MS (ng/ml)	Deviation[a] (%)	LC–MS/MS Reference (ng/ml)	LC–MS (ng/ml)	Deviation[a] (%)
01	<0.50[b]	<1.00[b]	–	1.39	1.54	10.8
02	3.72	4.14	11.3	1.24	1.47	18.5
03	4.09	3.70	−9.5	1.53	1.60	4.6
04	6.51	7.11	9.2	5.12	5.58	9.0
05	2.14	2.13	−0.5	2.47	2.44	−1.2
06	3.84	3.87	0.8	2.76	2.75	−0.4
07	8.93	9.13	2.2	3.37	3.47	3.0
08	<0.50[b]	<1.00[b]	–	4.02	4.09	1.7
09	6.47	6.93	7.1	4.39	4.29	−2.3
10	9.92	10.8	8.9	5.60	5.45	−2.7
11	30.5	30.1	−1.3	8.29	7.80	−5.9
12	50.2	51.8	3.2	14.6	13.1	−10.3
13	52.2	55.4	6.1	14.0	12.7	−9.3
14	29.5	32.2	9.2	17.2	15.7	−8.7
15	38.5	41.5	7.8	21.9	20.6	−5.9
16	36.7	38.1	3.8	25.9	25.3	−2.3
17	7.44	8.16	9.7	32.6	32.6	0.0
18	27.4	29.8	8.8	35.9	34.0	−5.3
19	20.7	22.4	8.2	40.2	39.8	−1.0
20	10.5	11.3	7.6	45.5	44.1	−3.1
21	11.0	12.0	9.1	50.8	49.6	−2.4
22	9.00	9.55	6.1	49.1	48.0	−2.2
23	13.0	14.1	8.5	52.1	52.0	−0.2
24	14.6	15.9	8.9	57.5	56.0	−2.6
25	13.2	15.2	15.2	54.5	54.4	−0.2

[a] Percentage deviation between concentrations obtained by LC–MS and LC–MS/MS.
[b] Below limit of quantification.

Table 7
Stability of I, II, and III in stabilised[a] rat EDTA plasma ($n = 5$)

Compound	Storage conditions	Concentration added (ng/ml)	Change of conc. after storage (%)	Remarks
I	1 h at 25°C	50	−10.2	Stable
	1 h at 25°C	1000	−3.0	Stable
	5 h at 25°C	50	−99.9	Unstable
	5 h at 25°C	1000	−99.9	Unstable
	3 months at −80°C	50	−1.2	Stable
	3 months at −80°C	1000	−3.5	Stable
	6 months at −80°C	50	−5.7	Stable
	6 months at −80°C	1000	−3.2	Stable
	10 months at −80°C	50	−7.5	Stable
	10 months at −80°C	1000	−4.2	Stable
II	1 h at 25°C	50	−1.8	Stable
	1 h at 25°C	1000	7.3	Stable
	5 h at 25°C	50	63.9	[b]
	5 h at 25°C	1000	75.7	[b]
	3 months at −80°C	50	−8.4	Stable
	3 months at −80°C	1000	−9.2	Stable
	6 months at −80°C	50	−4.7	Stable
	6 months at −80°C	1000	−7.4	Stable
	10 months at −80°C	50	−12.7	Stable
	10 months at −80°C	1000	−9.9	Stable
III	1 h at 25°C	50	−2.3	Stable
	1 h at 25°C	1000	1.3	Stable
	5 h at 25°C	50	−2.7	Stable
	5 h at 25°C	1000	0.6	Stable
	3 months at −80°C	50	−2.0	Stable
	3 months at −80°C	1000	−2.3	Stable
	6 months at −80°C	50	−4.8	Stable
	6 months at −80°C	1000	−5.2	Stable
	10 months at −80°C	50	−0.2	Stable
	10 months at −80°C	1000	−5.4	Stable

[a] Stabilised with dichlorvos (1 mg/ml plasma).
[b] No judgement possible.

2.4. Sample preparation

For dog and rat plasma in the low calibration range, plasma specimens of 250 μl were mixed subsequently with the appropriate internal standard solution (50 μl) and perchloric acid (250 μl, 0.5 M). After centrifugation (ca. 15 000 × *g*, 6 min), the supernatant was quantitatively transferred into an autosampler vial (1.5 ml, Eppendorf). After mixing the supernatant with ammonium formate solution (1 ml, 1 M), the sample was ready for chromatography. The same procedure was applied to human plasma samples.

For dog and rat plasma in the high calibration range, plasma specimens of 50 μl were subsequently mixed with water (450 μl), the appropriate internal standard solution (50 μl) and perchloric acid (500 μl, 0.5 M). After centrifugation (ca. 15 000 × *g*, 6 min), an aliquot of the supernatant (0.1 ml) was transferred into an autosampler vial (1.5 ml, Eppendorf). After mixing the supernatant with ammonium formate solution (1.25 ml, 1 M), the sample was ready for chromatography.

Because of the limited stability of I in rat plasma at room temperature (see below), all handling steps with thawed rat plasma samples had to

be organised in such a way that multiple thaw–freeze cycles could be avoided, and the time period for sample preparation could be kept below 1 h. (Dog and human plasma samples could be processed without special precautions.) For this reason, a rat plasma sample was only thawed once, divided into three aliquots and analysed simultaneously in different calibration ranges; the first in the low range, the second in the high range and the third, after dilution with water, also in the high range. At least one valid value was obtained during analyses for each analyte, using this procedure, regardless of the concentrations of I, II, and III present in plasma, avoiding any further freeze–thaw cycles. Due to the simple sample clean-up procedure, all handling steps, namely thawing of samples, transferring aliquots, adding internal standards and precipitating plasma proteins could be performed in less than 10 min. The critical time interval (ca. 1 h), in which thawed rat plasma samples could be handled without problems was never exceeded, even when sequences of 130 samples or more were processed simultaneously. Once the analysis was completed,

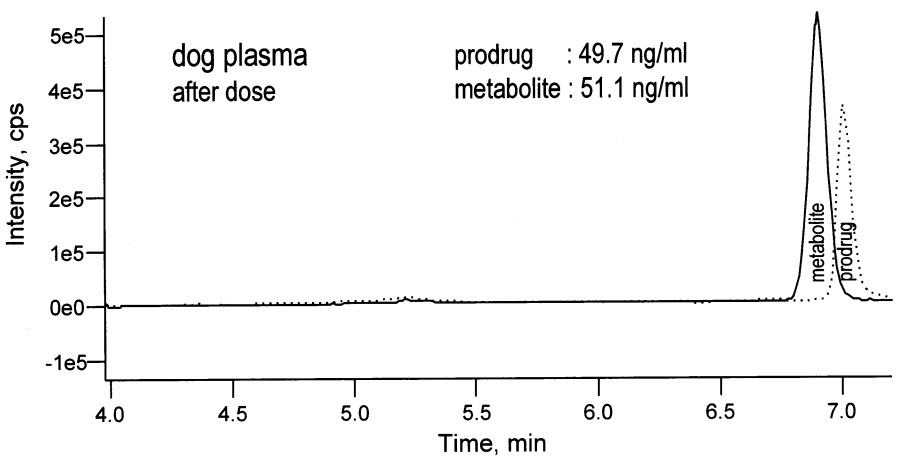

Fig. 3. Chromatogram of a dog plasma sample, using EDTA as anticoagulant. First period containing the protonated ions of II (prodrug) and III (metabolite).

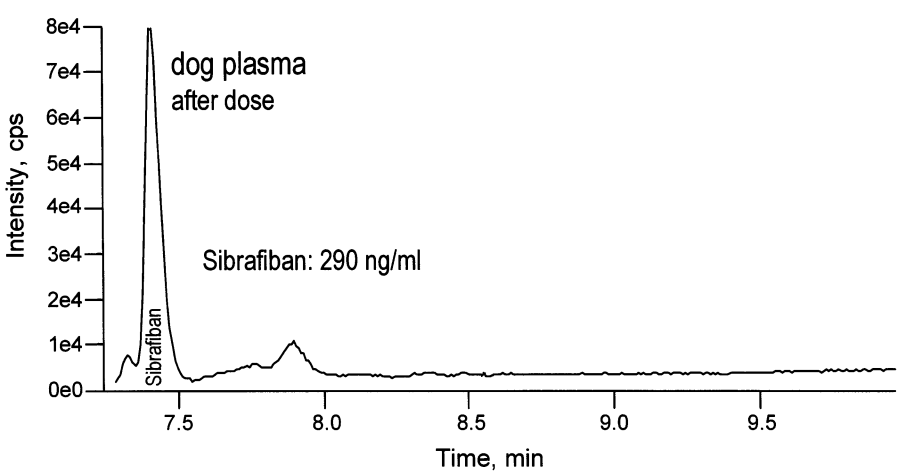

Fig. 4. Chromatogram of a dog plasma sample, using EDTA as anticoagulant. Second period containing the protonated ion of I (Sibrafiban).

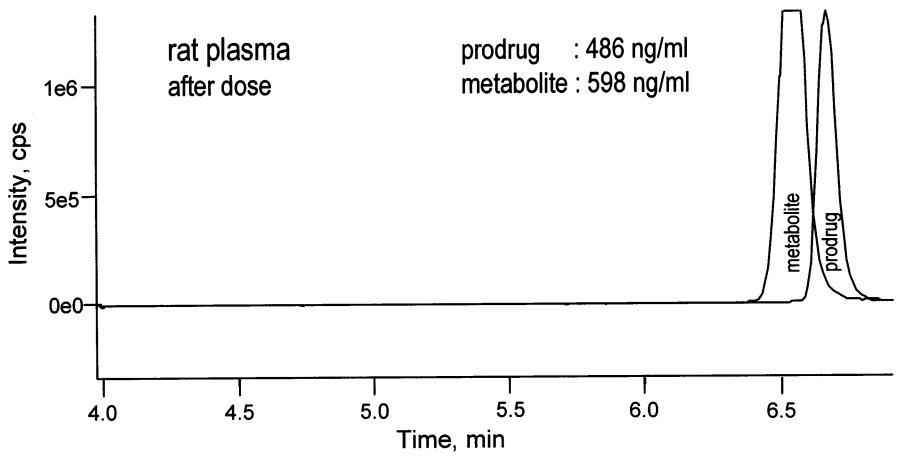

Fig. 5. Chromatogram of a rat plasma sample, using EDTA as anticoagulant. First period containing the protonated ions of II (prodrug) and III (metabolite).

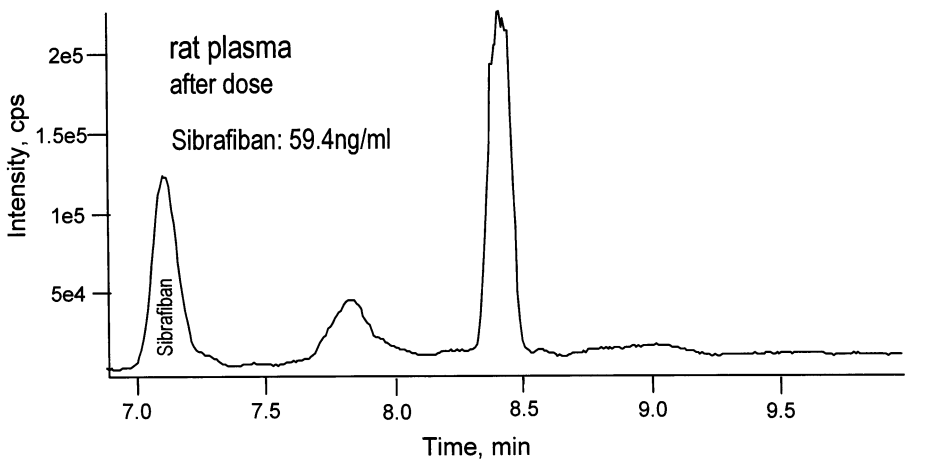

Fig. 6. Chromatogram of a rat plasma sample, using EDTA as anticoagulant. Second period containing the protonated ion of I (Sibrafiban).

a sample was immediately re-frozen by first dipping into dry ice and then storing at −80°C.

2.5. Chromatographic system

The complete system (see Fig. 2) consisted of a first gradient pump, P1 (Model L-6200; Merck–Hitachi, Darmstadt, Germany) delivering the solvents A1 and B1 at a flow-rate of 2.5 ml/min; an autosampler, AS (Model AS-4000, Merck–Hitachi), with an injection volume of 1 ml, equipped with a 5-ml syringe, a 2-ml loop, and operating in the 'slow needle-down-speed'-mode; a second gradient pump, P2 (Model L-6200A; Merck-Hitachi), in connection with a dynamic mixing chamber, DMC (Model MICA 75; Portmann, Biel–Benken, Switzerland), delivering the gradient mobile phase A2–B2 at a flow-rate of 0.2 ml; and a high-pressure switching valve V (Model 7000E; LabSource, Reinach, Switzerland).

A cartridge holder (20 mm; Supelco, Bellefonte, PA, USA), assembled with cartridges (20 mm; Supelco), packed with LC-ABZ (5 μm; Supelco) was used as trapping column, TC. A column

(150 × 2.1 mm; GL Sciences, Tokyo, Japan) packed with Inertsil ODS-2 (5 μm; GL Sciences) was used as analytical column, AC. Two mobile phases were delivered by pump P1; A1, ammonium formate (pH 3.6; 5 mM) and B1, ammonium formate (pH 3.6; 5 mM)–ethanol (10:90, v/v). The gradient mobile phase prepared by pump P2 consisted of two components; A2, formic acid (1%)–water–methanol (10:80:10, v/v) and B2, formic acid (1%)–methanol (10:90, v/v).

2.6. Column switching procedure

An analytical run included three subsequent column switching steps and lasted 7 or 10 min, depending on the number of analytes to be determined simultaneously in plasma (see Tables 1 and 2 and Fig. 2).

2.7. Mass spectrometry

A single-quadrupole mass spectrometer MS (Model API 150; Sciex, Concord, Canada) equipped with a turbo ion spray source was applied, using the following electrical settings: sprayer voltage, + 5000 V; ring electrode, + 190 V; orifice, + 20 V; quadrupole Q0, − 5 V; and multiplier, low energy settings. The whole effluent (0.2 ml/min) of the analytical column was introduced into the sprayer, producing $[M + H]^+$-ions at atmospheric pressure, using nitrogen both as nebuliser and curtain gas. The evaporation process was supported by heated air at a flow rate of 8000 ml/min and a temperature of 300°C.

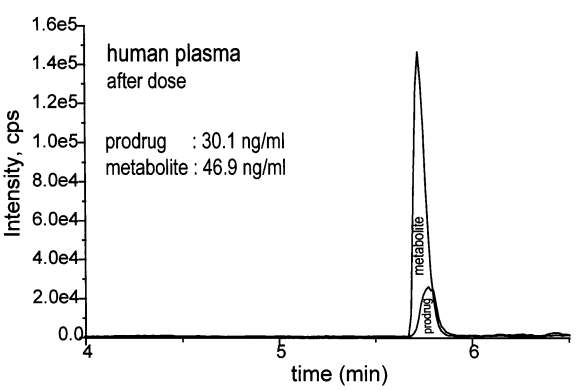

Fig. 7. Chromatogram of a human plasma sample, using EDTA as anticoagulant and containing the protonated ions of II (prodrug) and III (metabolite).

The mass calibration of the mass spectrometer was tuned weekly up to 1000 Da at unit mass resolution by infusion of a standard solution of quaternary alkylammonium salts in acetonitrile (80 fmol/μl), using a syringe pump at 50 μl/min (Harvard, Southnatick, USA). The sprayer position was optimised occasionally for maximum response at a flow rate of 0.2 ml/min by means of flow injection of a standard solution containing I, II, and III at a concentration of 1 ng/μl.

The selected mass-to-charge ratios (m/z) of the protonated analyte and internal standard ions $[M + H]^+$ used in the selected ion mode (SIM) were as follows: I, 421; II, 393; III, 377; IV, 424; V, 396; and VI, 380. In the case of the simultaneous determination of I, II, and III (rat and dog plasma), two periods for data acquisition were employed for adaptation to optimal dwell time. During the first period (from 4 to 7 min), the selected ions monitored were 393, 396, 377 and 380. During the second period (from 7 to 10 min), the selected ions 421 and 424 were monitored. The dwell times were set at 250 ms for the analytes and at 80 ms for the deuterated internal standards. When only II and III were determined simultaneously (human plasma), all ions were monitored in a single period.

2.8. Calibration and calculation

A Power Macintosh 7500/100 computer was used for instrument control and data acquisition. Data acquisition and integration of SIM chromatograms were performed running the proprietary software packages SAMPLE CONTROL (Version 1.3) and MACQUAN (Version 1.5) from PE Sciex. Processed data, such as retention times and peak areas, were transferred to a pharmacokinetic Laboratory Information Management System (LIMS) for final data reduction and reporting [5,6].

Along with the biological samples at least seven plasma standards covering the expected concentration range were processed. The standard curves for the analytes were obtained by weighted least-

squares regression (weighting $= 1/x^2$) of the measured peak area ratios analyte/internal standard versus the analyte concentrations added to the plasma. The standard curves were then used to calculate concentrations of the analytes in unknown and QC samples from the measured peak area ratios.

2.9. Cross-validation experiments

Twenty five plasma pools originating from human plasma samples obtained during a clinical pharmacology trial in healthy volunteers were prepared. The drug concentrations in the samples ranged from less than 1 to approx. 60 ng/ml for both II and III. Aliquots of these samples were distributed to the laboratories participating in the cross-validation experiments. At each laboratory the samples were analysed in duplicate for II and III by using either LC–MS/MS [2] or the LC–MS method described in this paper. Duplicate concentrations were averaged and coefficients of variation calculated. Final results were collected at a central location within the company, which was not involved in the experimental procedures. The data obtained by the existing LC–MS/MS method were defined as reference data. Inaccuracy data were calculated by comparing concentrations obtained by LC–MS and LC–MS/MS.

3. Results and discussion

3.1. Recovery

In order to investigate the proper release of the analytes from plasma proteins during the sample preparation, the recoveries of I, II, and III from human, dog, and rat plasma relative to those from water were determined at two different concentrations. The results in Table 3 demonstrate that the three compounds were completely released from human and dog plasma proteins during protein precipitation. Additionally, the recoveries from citrated and EDTA treated plasma were almost identical, thus simplifying the calibration procedure by allowing the analysis of unknown citrated plasma samples versus EDTA-treated plasma standards. The excellent accuracy data obtained for citrated QC samples demonstrated the validity of this procedure (Table 5).

In contrast, the recoveries of I, II, and III from rat plasma were somewhat incomplete (Table 3). However, the precision and accuracy data obtained for rat plasma QC samples during routine analyses were always good (Table 5), suggesting that the use of deuterated internal standards almost totally compensated for the incomplete recovery.

3.2. Limit of quantification

The limit of quantification, defined here as the minimum concentrations that could be measured routinely with acceptable precision ($< 20\%$) and inaccuracy ($< 20\%$), was 1 ng/ml for all analytes in all plasma types (see Tables 4 and 5).

3.3. Linearity

In contrast to the existing LC–UV method [3], where extremely wide concentration ranges could be calibrated, the linear dynamic range of the new LC–MS method was limited to approximately three orders of magnitude. To cope with concentrations between 1 ng/ml (LOQ) and the highest toxicokinetic levels observed so far, two standard curves had to be established for rat and dog plasma. Plasma concentrations from clinical studies rarely exceeded a value of 100 ng/ml and, for this reason, a single standard curve could include all therapeutic concentrations from human plasma.

3.4. Selectivity

The single LC–MS method was very specific with respect to endogenous plasma components and to co-administered drugs. In the thousands of clinical and pre-clinical blank plasma samples (pre-dose, placebo) from the hundreds of patients and test animals analysed so far, unexpected signals were observed in only very few cases.

The cross-validation experiments described under Experimental demonstrated that the new LC–MS method produces reliable data, which were

comparable with the existing LC–MS/MS method [2]. The mean deviation between concentrations obtained by LC–MS and LC–MS/MS (reference) was 6.4% for II and −0.55% for III. (Table 6).

3.5. Precision and accuracy

The precision (relative standard deviation of replicate analyses) and the inaccuracy (percentage deviation between found and added concentration) were evaluated for toxicokinetic and clinical concentrations in rat, dog, and human plasma. For each level (1–10 000 ng/ml, rat and dog plasma and 1–100 ng/ml, human plasma), a spiked sample was analysed on *n* different days, using a separate calibration line on each day. The data shown in Tables 5 and 6 demonstrate the good precision and accuracy in plasma over both concentration ranges investigated.

3.6. Stability in rat plasma

The stability of I, II, and III in dog plasma, human plasma and humun urine have already been reported [2,3]. Early stability experiments in rat EDTA plasma showed extreme instability of I at 25 and −20°C (data not published). For this reason, additional stability experiments were performed with the new LC–MS method, using the esterase inhibitor dichlorvos as stabilising agent. Rat EDTA blank plasma was stabilised with dichlorvos (1 mg/ml), spiked with I, II, and III at concentrations of 50 and 1000 ng/ml and stored for different time intervals at 25 and −80°C. After storage, the samples were analysed with an equal number of freshly prepared samples to provide the 100% values. The statistical interpretation [7] of the data presented in Table 7 indicated that the storage of frozen samples was not critical, provided enough dichlorvos had been added to the plasma (at least 1 mg/ml) and the samples were stored at very low freezer temperatures (at least at −80°C) Even in the presence of such high dichlorvos concentrations, the ester I hydrolysed almost completely to the acid II when stored for more than 1 h at room temperature, while II and III were stable under these conditions. For this reason extreme care had to be taken during handling of thawed samples, as described in Section 2.

3.7. Application to biological samples

The practicability of the new assay has been demonstrated by the analysis of several hundred rat and dog plasma samples from toxicokinetic investigations and approximately 14 000 patient plasma samples from a phase III clinical study performed with patients. Figs. 3–7 show representative chromatograms from these studies.

4. Conclusions

In many laboratories a shift from traditional analytical techniques to LC–MS/MS methods has occurred. With triple quadrupole instruments, ultimate sensitivity and selectivity can be achieved with very short analysis times, assuming that no significant matrix suppression occurs during the ionisation process. Similar sensitivity should be expected with single quadrupole LC–MS as for LC–MS/MS. The achievable LOQ with biological extracts using LC–MS will, however, also be dependent on the selectivity of the chromatographic and sample preparation procedure. Nevertheless, during the development program of a new drug candidate, ultimate sensitivity may not be required at all stages, and LC–MS could be a cost effective alternative to LC–MS/MS and a more efficient and faster alternative to LC–UV.

Compared with the available LC–UV method [3], the quantification limit of the new assay could not be improved. However, the accuracy and selectivity of the LC–MS method near the LOQ was shown to be significantly better. Even more important, all three analytes could now be determined in a single chromatographic run, while the LC–UV method was restricted to the analyses of II and III.

Compared with LC–MS/MS [2] the new method was about five times less sensitive. However, experience with various clinical studies in phases I, II, and III showed that an LOQ of 1 ng/ml was sufficient for proper evaluation of pharmacokinetic parameters. Since the chromatographic run times were comparable, the sample throughput per day was similar for both methods (ca. 130 samples/day)

and much better than for LC–UV (ca. 70 samples/day). Cross-validation experiments with clinical samples showed that LC–MS and LC–MS/MS provided comparable results. Concerning costs per sample, the LC–MS method was superior to LC–MS/MS because of less expensive instrumentation (single instead of triple quadrupoles), this being an important factor when considering the analysis of large phase III studies in the clinic with thousands of patient samples.

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