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Simultaneous determination of bosentan and its three major metabolites in various biological matrices and species using narrow bore liquid chromatography with ion spray tandem mass spectrometric detection.

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Abstract

An analytical method was developed for the determination of bosentan and its three main metabolites in various matrices and species with focus on robustness. The drug assay involved protein precipitation, followed by liquid-liquid extraction and column switching in combination with narrow bore HPLC-MS-MS. Deuterated analogues of the analytes were used as internal standards. The sample preparation procedure was optimised with respect to minimise the suppression effects from different matrices. The drug and its metabolites could be analysed in plasma, serum, bile, and liver samples from man, dog, and rat with a run cycle time of 10 min. The method used always calibration samples made up in human plasma, whereas quality control samples were prepared in human plasma as well as in the identical matrix as the unknown samples. Calibration graphs for the drug and for the metabolites were linear in the range from 1 or 2 to 2000 or 10 000 ng/ml using a sample volume of 0.25 ml. Mean inter-assay precision and accuracy were 3.0% and 98.4%, respectively. Two additional methods were derived from the main method for the analysis of plasma samples only with focus on reduced manual effort and instrumental run cycle time. The modified methods showed a mean inter-assay precision and accuracy of 5.0% and 99.9% for the method using column-switching, and 3.5% and 98.8% for the method using off-line SPE, respectively. All methods proved to be robust, sensitive, and selective during the analysis of several thousand samples.

Keywords: Bosentan; LC-MS-MS; Quantitative analysis; Sulfonamide drugs; Bioanalysis

1. Introduction

Bosentan is a non-peptidic endothelin receptor antagonist, which is under development for the treatment of hypertension and chronic heart failure. It acts on the known human endothelin receptors, ET_A and ET_B. ET_A is present in smooth muscle cells and mediates vascoconstriction and proliferation. ET_B is present in astrocytes and neurons, endothelial/epithelial cells and certain smooth muscle cells, and can mediate both, endothelium-dependent relaxation, vascoconstriction, and brochoconstriction. Metabolism of bosentan (I) occurs mainly in the liver

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by the action of cytochrome P (CYP) 450 3A4 and 2C9, which produces three metabolites: the hydroxylated (hydroxy) metabolite (II), the demethylated (phenol) metabolite (III), and the hydroxylated and demethylated (hydroxy-phenol) metabolite (IV) (Fig. 1) [1–5]. In an early phase of drug development, a high-performance liquid chromatography assay with ultraviolet detection (HPLC-UV) was used for the quantification of the drug alone, with a limit of quantification (LOQ) of 5 ng/ml using a 1 ml plasma aliquot [6]. Since the LOQ and the specificity of the HPLC-UV assay was not sufficient for monitoring kinetic profiles, a liquid chromatography tandem mass spectrometric (LC-MS-MS) method with a detection limit of 0.5 ng/ml and a run cycle time of 5 min using 0.5 ml plasma was developed. The LC-MS-MS method used, after protein precipitation, liquid-liquid extraction under basic conditions with subsequent separation on narrow bore HPLC and tandem mass spectrometric detection [7]. In a later stage of the development, it became necessary to monitor, in addition, the metabolites in order to follow the fate of the drug in different tissues. Due to the chemical diversity of the metabolites and the drug itself the method applied to the quantification of bosentan only failed [8]. The aim of the presented

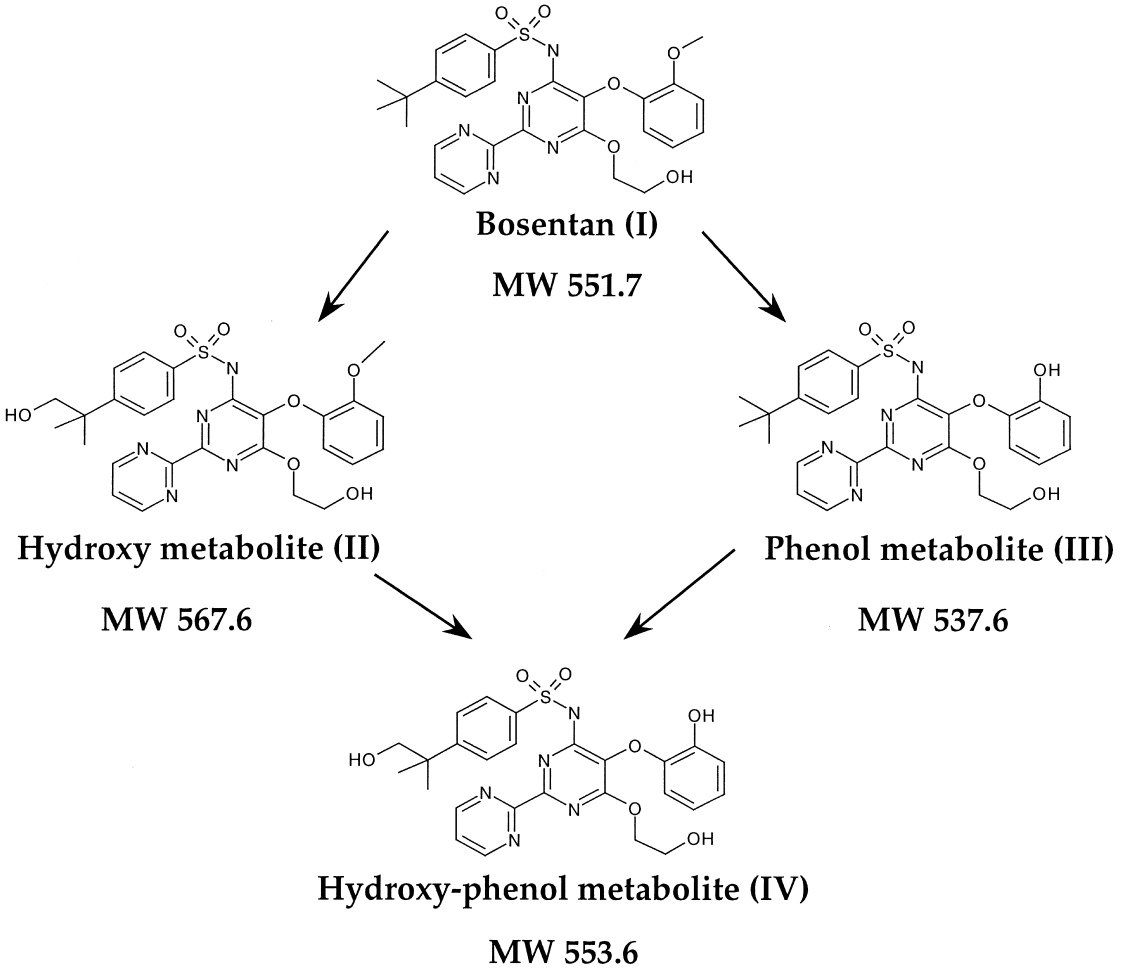

Fig. 1. Structure of the analytes and metabolic pathway of bosentan.

work is to describe the development and the performance of a versatile drug assay for the simultaneous determination of bosentan and of its three main metabolites in different tissues from various species. During method development, it became evident that bosentan and its metabolites were very sensitive to matrix effects during the ionisation process. Therefore, special emphasis was given to the selectivity of the method rather than to the sensitivity or speed. To cope with higher sample throughput required to support clinical investigations the method was appropriately modified to allow the quantification of bosentan and its metabolites in human plasma. All methods were validated and showed long term robustness by its application to several thousands of samples.

2. Experimental

2.1. Chemicals

Bosentan (I, purity 99.7%), the metabolites (II, purity 94.7%), (III, purity 98.5%), (IV, purity 97.7%) and the four corresponding $^{2}H_{4}$-internal standards (purity: 99.5% I.S. of I, 97.7% I.S. of II, 99.5% I.S. of III, 98.3% I.S. of IV) were obtained from F. Hoffmann-La Roche Ltd., Basel. The stable isotope labeled analytes used as internal standards differ from the analytes by four deuteriums on the ethyleneglycol moiety of the molecule. *n*-Chlorobutane was obtained from Fisons (reagent grade), and dichloromethane and triethylamine were delivered by Fluka (reagent grade). Acetonitrile, methanol, ethanol, acetic acid, ammonium acetate, sodium carbonate, and buffer solution pH 4 (Titrisol citrate/HCl) were purchased from Merck. Water was either double distilled or was purchased from Merck (chromatography grade).

2.2. Sample preparation

To the plasma sample (0.25 ml), 50 μl of the internal standard solution (containing 100 ng of each internal standard in acetonitrile–5 mM ammonium acetate–acetic acid (10:90:1, v/v/v)) was added. A volume of 1 ml acetonitrile–ethanol (1:1, v/v) was added for protein precipitation. The supernatant was, after centrifugation (14000 *g* for 10 min), transferred to another tube, and 1 ml buffer (pH 4.0) was added. Liquid-liquid extraction was carried out with 6 ml *n*-chlorobutane–dichloromethane (8:2, v/v) for 20 min on a rotating mixer (40 rpm). The organic layer was transferred to another tube and evaporated to dryness by means of a vacuum centrifuge. After reconstitution of the sample with 1 ml acetonitrile–5 mM ammonium acetate–acetic acid (10:90:1, v/v/v), 0.95 ml were injected onto the column-switching HPLC system. The liver samples were homogenised prior to analysis by adding two parts of 0.1 M phosphate buffer to one part of liver. The mixture was then treated with a ''Polytron'' homogeniser at 8000 rpm for 10 to 15 sec.

2.3. High performance liquid chromatography

The chromatographic system consisted of two pumps. An L-6200A (Merck-Hitachi, Darmstadt, Germany) was used to provide a gradient for the analytical column and the second one an L-6200 was used for solvent delivery to the trapping column. The column-switching system consisted of a 25×4 mm Superspher RP-Select B trapping column (5 μm particle size, 60 Å pore size) and an 150×2.1 mm Symmetry RP-18 column analytical column (protected by a 10×2 mm Superspher RP-18 guard column), which were separated by a six port switching valve 7000E (LabSource, Riehen, Switzerland). The sample solution was injected by means of an AS4000 autoinjector (Merck-Hitachi). After injection, the trapping column was washed for 2 min at a flow-rate of 1 ml/min with 5 mM ammonium acetate containing 1% acetic acid. Then, the valve was switched in line with the analytical column in order to elute the analytes and internal standards from the trapping column. The gradient started with 50% B, and the proportion of B was increased to 90% within 4.5 min at a flow-rate of 0.25 ml/min. Solvent A consisted of a mixture acetonitrile–methanol–5 mM ammonium acetate–acetic acid in the ratio 25:25:50:1 (v/v/v/v), whereas solvent B consisted of the same components as A, but in the ratio 45:45:10:1 (v/v/v/v). After elution of the analytes, the analytical column was flushed with acetonitrile containing 1% acetic acid for 1 min at an increased

flow-rate of 0.35 ml/min. Reconditioning of the analytical column with the initial solvent composition was carried out for additional 0.5 min, also at 0.35 ml/min. The trapping column was washed after elution of the analytes for 3.5 min in backflush and frontflush mode (acetonitrile–methanol–5 mM ammonium acetate–acetic acid, 45:45:10:1, v/v/v/v) and reconditioned for an additional 2.5 min (5 mM ammonium acetate containing 1% acetic acid), both at a flow-rate of 1 ml/min.

2.4. Column-switching without liquid-liquid extraction

To the plasma sample (0.25 ml) 0.75 ml of an organic mixture (acetonitrile–ethanol, 1:1, v/v, containing 100 ng of I.S.) was added for protein precipitation. After reduction of the volume to approximately 0.25 ml and addition of 0.3 ml (acetonitrile–5 mM ammonium acetate–acetic acid, 10:90:1, v/v/v) 0.5 ml were injected onto the system. The column-switching set-up and HPLC conditions were similar to that described previously with following modifications. The trapping column was washed first with a mixture of methanol–water (2:8, v/v) for 2 min, then with 5 mM ammonium acetate containing 1% acetic acid for two additional min (1.5 min in frontflush and 0.5 min in backflush mode).

2.5. Off-line solid-phase extraction

To the plasma sample (0.25 ml) 50 μl I.S. were added. Protein precipitation was performed by the addition of 0.75 ml methanol. The supernatant was diluted with 2 ml (50 mM ammonium acetate buffer pH 10, adjusted with ammonia), and applied to the Oasis SPE cartridges (30 mg). The cartridge was washed subsequently with water (1 ml), 20 mM phosphoric acid (2 ml), methanol–water (2:8, v/v, 2.1 ml) and finally with water (1 ml). Elution was carried out with a mixture of acetonitrile–methanol 3:7, v/v containing 2% triethylamine. After evaporation the sample was reconstituted with 0.15 ml acetonitrile–5 mM ammonium acetate–acetic acid (10:90:1, v/v/v). Chromatographic separation is carried on a 125×2 mm Superspher (Merck) column in isocratic mode with acetonitrile–methanol–5 mM ammonium acetate–acetic acid (37.5:37.5:25:1, v/v/v/v) at a flow-rate of 0.25 ml/min.

2.6. Mass spectrometry

Mass spectrometric detection was performed on an API 300™ triple quadrupole mass spectrometer from Perkin-Elmer Sciex (Concord, Ontario, Canada) operated in electrospray ionization (ESI) positive ion mode, and equipped with the TurboIon™ spray ion source. During method development, the mass spectrometer was upgraded to an API 365™. In parallel, the design of the TurboIon™ spray source was changed from a fused-silica based sprayer to a stainless steel based sprayer, which improved, considerably, stability and robustness during long term operation. Product ion spectra were produced by collision induced dissociation (CID) using nitrogen as collision gas at a device collision gas thickness setting of 3, and a collision energy, calculated from the difference between the quadrupole 2 (q2) voltage and quadrupole 0 (Q0) voltage settings, of approximately 48 eV. Product ion spectra were recorded by infusion of a standard solution of each analyte at a concentration of 1 ng/μl (diluted in acetonitrile–methanol–5 mM ammonium acetate–acetic acid 40:40:20:1, v/v/v/v) at a flow-rate of 20 μl/min. For quantitative purposes, the mass spectrometer was operated in the selected reaction monitoring (SRM) mode. Both quadrupoles were tuned at unit mass resolution. Collision-induced dissociation of compound I and II and their tetra-deuterium-labelled internal standards yielded the same product ion at m/z 202, while compound III and IV and their tetra-deuterium-labelled internal standards yielded the same product ion at m/z 189. Therefore, the mass transitions m/z 552–202 (I), m/z 556–202 (IS I), m/z 568–202 (II), m/z 572–202 (IS II), m/z 538–189 (III), 542–189 (IS III), m/z 554–189 (IV), m/z 558–189 (IS IV) were used for SRM experiments.

The use of an API 300 or, later, an API 365 mass spectrometer with the corresponding software package allows to set individual dwell times for each analyte and each internal standard, leading to an improvement of the signal-to-noise ratio. Typical

settings for the dwell times of the analytes were in the 300 msec range, whereas for the internal standards a dwell time of approximately 100 msec was used. Pause times of about 20 msec were set between all analytes dwell times, resulting in a overall cycle time of 0.88 sec for one scan. In the first period of the ion chromatogram, from 2 to 6 min, elution of the hydroxy phenol metabolite (IV), the most hydrophilic compound, and the hydroxy metabolite (II) occurred, whereas in the second period, from 6 to 9 min, the phenol metabolite (III) and bosentan (I) were eluted.

2.7. Preparation of calibration and quality control samples

Calibration samples were prepared in human plasma to cover the range 1 ng/ml to 10000 ng/ml for bosentan and 1 ng/ml to 2000 ng/ml for the metabolites II to IV. Stock solutions of all analytes were prepared in methanol–50 mM sodium carbonate (1:1, v/v). Spike solutions were produced from the stock solutions by combining an appropriate volume of each individual stock solution into one volumetric flask and subsequent sequential dilution of this spike solution. Human plasma calibration samples were prepared by spiking a volume of each spike solution, which was equal or less then 2% of the plasma volume, to drug free human plasma. After mixing for 10 min to achieve homogeneity, the calibration samples were aliquoted and stored in a deep freezer. Quality control (QC) samples were prepared from independently weighed amounts of each analyte in the same way. QC samples were prepared in human plasma as well as in all other matrices investigated at least two different concentrations within the calibration range.

2.8. Data acquisition and data processing

A Macintosh Power PC 7500 computer was used for instrument control, data acquisition, and data processing. Multilevel calibration curves were constructed using MacQuan software (PE Sciex). The peak area ratios of the analyte and its internal standard were fitted against the respective calibration concentration using weighted $(1/x^2)$ last-squares linear regression. The linear regression equations were used for calculation of drug and metabolite concentrations.

3. Results and discussion

3.1. Mass spectrometry

Drug assay development using LC-MS-MS starts almost with the optimisation of the mass spectrometric parameters to maximise analyte response in order to achieve the lowest possible detection limit. Full scan and product ion spectra were recorded using standard solutions of the analytes dissolved in solvents containing a high organic content and acetic acid. The product ion spectra of the analytes show equally abundant fragment ions for the drug (I) and the hydroxy metabolite (II) at m/z 202, as one pair, and for the phenol and the hydroxy-phenol metabolites (III–IV) at m/z 189, as another pair (Fig. 2). The fragmentation pathway of bosentan and derivatives has been intensively investigated by Hopfgartner et al. [9,10]. They found unexpected fragmentation behaviour due to the formation of an even-electron cation as an intermediate. The stable isotope labeled analytes used as internal standards differ from the analytes by four deuteriums on the ethyleneglycol moiety of the molecule. After collision induced fragmentation, this side chain leaves the molecule first, and gives rise, after successive fragmentation, to the same abundant fragments as found for the corresponding analytes. Therefore, attention was paid during set-up of the mass spectrometric conditions to prevent ''cross talk'' in the collision cell. Cross talk is a phenomenon, which is observed on triple stage mass spectrometers equipped with high-pressure collision cells and for analytes with different m/z generating identical fragments. The residence time of the fragments in the collision cell is increased in the due to the ''high collision gas pressure''. The ions generated from one precursor ion are still ejected during the time, while the other analyte is monitored. To avoid cross talk on the API 300, dummy ions (ions that are not in the mixture) are set in between the ion transitions of the analytes

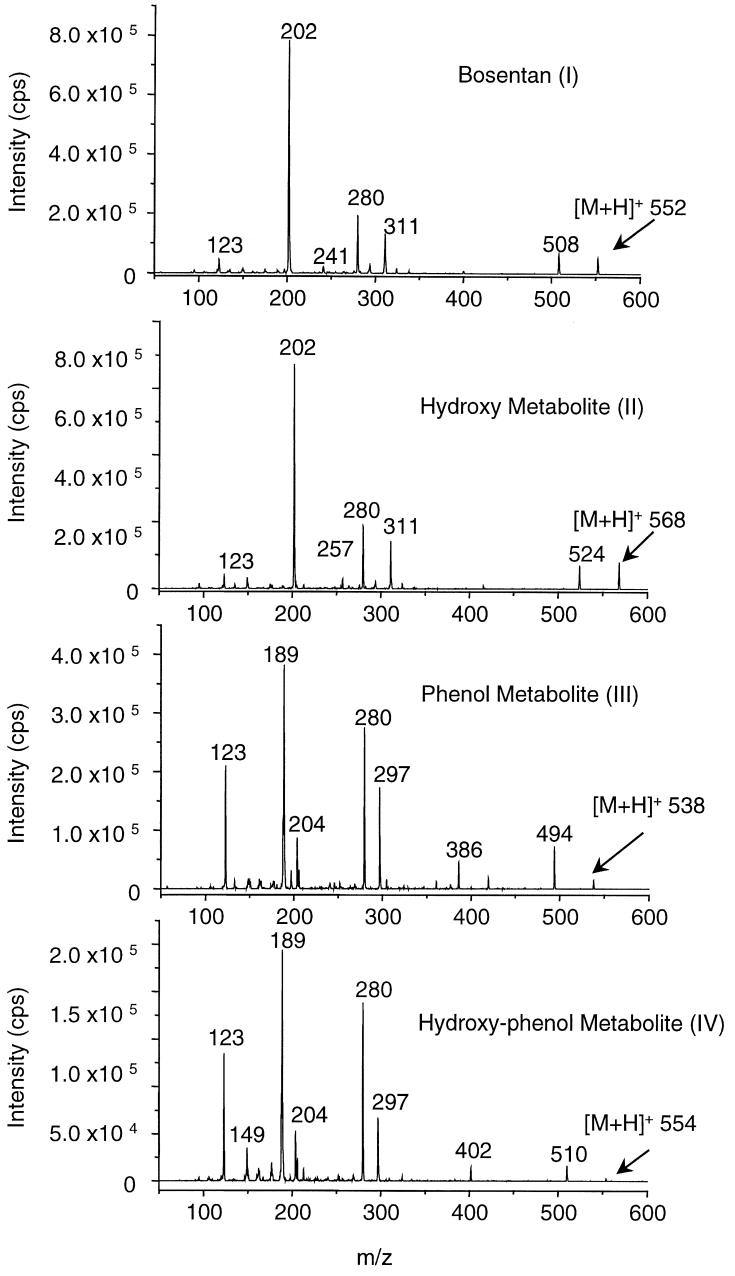

Fig. 2. Product ion spectra of bosentan and its metabolites.

and internal standards. With the API 365, the ''settling'' procedure was switched on by an automatic routine whenever identical fragments following each other were recognised by software.

3.2. Sample clean-up and liquid chromatography

The usage of a mass spectrometer especially in tandem MS mode is highly selective versus classical

UV detection. Unfortunately, co-eluting components from the matrix or salts can suppress or enhance the analyte signal during the ionisation process. This phenomenon is compound and matrix dependent, but does not affect the chromatographic peak shape. These effects are not obvious to detect and they are rather expressed as imprecise and inaccurate data. Therefore, sample preparation remains with LC-MS-MS an essential step in the analytical procedure [11–13]. During method development and from previous experience it became evident that bosentan and its metabolites are very sensitive to matrix suppression effects. In this case, where the goal was to develop an assay for various matrices (plasma, serum, bile, and liver) the emphasis was set to use a combination of sample preparation techniques such protein precipitation, liquid-liquid extraction, column-switching and gradient elution in order to minimise matrix suppression effects.

Gradient elution was necessary to optimise the run cycle time and achieve a separation of the analytes. For each analyte a deuterated internal standard was used, which improved significantly the assay performance. Moreover, deuterated internal standards have similar extraction recovery as the non-labelled compounds. The resulting advantage of the use of labelled I.S. is that for all matrices and species calibration samples were made up in human plasma while only the QC samples were prepared in the matrix of the species under investigation. Such kind of approaches allows minimising validation work.

3.3. Inter-assay precision and accuracy

The inter-assay accuracy and precision calculated from independently processed sets of calibration samples during the method validation phase are listed in Table 1. For this, two sets of calibration samples were prepared and analysed together on six different occasions. One set of calibration samples was used for calculating the weighted linear regression equation (weighting factor $1/x^2$) and the other was used for the calculation of the method parameters. As can be seen from Table 1, all values for precision and inaccuracy are below the 15% limit. Most of the precision and the inaccuracy values were below 10%, and only a few values were in between 10 and 15%. The inter-assay precision and accuracy calculated from independently prepared QC samples are listed in Table 2. The values for precision and accuracy are randomly distributed over all matrices and analytes, leading to the conclusion that the method was equal applicable to all the matrices tested. Depending on the type of the study and the drug concentration expected, two to four QC samples

Table 1
Inter-assay precision and inaccuracy of human plasma calibration samples declared as QC samples

		Bosentan (I)			Hydroxy Metabolite (II)			Phenol Metabolite (III)			Hydroxy-phenol Metabolite (IV)			
	Amount added (ng/ml)	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	*n*
C01	1.0	1.1	8.4	110.3										6
C01	2.0	2.0	9.4	98.2	2.1	6.8	105.0	2.1	8.1	104.8	2.1	4.6	105.3	6
C03	5.0	5.1	4.7	100.9	4.7	10.5	93.4	5.1	7.7	101.6	5.2	8.5	104.7	6
C04	10.0	10.0	5.1	100.1	9.6	4.7	96.4	10.1	4.2	101.3	10.3	4.0	103.0	6
C05	20.0	19.8	3.3	98.8	20.1	4.0	100.5	20.6	5.0	103.2	20.6	6.4	103.2	6
C06	50.0	50.9	3.2	101.7	50.8	2.1	101.6	51.4	4.6	102.9	49.8	6.4	99.6	6
C07	100.0	101.5	4.3	101.5	102.4	1.7	102.4	100.3	0.4	100.3	100.9	4.5	100.9	6
C08	200.0	199.6	2.8	99.8	200.3	2.2	100.2	197.4	2.6	98.7	194.3	4.5	97.2	6
C09	500.0	498.5	3.6	99.7	505.2	2.1	101.0	506.3	3.7	101.3	482.3	2.1	96.5	6
C10	1000.0	1000.5	2.7	100.1	985.4	3.9	98.5	999.8	4.9	100.0	948.0	3.8	94.8	6
C11	2000.0	1939.2	2.1	97.0	1943.4	2.9	97.2	2091.4	14.4	104.6	1946.6	4.6	92.3	6
C13	5000.0	4702.2	1.4	94.1										6
C14	10 000.0	8695.5	3.2	89.6										6

Table 2
Inter-assay precision and inaccuracy of QC samples in various matrices and species

	Bosentan (I)			Hydroxy Metabolite (II)			Phenol Metabolite (III)			Hydroxy-phenol Metabolite (IV)			
Amount added (ng/ml)	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	Amount found (ng/ml)	RSD (%)	Accuracy %	*n*
Human plasma													
10.0	10.4	2.2	104.0	9.4	1.8	94.1	9.5	5.3	95.4	9.5	6.3	95.1	8
100.0	101.5	2.5	101.5	94.1	1.1	94.0	93.4	2.0	93.4	95.2	1.2	95.2	8
1000.0	995.9	1.7	99.6	937.5	1.4	93.8	930.9	2.2	93.1	935.8	1.7	93.6	8
5000.0	4705.7	1.3	94.1										8
Human serum													
50.0	50.9	3.8	101.8	50.7	3.0	101.4	51.3	3.0	102.6	50.2	4.0	100.4	6
500.0	499.1	2.5	99.8	501.9	3.3	100.4	499.1	3.3	99.9	494.4	2.6	98.9	6
Dog liver													
100.0	101.5	4.3	101.5	102.4	1.7	102.4	100.3	0.4	100.3	100.9	4.5	100.9	6
200.0	199.6	2.8	99.9	200.3	2.2	100.2	197.4	2.6	98.7	194.3	4.5	97.2	6
Dog bile													
500.0	498.5	3.6	99.7	505.2	2.1	101.0	506.3	3.7	101.3	482.3	2.1	96.5	6
1000.0	1000.5	2.7	100.1	985.4	3.9	98.5	999.8	4.9	100.0	948.0	3.8	94.8	6
Dog plasma													
10.0	9.2	1.7	91.8	9.3	1.5	92.7	10.1	2.3	100.6	9.9	3.9	98.9	5
100.0	101.2	1.8	101.2	97.4	1.7	97.4	92.7	0.9	92.7	93.6	9.6	93.6	5
1000.0	1012.8	1.1	101.3	973.7	1.5	97.4	927.2	0.3	92.7	957.3	9.6	95.7	5
5000.0	4634.5	1.2	92.7										5
Rat plasma													
50.0	50.0	1.8	100.0	50.0	1.9	99.9	49.5	1.0	98.9	50.3	3.4	100.7	6
500.0	490.4	1.2	98.1	496.4	1.1	99.3	480.9	1.1	96.2	484.1	4.2	96.8	6

concentrations were prepared and analysed with each set of unknown samples.

3.4. Linearity and limit of quantification

For bosentan, a calibration range 1 to 10 000 ng/ml, and for the metabolites a range 2 to 2000 ng/ml could be established. The upper limit of quantification (ULQ) of the metabolites was set to 2000 ng/ml, because the metabolite concentrations in plasma reach, at most, 10% of the bosentan concentrations. The lower limit of quantification (LOQ) was set for bosentan to 1 ng/ml and 2 ng/ml for the metabolites to meet the criterion for precision and inaccuracy of less than or equal to 15%.

3.5. Plasma sample analysis

Extracted selected reaction ion chromatograms of a drug free human plasma, and a calibration sample prepared in human plasma containing 10 ng/ml of each analyte and 400 ng/ml of the corresponding internal standards (traces of the internal standards are not extracted and not shown in Figs. 3 to 8) are shown in Figs. 3 and 4. The chromatographic run was divided into two periods, in order to increase the dwell time and the signal-to-noise ratio for each pair of analytes. A detailed inspection of the extracted selected reaction ion chromatogram of a drug free plasma sample depicted in Fig. 3 shows only noise or very weak signals in the traces of the metabolites, demonstrating the selectivity of the method. For

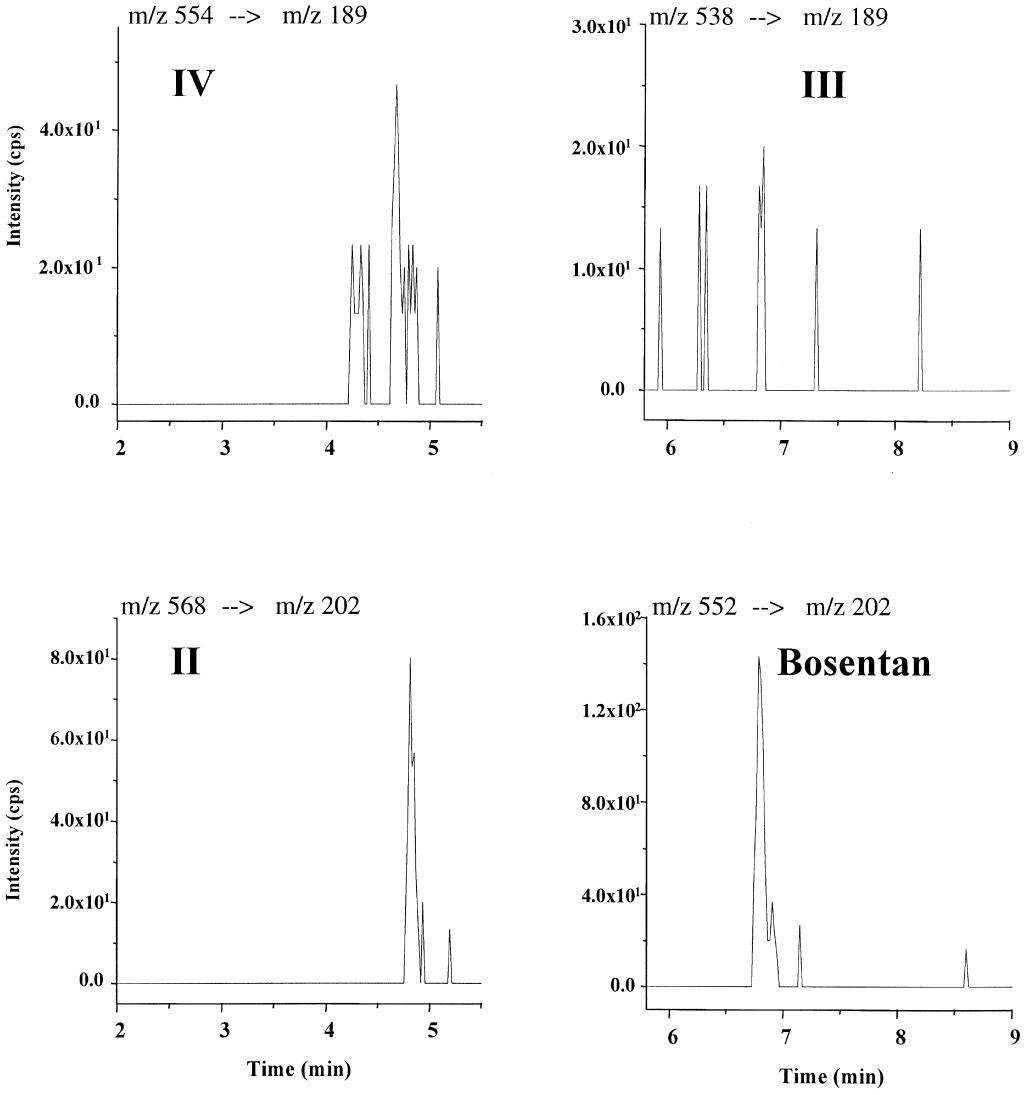

Fig. 3. Extracted selected reaction ion chromatograms of a blank human plasma sample.

bosentan, a small peak appears at Rt 6.8 min in the trace of the drug free plasma sample, originating from an impurity of approximately 0.5% in the internal standard with non-labelled compound. This was confirmed by a full scan spectrum recorded from a standard solution of the internal standard of bosentan (data not shown). The contribution of the I.S. was not found to be critical for the assay performance, because the lowest calibration sample counts for a peak height of about 1000 counts per second and the interference from the impure internal standard for only 160 counts per second. Carry-over from the autosampler can also contribute to peak signal. However, in the calibration range investigated the very small carry-over observed from the autosampler did not affect the LOQ of the assay. The robustness and specificity of the method has been proven by its application to samples from several clinical trials and non-clinical investigations, in which the concentration-time profiles of the drug and

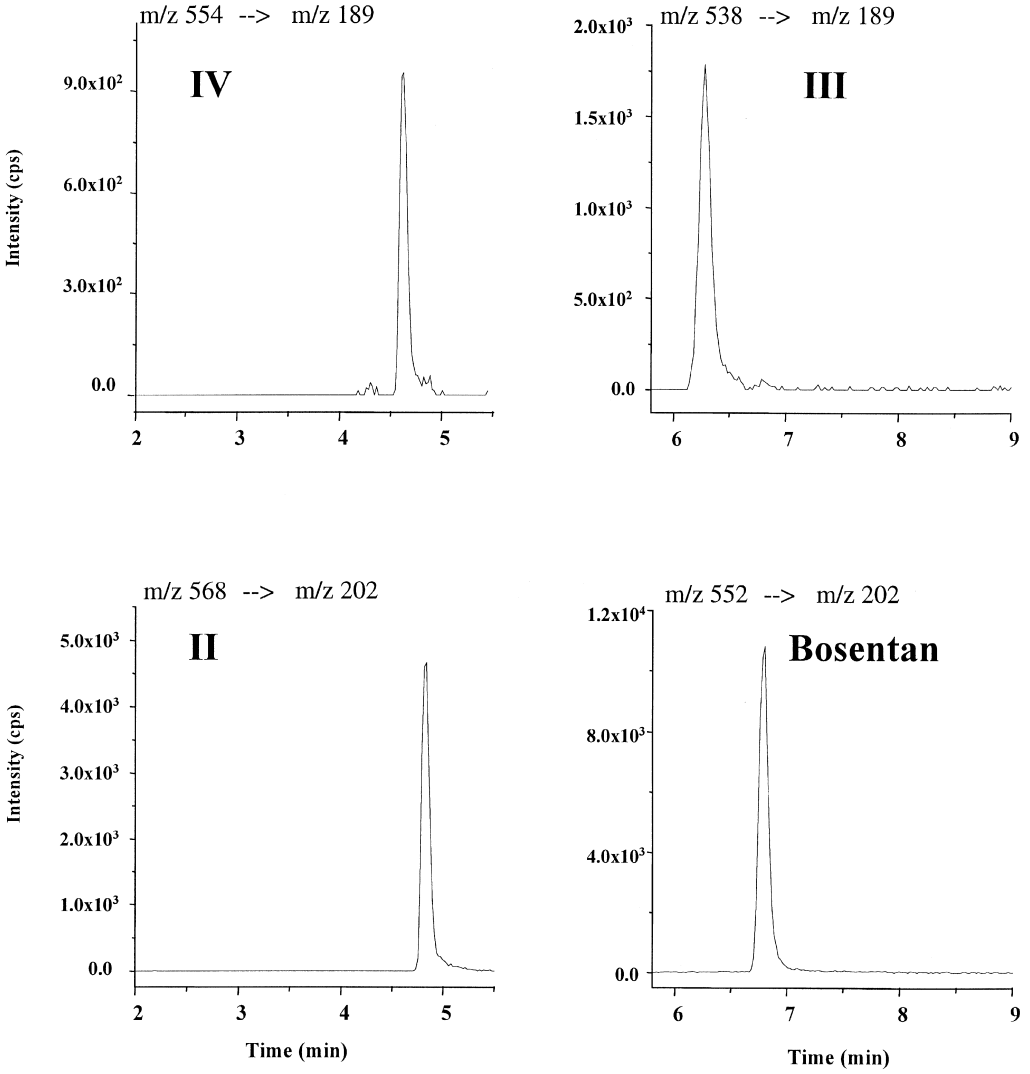

Fig. 4. Extracted selected reaction ion chromatograms of a human plasma calibration sample containing 10 ng/ml of each analyte.

its metabolites could be followed. Mainly plasma samples from men, dog, and rat were processed using this method.

3.6. Bile samples analysis

The versatility of the method is demonstrated by its application to a pilot toxicology study, where the influence of the drug on bile flow and bile composition was investigated. For this, the concentration of bosentan and its metabolites were determined in dog bile and plasma. In Fig. 5 the extracted selected reaction ion chromatograms of the analytes of a drug free dog bile sample show only weak peaks for bosentan (I) and metabolites (II to IV). Peaks appearing in the traces of bosentan and metabolite II originated either from impurities present in the internal standard (bosentan) or from an autoinjector carry over. In Fig. 6, the extracted selected reaction ion chromatograms of a bile sample collected in the

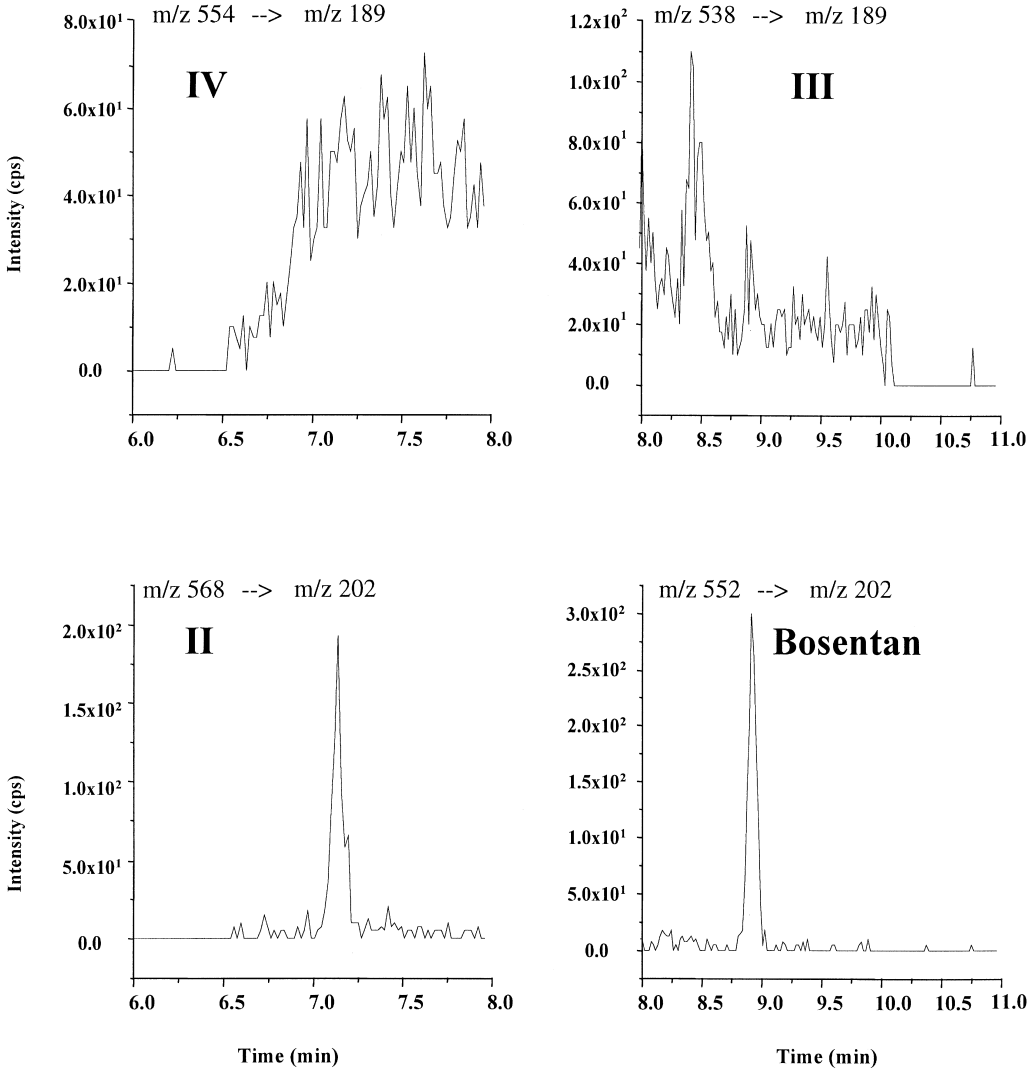

Fig. 5. Extracted selected reaction ion chromatogram of a drug free dog bile sample.

period 1 to 14 h after dosage of bosentan to a cannulated dog are shown. The peaks correspond to a concentration of 18.3 μg/ml of bosentan, 269 μg/ml of the hydroxy metabolite (II), 8 μg/ml of the phenol metabolite (III) and 31.8 μg/ml of the hydroxy-phenol metabolite (IV). To bring the samples into the calibration range, they were diluted 100 or 1000 times with drug free dog bile. The mean precision and accuracy found during the control phase of the assay was 10.9% and 104.7% for bosentan, 9.3% and 106.2% for metabolite I, 9.4% and 106.0% for metabolite II, and 7.2% and 106.0% for metabolite IV, respectively. To maintain a high analytical performance over the time necessary for the analysis of approximately 70 to 90 bile samples, it was mandatory to increase the wash time on the trapping column from 2 to 4 min, which, in turn, lead to an increased overall analysis time. In addition, after the analysis of one batch of bile samples, replacement of the trapping and guard column and

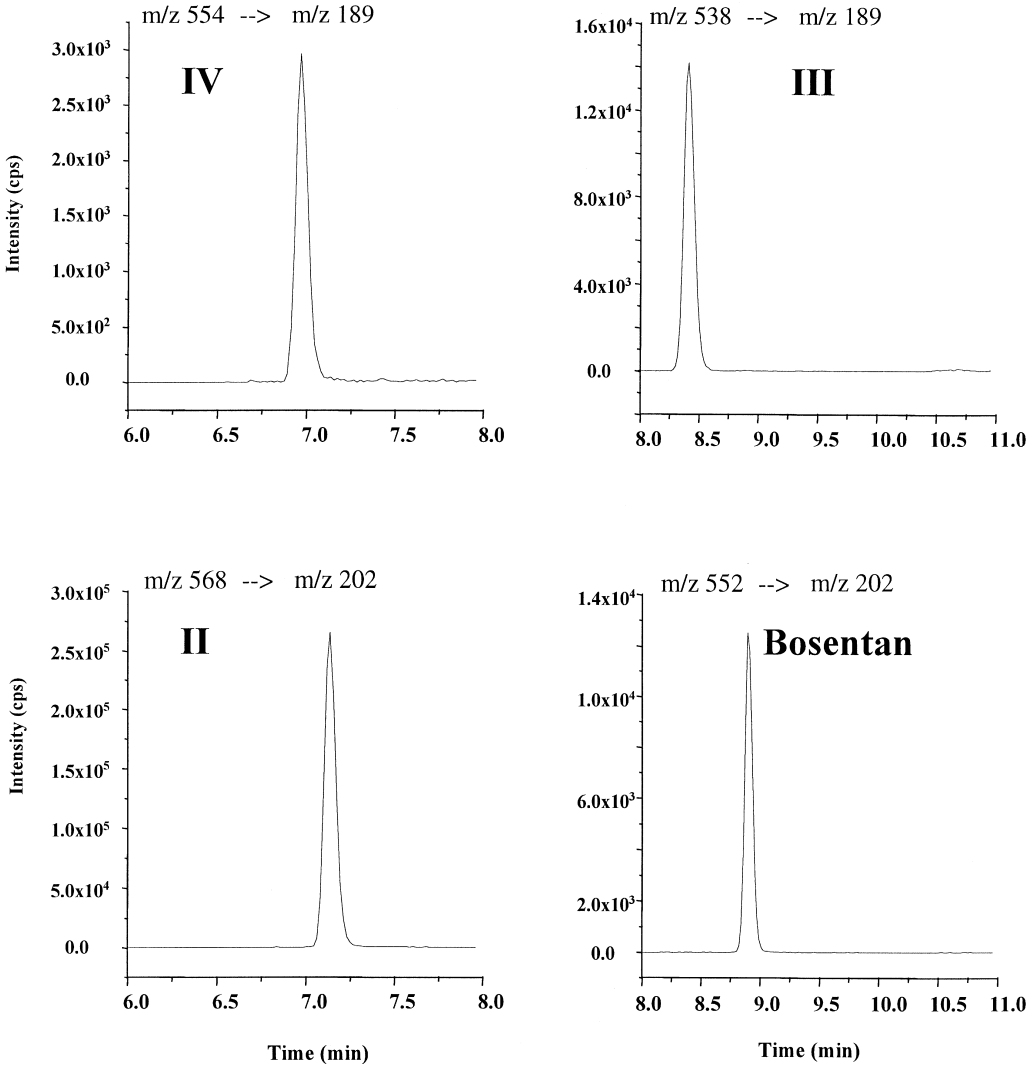

Fig. 6. Extracted selected reaction ion chromatograms of a dog bile sample taken after p.o. administration of 20 mg/kg/d of bosentan.

cleaning of the curtain plate of the mass spectrometer was necessary to maintain the analytical performance of the system.

3.7. Liver samples analysis

Another example demonstrating the specificity of the method, is the analysis of liver samples. Fig. 7 shows the extracted selected reaction ion chromatograms of a drug free dog liver sample, while Fig. 8 shows the extracted selected reaction ion chromatograms a dog liver sample taken one year after daily p.o. administration of 500 mg/kg of bosentan. Small peaks in the SRM traces (Fig. 7) of bosentan and the hydroxy metabolite (II) are originating from impurities in the bosentan I.S. and from carry-over in the autosampler. The peaks in the selected reaction ion chromatograms of the liver sample taken one year after daily administration of bosentan correspond to 5330 ng/ml bosentan, 4980 ng/ml for the hydroxy metabolite (II), 531 ng/ml for the phenol metabolite (III) and 218 ng/ml for the hydroxyphenol metabolite (IV). The liver sample was diluted three times with drug free liver homogenate to bring

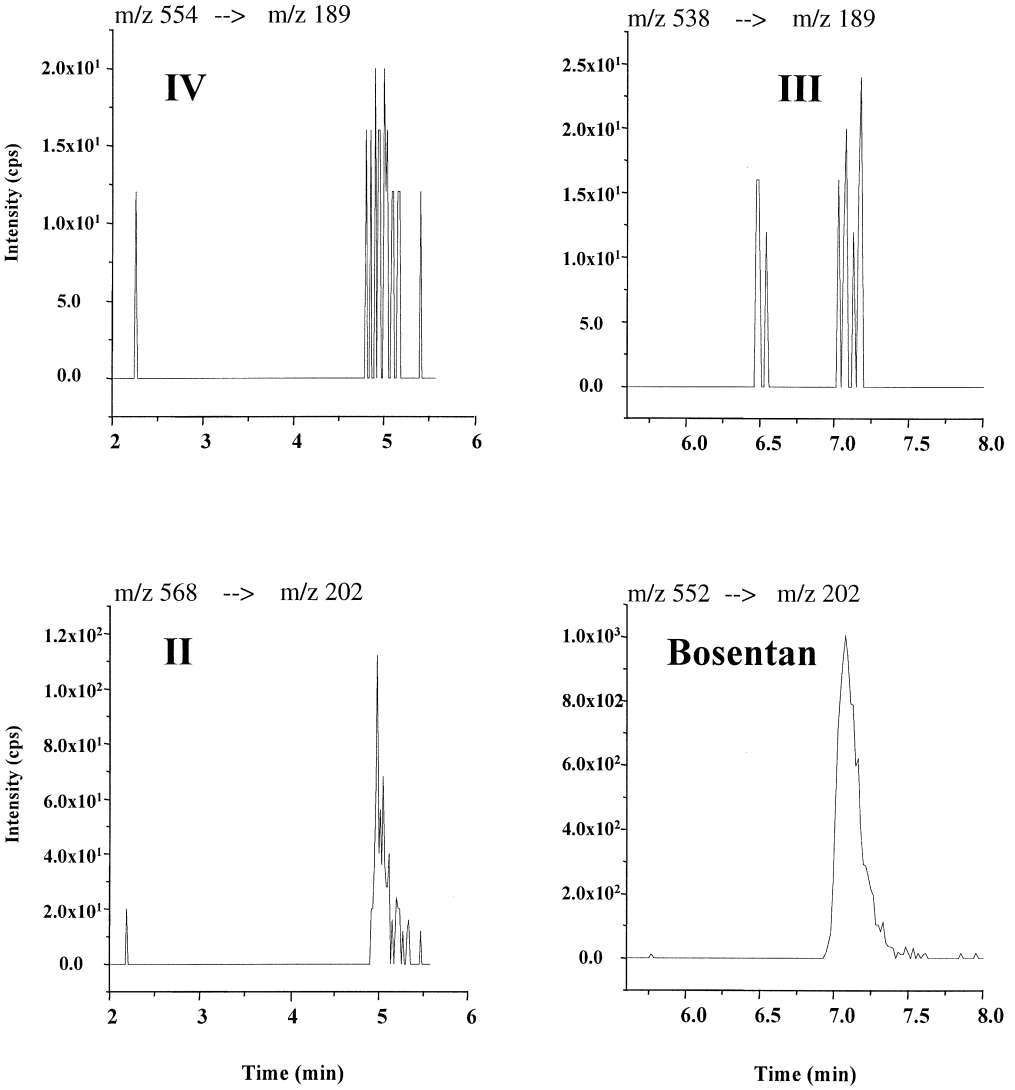

Fig. 7. Extracted selected reaction ion chromatograms of a drug free dog liver sample.

the samples into the calibration range of the metabolites. The quality of the results was monitored during the control phase by assessing the precision and accuracy of the quality control samples ($n=5$) which were prepared in drug free liver homogenate. The mean precision and accuracy derived for the quality control samples prepared at 50 and 500 ng/ml was 7.2% and 105.1% for bosentan, 3.3% and 101.6% for the hydroxy metabolite (II), 9.8% and 104.2% for phenol metabolite (III), and 6.0% and 104.7% for hydroxy-phenol metabolite (IV), respectively.

3.8. Extraction recovery

Extraction recoveries were determined in human plasma at 50 ng/ml ($n=6$) and were found to be 78% for bosentan, 79% for (II), 75% for (III), and 49% for (IV). The low extraction recovery found for the hydroxy-phenol metabolite (IV) was, in our opinion, related to

(i) the polarity of the compound, which was the most polar of the compounds under investigation,

(ii) the pH used for extraction, which may not

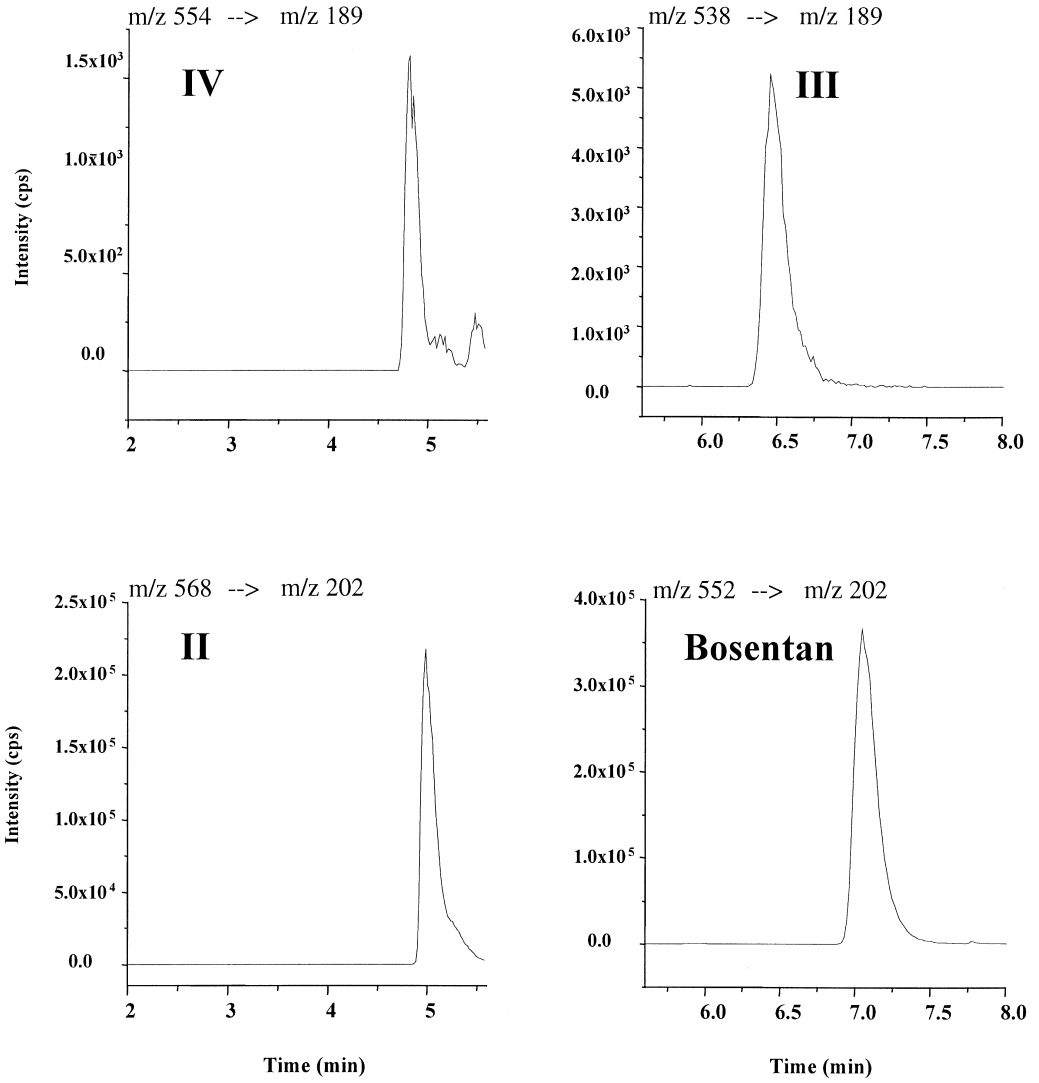

Fig. 8. Extracted selected reaction ion chromatograms of a dog liver sample taken after 1 year daily p.o. administration of 500 mg/kg of bosentan.

have been acidic enough to ensure that the phenol moiety was protonated,
(iii) the lower affinity of the compound to the support material of the trapping column.

Extraction recoveries were determined by extracting several spiked and a blank plasma samples and subsequent addition of the same amount of analytes to the blank plasma extracts before both sample types were further treated. Extraction recoveries were calculated from the peak area ratio analyte/I.S. rather then from the absolute peak area.

3.9. Drug stability

Stability of the drug and of its metabolites in human plasma was investigated at room temperature for a time needed for sample preparation (4 h), for freeze and thaw cycles, as well as for a storage period of one year at −20°C. All four analytes were

found to be stable under the conditions investigated. Stability investigations were performed at least two different concentrations (eight replicates). Compounds were declared stable if not more than a 15% decrease from the nominal concentration in comparison to a freshly prepared reference was observed [15].

4. Modifications of the method for human samples

4.1. Column-switching without liquid-liquid extraction

Due to the use of a very complex and time-consuming sample preparation procedure, the method was applicable to a variety of matrices and species. In order to reduce the tedious sample work-up, and to automate the assay, the liquid-liquid extraction step was replaced by a more sophisticated column-switching step, consisted of a sample washing procedure with two different solvents. The method was validated for human plasma and serum. The inter-assay precision and inaccuracy was found to be below 15% in the concentration range 2 to 10 000 ng/ml for bosentan and 2 to 2000 ng/ml for all three main metabolites (II–IV). Due to a less selective sample preparation the LOQ had to be increased for bosentan from 1 to 2 ng/ml, which was sufficient for supporting pharmacokinetic studies. Most of the values for precision and inaccuracy were, in fact, below 10% (5 out of 64 values were greater than 10%). Three of these values were related to the precision of the lowest calibration point for bosentan, and metabolites III and IV. All values for inter-assay precision and inaccuracy, derived from the QC samples, were below 10%. The overall process time on the HPLC-MS instrument was about 11 min for each sample, resulting in a total operation time of about 27 h for batches of 150 samples. Each day, one technician could easily prepare a batch of 150 samples, which was impossible using the LLE and column-switching method. Attempts to reduce the cycle time for one sample by using a dual column switching approach, as reported by others [14], were not taken into consideration because of the very complex trapping procedure. This modified drug assay was applied successfully to the analysis of several thousands of human plasma samples and hundreds of human serum samples.

4.2. Off-line solid-phase extraction

A run cycle of about 11 min even with four analytes blocked the mass spectrometer for too long with just one task. Also at later stage of drug development analysis of mainly human plasma samples is required. Therefore, we replaced the column-switching method by an off-line SPE method, which allowed a more efficient use of the expensive triple stage mass spectrometer. The off-line SPE method was developed on a Zymark RapidTrace™ SPE robot. Overall run LC-MS-MS cycle time was reduced from 11 min to 4 min resulting in an approximately 2.7 fold increased sample throughput. The differences in retention times between the main method (A), column-switching (B), and off-line SPE (C) are depicted in Fig. 9. For the off-line SPE method (Fig. 9C) the hydroxy (II) and the hydroxy-phenol metabolite (IV) coeluted. Since the two analytes have different fragment ions cross-talk was not found to be critical.

The method was validated for human and rat plasma in the concentration range 1 to 2000 ng/ml for all four analytes. Inter-assay precision and inaccuracy did not exceed 20%. For most of the values precision and inaccuracy were in fact below 10% (3 out of 66 values were in the range 10 to 20%, data not shown). Although the peak height at the 1 ng/ml level was between 100 to 500 counts per second with a noise level of about 10 counts per second, attempts to lower the quantification limit to 0.5 ng/ml failed due to a significant deterioration in precision and accuracy. Inter-assay precision and inaccuracy derived from QC samples were below 10% for human and rat plasma. Extraction recoveries were determined identical to the main method at 50 ng/ml ($n=9$), and were found to be 92% for bosentan, 94% for the hydroxy metabolite (II), 90% for phenol metabolite (III), and 95% for the hydroxy-phenol metabolite (IV), respectively. The addition of triethylamine in the elution solution improved significantly the recovery of the analytes from the SPE cartridges. The RapidTrace SPE robot is particularly suitable for rapid method development and the

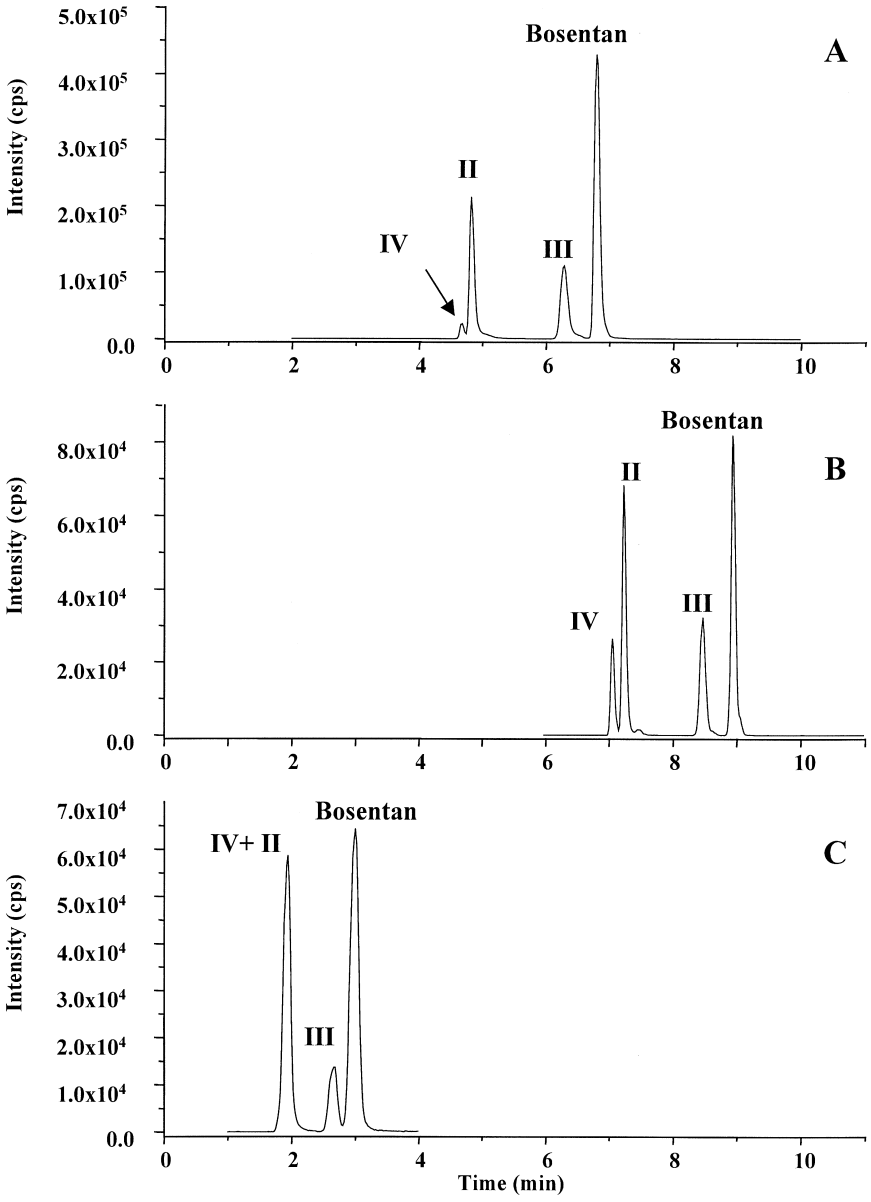

Fig. 9. Total ion chromatograms in SRM mode of calibration samples containing 200 ng/ml of bosentan and its metabolites processed with method A) LLE and column-switching B), column-switching and method C) off-line SPE.

analysis of small sample batches as occurring in on-line analytics rather than for large clinical trials. This assay can certainly easily be adapted to the 96-well plate format for high-throughput and automated sample processing using liquid handling devices such as Tecan Genesis or Multiprobe II.

4.3. Conclusions

Along with the drug development of bosentan for the treatment of hypertension and chronic heart failure, a drug assay was developed to support pharmacology and to follow the fate of the drug in a

variety of matrices and species originating from clinical as well as from non-clinical investigations. The assay was developed to quantify the drug as well as its three main metabolites and used a combination of liquid-liquid extraction and column switching LC-MS-MS. The assay used always human plasma calibration samples and was applied to plasma, liver and bile of men, rat and dog, whereas, QC samples were made up in human plasma as well as in the particular matrix of the unknown samples. The assay was simplified for application to plasma samples only, by replacing the liquid-liquid extraction by a more sophisticated column-switching step. Further, the instrumental analysis time was reduced by replacing the column switching step by off-line solid-phase extraction. Calibration ranges from 1 or 2 to 2000 or 10000 ng/ml using 0.25 ml sample aliquots were established, with values for precision and inaccuracy mostly below 10%.

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