ELSEVIER

Journal of Chromatography B, 769 (2002) 9–26

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Application of column-switching liquid chromatography-tandem mass spectrometry for the determination of pharmaceutical compounds in tissue samples

Katja Heinig*, Franz Bucheli

F. Hoffmann-La Roche Ltd., Pharmaceuticals Division, Non-clinical Drug Safety, Bioanalytical Section, Building 68/101, CH-4070 Basle, Switzerland

Received 20 September 2001; received in revised form 5 December 2001; accepted 5 December 2001

Abstract

Information on plasma-tissue distribution which is important for drug development may be obtained by ''in silico'' prediction tools. To support the validation of computer models, drug concentrations in rat plasma and tissues (brain, liver, kidney, testes, spleen, gut, lung, heart, muscle, skin and fat) had to be determined. In our work, we established analytical assays for a variety of substances including nicardipine, nitrendipine, felodipine and benzodiazepines. Sample preparation had to be simple and method development as well as analytical run time short to allow a high sample throughput and to minimize resources. Column-switching HPLC after homogenization and protein precipitation served as an efficient, easy and rapid sample preparation method, followed by selective MS–MS detection. Optimization of the trapping procedure was performed in order to reduce the influence of endogenous interferences and to obtain good recovery. Chromatographic separation was necessary to increase the selectivity. The use of small analytical column dimensions (2.1×10 mm) was investigated to achieve higher sample throughput without compromising the assay quality. Mass spectrometric parameters, such as ionization modes (positive vs. negative) and ion source types (TurboIonSpray vs. APCI) were screened to find suitable conditions for sensitive analysis of the compounds. Matrix suppression effects were taken into consideration. Calibration samples were prepared in plasma only, whereas quality control samples were prepared in both plasma and tissues to save animals and time. Accuracy and precision were in the range of 84.4–119.1% and 1–16.5%, respectively. Limits of quantification were in the range of 0.5–2.5 ng/ml for plasma and 2–10 ng/ml for tissues. Run times as short as 2.2 min could be achieved.

Keywords: Column-switching; Pharmaceuticals

1. Introduction

The knowledge about pharmacodynamic and pharmacokinetic behavior of substances in living organisms is essential in the drug development process. In order to reduce or replace animal experiments, various efforts are taken to develop computer models to simulate drug pharmacokinetics (adsorption, distribution, metabolism, elimination) in the body [1]. A physiologically-based pharmacokinetic model for prediction of drug distribution was established recently [2]. Using data on lipophilicity, protein binding and clearance it is possible to predict in vivo

*Corresponding author. Fax: +41-61-688-2908.
E-mail address: katja.heinig@roche.com (K. Heinig).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00600-6

drug concentrations in plasma and tissues and extrapolate these values to humans. However, it is necessary to validate the ''in silico'' tool before application using real animal data. For that purpose, rats were dosed intravenously with a variety of well-known pharmaceutical compounds, including nicardipine, felodipine, nitrendipine, oxazepam and lorazepam (Table 1). Plasma and tissues (brain, liver, kidney, testes, spleen, gut, lung, heart, muscle, skin and fat) needed to be analyzed to obtain drug concentrations for comparison with the simulated results.

Complex matrices, such as tissues and organs, require sample preparation methods to remove matrix components and therefore, to increase the sensitivity and selectivity. Literature describes a variety of laborious and time-consuming off-line procedures involving SPE and liquid–liquid extraction or, especially when using less selective detectors such as UV or single MS, a combination of clean-up methods. Although liquid chromatography–tandem mass spectrometry (LC–MS–MS) provides high selectivity and low detection limits for the determination of drugs in biological samples [3–7], it is often also necessary to remove matrix components which can cause signal suppression. Selective sample extraction is mostly preceded by homogenization with aqueous solutions or organic solvents (e.g. brain with methanol [6], brain and liver with acetone [7]). For the extraction of a narcotic drug from brain, SPE Bond Elute Certify MS–MS was used [8]. Liquid–liquid extraction was applied to pig muscle and kidney, followed by clean-up on an Oasis SPE cartridge and LC–MS–MS analysis [9]. Antibiotics were determined in bovine tissues (liver, kidney and muscle) using LC–MS–MS after combination of clean-up steps on ion-exchange cartridges [10]. Another method for antibiotics in tissues involved extraction in Tris buffer, protein precipitation with tungstate and clean-up on an Oasis SPE column before MS–MS detection [11]. Porcine muscle, liver, kidney, fat and skin were analyzed for levamisole using tandem MS with atmospheric pressure chemical ionization (APCI). Sample preparation consisted of extraction of the homogenates with hexane–isoamylalcohol and NAOH, acidification, back-extraction into aqueous medium and purification on SCX material [12]. Chicken muscle, liver and kidney were homogenized with acetonitrile/sodium sulfate, followed by clean-up on alumina and ion-exchange columns and LC–MS analysis [13]. Cocaine and metabolites were determined in rat placental and fetal tissue homogenates using extraction on silica and LC–MS–MS [14]. Microdialysis of liver for LC with fluorescence detection saved time compared to a laborious multi-step extraction method [15]. Automated accelerated solvent extraction (including defatting and extraction steps) and LC–MS–MS was described for corticosteroid residues in bovine liver [16]. On-line SPE using a column-switching system (with or without prior protein precipitation) is an automated, less time consuming approach for LC with UV, fluorescence, single MS or tandem MS detection [17–21]. Combination of off-line extraction of tissues and column switching was performed for LC with UV detection [22]. A column-switching system was applied for the LC fluorescence determination of D- and L-leucine in brain combining an ODS and a chiral column [6]. NMDA receptor blockers were determined in brain after homogenization in ethanol using column-switching HPLC with UV or fluorescence detection [23,24].

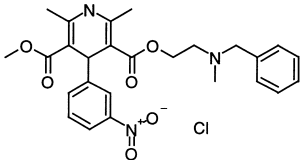
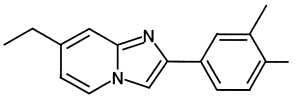
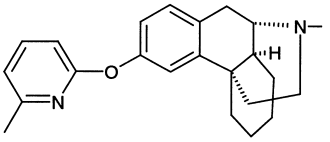
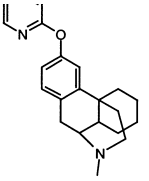
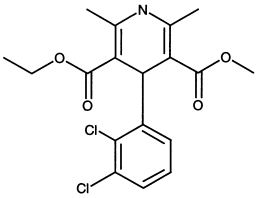
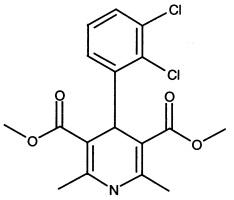
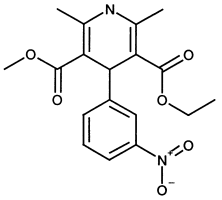
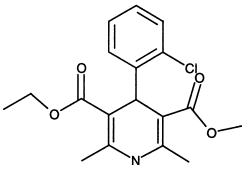
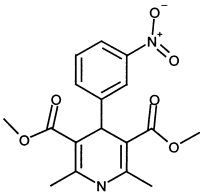
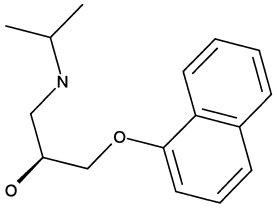
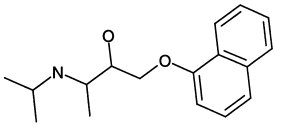
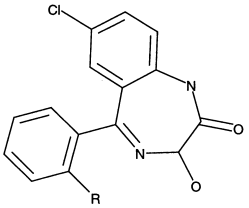
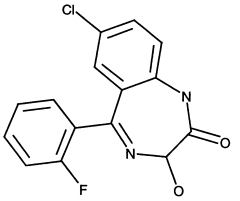
This paper deals with the development of analytical methods to determine nicardipine, felodipine, nitrendipine, Ro 24-6173, oxazepam, lorazepam and propranolol quantitatively in rat plasma and tissues. LC–MS–MS was chosen due to its sensitivity and selectivity, which is needed for complex sample matrices. On-line solid-phase extraction was applied using a column-switching HPLC system originally developed for LC–UV analyses [23,24] and established for a different LC–MS–MS method [25]. The authors show the suitability of this simple and robust technique for a variety of analytes and matrices. Method development details are presented, including the search for the optimum stationary phases and solvents to minimize matrix effects. Data on stability of the substances and recovery from different biological materials as well as accuracy and precision of the assays will be discussed.

2. Experimental

2.1. Chemicals and reagents

The analytes nicardipine, nitrendipine and felodipine were purchased from ICN Biomedicals

Table 1
Structures and MS–MS transitions of analytes and internal standards

Analytes		Corresponding internal standards	
Nicardipine *m/z* 480.3→315.2		ISTD 1 *m/z* 251.2→236.3	
Ro 24-6173 *m/z* 349.3→224.2		ISTD 2 *m/z* 335.3→210.2	
Felodipine *m/z* 384.1→338.1		ISTD 3 *m/z* 370.2→338.1	
Nitrendipine *m/z* 361.2→315.1 (positive) *m/z* 359.2→122.0 (negative)		ISTD 4A *m/z* 348.2→320.2 (positive)	ISTD 4B *m/z* 345.2→122.0 (neg.)
			
Propranolol *m/z* 260.2→116.1		ISTD 5 *m/z* 274.2→130.1	
Oxazepam (R = H) *m/z* 287.1→241.1 Lorazepam (R = Cl) *m/z* 321.1→275.1		ISTD 6 *m/z* 305.1→259.1	

(Aurora, OH, USA). Oxazepam, lorazepam, Ro 24-6173 and the internal standards were obtained from F. Hoffmann-La Roche (Basle, Switzerland). Acetonitrile (ACN) HPLC grade S was purchased from Rathburn (Walkerburn, UK). Methanol (MeOH), ethanol, acetic acid (HOAc), formic acid and ammonium acetate (NH_4Oac) p.a. were obtained from Merck (Darmstadt, Germany). All solutions were prepared using in-house generated doubly distilled water.

2.2. Solutions and standards

Ammonium acetate solution of 1 *M* was prepared by dissolving 77.08 g with distilled water to 1000 ml. Stock solutions of internal standards and analytes (500 μg/ml) were prepared in ethanol. Internal standard working solutions (10–100 ng/ml) were prepared by diluting stock solutions with acetonitrile/ethanol 50:50 (ISTD 3) or with pure ethanol. For dilution solutions (DS), the appropriate volumes of internal standard working solution and doubly distilled water were mixed. The analyte stock solutions were diluted with ethanol/water 70:30 to obtain spiking solutions. The solutions were stored at 4 °C and could be used for several months. Calibration standards in the range 1 to 500 or 1000 ng/ml were prepared by spiking the analytes to human plasma (volume of spiking solution <2% of matrix volume). Quality control (QC) samples at low (4 or 8 ng/ml), medium (40 ng/ml) and high (400 or 800 ng/ml) levels were prepared by spiking the matrix to be assayed.

2.3. Sample preparation (off-line)

Biological samples were thawed at room temperature together with calibration and QC samples. Tissue samples were homogenized with a threefold volume of water (exception: skin was homogenized with a ninefold amount) using a Heidolph RZR 2051 control stirrer with a glass piston and homogenizer (GlasKeller, Basle, CH). To aliquots of 200 μl (or less) sample the threefold volume of internal standard solution was added. After short mixing, the samples were stored for 5–10 min in the deep freezer at approximately −20 °C to obtain optimal protein precipitation. The samples were centrifuged for 6 min at 15 000 rpm (ca. 18 000 *g*) in a Heraeus Sepatech Megafuge 2.0 R with the temperature set to 10 °C. If the expected sample concentration exceeded the calibration range, the supernatant was diluted with dilution solution after centrifugation. High QC samples were treated the same to provide dilution QC samples.

2.4. Apparatus

2.4.1. HPLC system

An in-house assembled column-switching LC–MS–MS system (Merck-Hitachi, Tokyo, Japan) was used (Fig. 1). The L-6200 Intelligent Pump (P1)

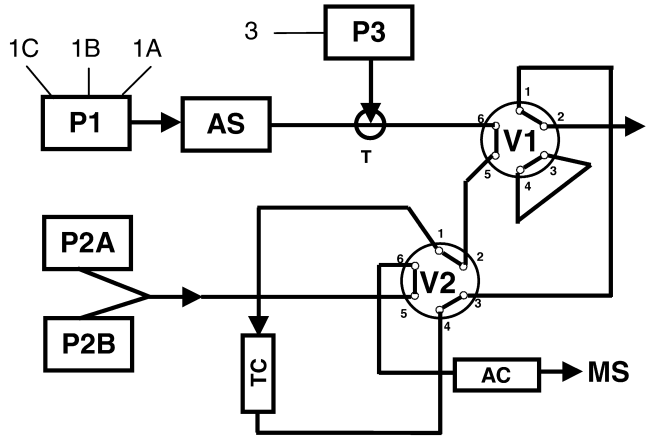


Fig. 1. Scheme of column-switching LC–MS–MS system.

with a low-pressure gradient module controlled the pump L-6000 (P3). The L-6200A Intelligent Pump (P2A) and pump L-6000A (P2B) operated in high-pressure gradient mode using a 75-μl dynamic mixer (Labsource, Reinach, Switzerland). The autosampler was a Merck AS 4000. The solvent degassing unit SDU 2003 and the switching valves V1 and V2 (High Speed Valve 7000E) were from Labsource. As trapping columns (TC) the Nucleosil 100-5 RP_{18} HD (8×4 mm, 5 μm) from Macherey-Nagel (Düren, Germany), a Lichrospher RP18 ADS (25×4 mm, 25 μm) from Merck or the XTerra™ RP18 (10×2.1 mm, 5 μm) from Waters (Milford, MA, USA) were employed. The analytical column (AC) was a Symmetry Shield RP_8 (50×2.1 mm, 3.5 μm) or XTerra MS™ C_{18} (50×2.1 mm or 10×2.1 mm, 3.5 μm) from Waters. The analytical columns were protected using a C_{18} 4×2 mm SecurityGuard™ cartridge (Phenomenex, Torrance, CA, USA). Solvent 1A composition and trapping flow rates are shown in Table 2. Solvent 1B consisted of water/ethanol 20:80 and solvent 1C of pure ethanol. Mobile phase compositions for elution and separation (2A and 2B) are also shown in Table 2. Flow rate on the analytical column was 0.4 ml/min.

2.4.2. Mass spectrometer

The API III^+ triple quadrupole mass spectrometer from PE Sciex (Concord, Ont., Canada) equipped with a TurboIonSpray™ or an APCI interface was used. Positive ion selected reaction monitoring mode (SRM) was applied, except for nitrendipine. Nitrogen served as nebulizer, auxiliary and curtain gas, argon as collision gas. Gas flow rates, temperatures, IonSpray voltages and collision energies were optimized for every compound by infusion of 1 ng/μl standard solutions in methanol/water/acetic acid 50:50:1 at 20 μl/min and by flow-injection analysis (FIA) at the LC flow rate. The MS–MS transitions for analytes and internal standards are listed in Table 1. Data acquisition was performed with dwell times of 100–150 ms. The quadrupoles Q1 and Q3 were operated at unit mass resolution (<0.7 Da peak width at half height). Calibration of mass axis and optimization of resolution was performed daily using a mixture of quaternary ammonium salts. The Tune software from PE Sciex was used for calibration and tuning, RAD served to control data acquisition. The integration of the LC–MS–MS chromatograms was performed using MacQuan.

2.5. Automatic trapping procedure

The column-switching system which has been described before for UV detection [23,24] was adapted to tandem mass spectrometric detection (Fig. 1). The autosampler (AS) loads the sample, waits for the MS ready signal, injects the sample and starts afterwards the program of pump P1 and MS–MS data acquisition. P1 controls the flow of pump P3, the two switching valves V1 and V2 and gives the start signal to pump P2A. At the beginning of the trapping program, valves 1 and 2 are switched to the initial positions (TC to waste, forward flush). P1 serves for sample transfer and flushing of the trapping column (solvent 1A). P3 is used for on-line dilution of the injection solution with solvent 1A. During sample trapping and rinsing the analytical column is equilibrated with the initial mobile phase using pumps P2A and P2B. Then V1 is switched, and the trapping column is rinsed for 0.1 min in back-flush mode with solvent 1A, followed by switching of valve V2 and isocratic elution of the analytes using pumps P2A and B. Trapping column and analytical column are decoupled (switch V2) as soon as the analytes are transferred onto the analytical column. P1 rinses the tubing and, after elution, the trapping column in back-flush mode with solvents 1B (water/ethanol 20:80) and 1C (100% ethanol), respectively. The analytes are separated on the analytical column using a high-pressure gradient program. The trapping column is reequilibrated with solvent 1A using P1 (switch V1 to forward flush). Pumps P2A and P2B reestablish the initial mobile phase composition shortly before the chromatographic run is finished. In the meantime, the autosampler loads the next sample and waits for injection.

The general trapping procedure was similar for all compounds. Depending on chromatographic behavior and MS response, the injection volume, TC material, transfer/dilution flow, transfer and rinsing time and solvents as well as elution time and solvents were varied. Table 2 shows the optimized parameters for all analytes.

Table 2
Analysis conditions for all analytes

	Inj. vol.	Trapping column (TC)	Analytical column (AC)	Trapping	Elution	Run time
Nicardipine	10 μl	Nucleosil C_{18} HD 4×8 mm	XTerra MS C_{18} 2.1×50 mm	20 m*M* NH_4OAC+2% ACN+2% HOAc (0.5 min), P1/P3 2/2 ml/min	20% 2B* 0.6 min, to 85% 2B* within 1.3 min	2.7 min
Ro 24-6173	50 μl	Nucleosil C_{18} HD 4×8 mm	SymmetryShield RP8 2.1×50 mm	20 m*M* NH_4OAC+2% ACN+2% HOAc (1 min), P1/P3 0.5/2 ml/min	100% 2A 0.8 min, to 70% 2B within 1.4 min	3.5 min
Felodipine	150 μl	Lichrospher RP18 ADS 4×25 mm, 25 μm	SymmetryShield RP8 2.1×50 mm	20 m*M* NH_4OAC+10% MeOH (1 min), P1/P3 1/5 ml/min	40% 2B 1.2 min, to 100% 2B within 1.2 min	3.8 min
Nitrendipine (positive)	100 μl	Lichrospher RP18 ADS 4×25 mm, 25 μm	SymmetryShield RP8 2.1×50 mm	20 m*M* NH_4OAC+10% MeOH (1 min), P1/P3 1/5 ml/min	40% 2B 0.8 min, to 100% 2B within 1.3 min	3.5 min
(negative)	10 μl	XTerra RP18 2.1×10 mm	XTerra MS C_{18} 2.1×50 mm	20 m*M* NH_4OAC+2% ACN (0.5 min), P1/P3 0.5/3 ml/min	20% 2B** 0.6 min, to 80% 2B** within 0.8 min	2.5 min
Oxazepam, Lorazepam	40 μl	XTerra RP18 2.1×10 mm	XTerra RP18 2.1×10 mm	20 m*M* NH_4OAC+2% ACN (0.5 min), P1/P3 0.5/3 ml/min	100% 2A 0.6 min, to 80% 2B within 0.8 min	2.2 min
Propranolol	20 μl 40 μl (APCI)	XTerra RP18 2.1×10 mm	XTerra MS C_{18} 2.1×50 mm	15 m*M* NH_4OAC (0.5 min), P1/P3 0.5/3 ml/min	100% 2A 0.6 min, to 100% $2B^+$ within 0.8 min	2.3 min

2A, 15 m*M* NH_4OAC+23% ACN+1% HOAc; 2B, 5 m*M* NH_4OAC+45% ACN+45% MeOH+1% HOAc; 2B*, 5 m*M* NH_4OAC+35% ACN+35% MeOH+1% HOAc; 2B**, 5 m*M* NH_4OAC+85% ACN+0.5% HOAc; $2B^+$, 5 m*M* NH_4OAC+45% ACN+45% MeOH+0.5% formic acid.

2.6. Quantitation and validation

The calibration curves were established by linear least-squares regression ($1/x^2$ weighting) from peak area ratios (analyte/internal standard) versus nominal concentrations. Six to seven calibration standards were prepared. Validation of the detection limits (lower and upper limit) was performed by analyzing sets of calibration standards in duplicate and using the second set as quality control on 3–5 different days. The results were reported as inter-assay precision and accuracy. The validation was successful if the samples were within 80–120% of their nominal values, with a coefficient of variation less than 20%. Quality control samples, prepared in plasma and 11 tissues at three levels, were analyzed ($n=3$–6) and calculated against one set of calibration standards to obtain data on intra-assay precision and accuracy.

During unknown analysis, one standard was allowed to be outside the validity criterion and could be excluded from the calculation. Concentrations of unknown samples were accepted only within the calibration range. At least six QC samples (two at each level) were analyzed with every matrix. The data for unknown samples were valid if at least two-thirds of the QC samples deviated by less than 20% from the nominal values and the precision did not exceed 20% RSD.

3. Results and discussion

LC–MS–MS methods were developed for the determination of nicardipine, nitrendipine, felodipine, Ro 24-6173, oxazepam, lorazepam and propranolol (Table 1) in plasma and tissues. Due to the relatively small number of samples per compound (≈200) but many different matrices (plasma and 11 tissues) it was not the goal to perform extensive method optimization but to provide sample analysis with sufficient accuracy, precision and robustness of the assays. It was preferred to apply the same sample preparation and analysis technique for all compounds and matrices with minor variations to save method development time and to allow a rapid switching from one assay to another. The required limit of quantification was 1 ng/ml for plasma and tissue homogenates. The run times should be kept as short as possible without compromising the quality of the assay. Because the homogenization of tissues is very time consuming, the further sample preparation should be fast and simple. Direct sample injection after protein precipitation was not considered a rugged approach due to the complex matrices. Therefore, automated column switching was performed. Preparation of calibration samples in human plasma was preferable to preparation of calibrations in each animal tissue. Human plasma is readily available, and the number of animals to be sacrificed for obtaining blank material can be reduced. For that purpose, the recovery from plasma should be the same as from tissue, or at least an internal standard should compensate the matrix differences. Method development consisted of selection of ionization mode (positive/negative) and ion source (TurboIonSpray/APCI) as well as optimization of trapping program (including trapping column) and gradient program on the analytical column. Table 2 summarizes the final assay conditions for all analytes. Fig. 2 shows typical LC–MS–MS chromatograms (selected ion current profiles) for nicardipine, including LOQ standard, blank fat, low QC in fat and internal standard ISTD 1 in fat. No interferences were observed in blank materials. Fig. 3 presents the LC–MS–MS chromatograms for the compound Ro 24-6173 in plasma and liver blanks and in plasma and liver study samples near LOQ with the corresponding internal standard ISTD 2. These examples are representative for all other analytes. Because tandem MS is a very selective detection technique, the only peaks present in the chromatograms were the analyte and internal standard. The chromatograms of blanks, LOQ, QC and study samples for the other analytes look similar and are not shown. Assay performance data are presented in Tables 3–5. Inter-assay accuracy and precision of calibration samples were in the range of 85–110% and 3–15%, respectively. Mean accuracy and precision for quality control samples in plasma and tissues were in the ranges of 84.4–119% and 1–16.5%, respectively. Method development strategies and performance details are discussed in the following sections.

3.1. Internal standard selection

Isotopically-labeled compounds were not avail-

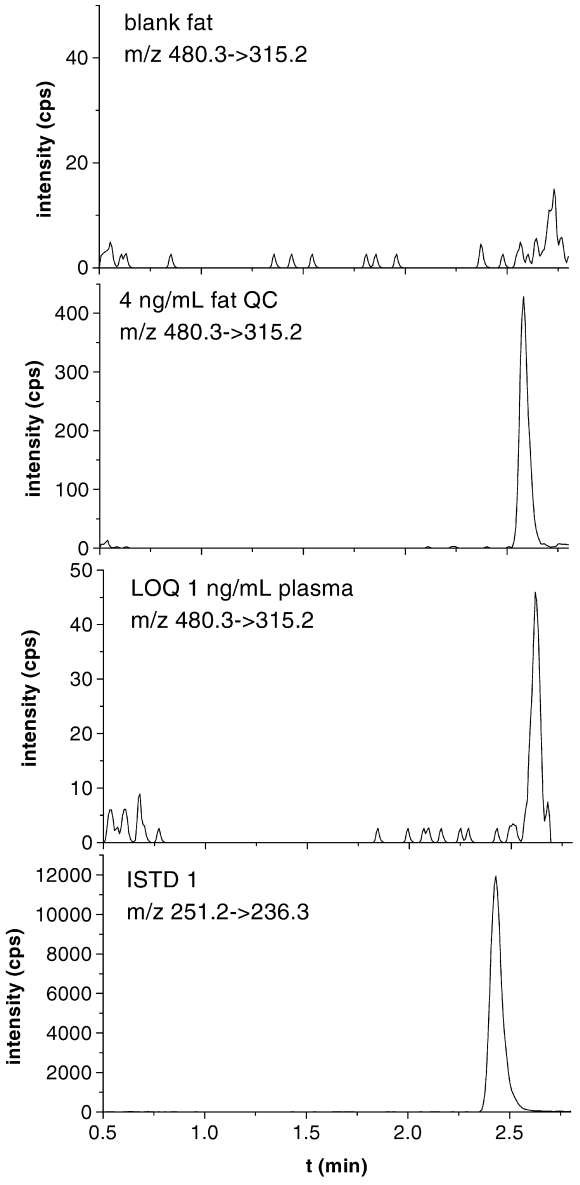


Fig. 2. LC–MS–MS chromatograms of nicardipine: 4 ng/ml QC in fat, ISTD 1 in fat, blank fat and LOQ 1 ng/ml in plasma.

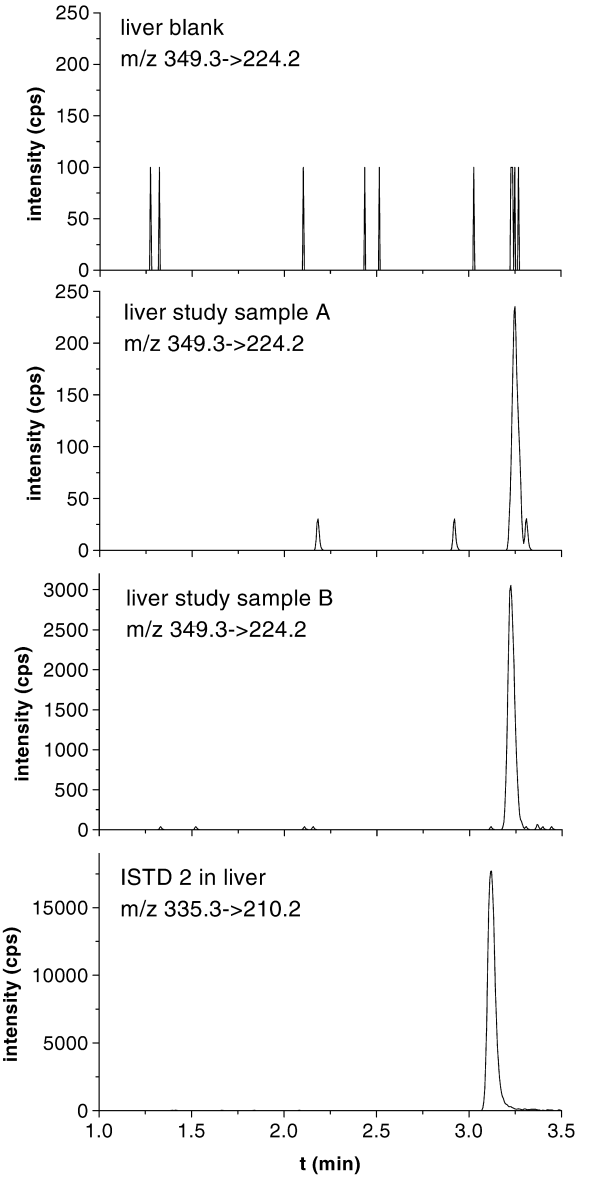


Fig. 3. LC–MS–MS chromatograms of Ro 24-6173 in liver blank and study samples; (A) low concentration (near LOQ), (B) higher concentration with corresponding internal standard ISTD 2.

able. Therefore, structural analogs were screened to find suitable compounds with similar chromatographic behavior which could correct for variations during analysis including suppression effects (Table 1 for finally used substances). No structurally related compound was available as internal standard for nicardipine. Other substances from the same class showed different retention behavior. Therefore, a substance with different structure but comparable chromatography was used (ISTD 1). Variations in signal intensity in the different tissues were successfully corrected by the internal standard, leading to a good precision and accuracy. Ro 24-6173 was analyzed applying a compound with similar structure

Table 3
Inter-assay accuracy and precision (%) of calibration samples

	Nicardipine	Ro 24-6173	Felodipine	Nitrendipine	Oxazepam	Lorazepam	Propranolol
1	103.5/10.2	100.1/12.4	95.6/7.9	96.7/10.9	102.5/9.0	–	98.8/9.6
2.5	103.7/10.7	95.5/14.6	103.5/9.7	105.1/10.8	104.2/3.4	100.2/13.2	99.7/6.0
10	106.6/8.6	108.4/10.1	100.6/10.3	109.7/7.1	95.9/6.9	95.0/10.0	103.3/5.9
50	107.5/8.7	112.1/5.5	101.5/1.8	108.4/8.5	102.0/5.8	96.7/8.4	98.0/9.0
200	105.3/7.8	97.8/9.7	101.5/3.4	105.2/8.2	103.7/3.2	102.8/3.2	108.7/4.4
500	102.8/12.4	84.6/3.5	100.6/4.1	92.7/12.1	100.1/4.4	98.6/3.5	87.7/10.4
1000	87.1/4.9	–	90.9/4.7	–	101.3/7.1	98.6/11.0	–

(ISTD 2, lack of the methyl group at the pyridine) which has been used before as internal standard in LC–UV analysis of the test substance. For felodipine, several compounds including nicardipine, nitrendipine, substances ISTD 3 and ISTD 4A were screened for applicability as internal standard, while ISTD 3 showed the closest chromatographic behavior. Due to the lack of one methyl group compared to felodipine it eluted earlier. Crosstalk between analyte and ISTD 3 due to the same monitored fragment ion was observed even when adding a dummy transition, making complete chromatographic separation necessary. Fig. 4 shows that an analyte peak appeared in the m/z trace of the internal standard, if it was present at high concentrations. A small peak for the internal standard showed in the selected ion current profile for the analyte. Nitrendipine was determined in positive mode using the compound ISTD 4A as internal standard. ISTD 4B served as internal standard in negative mode. The 2-fluorophenyl derivative of oxazepam (ISTD 5) was used as internal standard for both benzodiazepines. Methylated lorazepam was tested as internal standard for lorazepam, but the precision and accuracy were improved when calculating the area ratios using the fluorinated oxazepam. Several compounds were investigated as internal standards for propranolol. Substances with an additional methyl group or lacking a methyl group showed similar elution behavior than propranolol (slightly shorter or longer retention time). However, these substances contained a significant amount of the analyte as impurity. ISTD 6 was selected as internal standard because it contained the lowest concentration of propranolol and was used at a low concentration (10 ng/ml) to minimize analyte crosstalk.

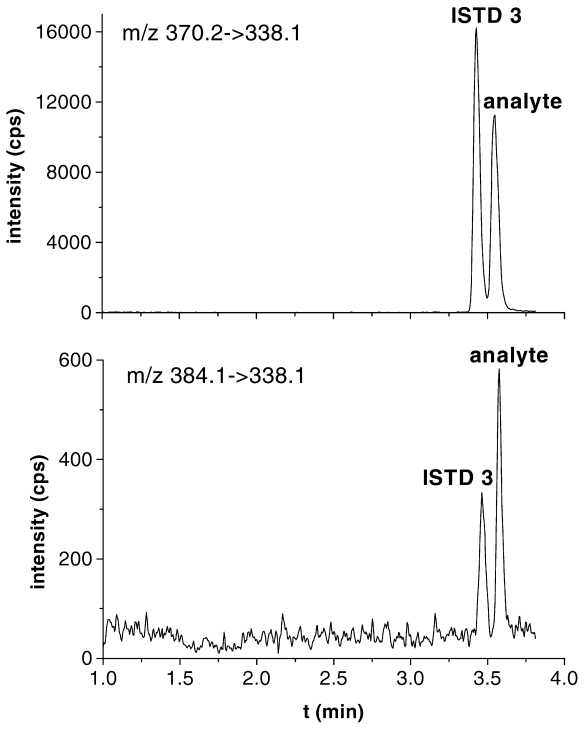


Fig. 4. Felodipine LC–MS–MS chromatograms: fat sample at ISTD 3 transition (m/z 370.2→338.1) and fat QC 4 ng/ml at analyte transition (m/z 384.1→338.1).

3.2. MS conditions

The majority of the analytes showed good response in positive ionization mode. The application of positive as well as negative ionization mode was possible for nitrendipine and nicardipine. Positive ionization mode was chosen for the analysis of nicardipine samples because the sensitivity was

Table 4
Intra-assay accuracy of QC samples (%)

	Nicardipine			Felodipine			Nitrendipine			Ro 24-6173			Oxazepam			Lorazepam			Propranolol		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Plasma	111.9	116.7	111.6	98.7	86.9	89.5	98.5	97.2	89.3	107.4	113.7	88.9	100.1	96.7	100.3	98.4	97.4	96.3	112.8	115.3	89.0
Brain	111.2	113.9	99.6	112.9	104	93.9	100.7	92.4	82.1	108.8	99.3	87.9	103.2	98.3	104.6	107.2	100.5	101.5	92.1	113.5	94.5
Fat	91.4	102.3	113.2	111.9	115.6	112	101.3	90.3	88.7	107	109.4	80.7	89.9	84.1	116.9	91.0	85.0	112.3	109.0	104.0	88.4
Gut	106.8	110.4	111.2	103.8	103.6	96.1	104.6	114.0	95.2	107.8	102.5	77.5	108	111.5	103.4	115.4	106.6	105.6	89.7	96.2	101.6
Heart	101.4	101.2	116.3	119	117.1	94.2	110.6	106.7	95.4	119.3	111.4	87.3	101.4	111.7	110.2	113.8	111.3	113.3	116.5	111.6	88.5
Kidney	113.4	98.8	91.6	99.5	109.8	108.9	95.3	90.8	81.2	99.5	117.1	80.2	99	106.8	106.8	101.8	116.7	97.1	94.3	105.6	97.7
Liver	91.2	104	97	111.5	97.1	108	113.0	114.3	96.4	91.5	119.1	86.5	93.4	101.7	98.8	111.8	97.9	106.1	98.4	102.5	96.2
Lung	111.2	107.5	96.4	94.9	88.9	85.6	97.2	90.4	86.5	115.1	119.2	92.8	92	99	106	106.0	98.9	111.1	117.0	105.7	98.1
Muscle	93.8	92.4	89.2	102.2	104.6	101.2	118.2	116.2	103.6	110	119.2	90.2	112.7	106.5	103.7	117.1	112.0	106.4	117.0	110.7	92.3
Skin	104.6	98.8	112.3	115.3	109.7	88.9	112.7	114.4	99.2	116.8	110.3	86.9	112.8	112.3	98.4	109.3	102.5	103.5	117.9	116.3	90.8
Spleen	89.8	–	103.3	108.2	109.7	107.8	90.3	93.2	87.2	106.4	93.5	83.8	109.6	97.7	112.2	113.5	101.9	113.1	85.7	97.5	99.8
Testes	115.6	94.3	95.9	100.3	109.5	98	100.7	99.7	86.2	104.2	111.3	98.6	107.6	103.9	105.9	129.6	110.5	104.2	118.3	119.1	85.4

Table 5
Intra-assay precision of QC samples (%)

	Nicardipine			Felodipine			Nitrendipine			Ro 24-6173			Oxazepam			Lorazepam			Propranolol		
	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High
Plasma	8.3	4.3	6.3	14.0	7.3	14.4	9.9	4.7	9.2	10.2	5.4	3.5	6.91	5.4	5.9	10.1	7.3	9.8	3.8	3.3	5.9
Brain	10.5	5.2	2.5	12.6	7.2	9.1	10.7	1.9	6.5	15.1	3.1	13.1	3.12	5.4	6.3	6.9	7.0	9.6	4.0	2.3	13.0
Fat	15.3	4.7	6.7	9.1	5.4	5.4	5.2	8.3	9.7	6.3	11.1	5.2	5.3	9.0	8.0	13.6	4.7	3.8	5.8	15.4	6.0
Gut	1.7	7.4	6.9	14.5	14.6	13.5	6.1	4.4	5.6	5.1	13.3	8.1	14.2	6.3	10.7	3.8	6.1	3.1	1.4	5.1	6.0
Heart	7.9	12.8	4.4	0.5	3.7	5.3	2.0	0.4	8.1	4.4	9.6	9.0	7.4	2.8	1.5	2.4	4.6	2.1	2.9	5.6	5.3
Kidney	0.5	4.3	7.3	27.1	6.8	9.1	2.8	5.4	1.9	10.5	2.0	3.6	8.4	5.0	10.6	5.9	2.9	10.4	5.7	3.7	8.3
Liver	5.3	8.9	9.9	9.8	23.3	3.8	13.8	5.1	13.2	19.5	9.2	11.7	6.9	10.0	5.2	8.8	10.1	15.8	7.7	7.5	15.5
Lung	7.5	6.2	11.4	6.8	13	2.2	7.4	4.8	4.3	7.9	2.6	8.2	7.9	12.3	8.2	13.3	14.4	10.6	4.8	14.6	7.1
Muscle	9.7	14.2	8.9	9.4	13.5	14.1	0.6	2.5	3.0	10.5	2.0	21.3	4.3	2.8	10.7	4.7	2.8	8.7	1.5	6.6	6.5
Skin	7.8	10.7	11.5	11.4	10.9	8.8	5.0	8.2	0.7	15.1	10.6	3.7	14.5	15.3	10.3	12.5	14.4	8.1	1.8	6.7	11.8
Spleen	10.9	–	6.2	6.2	6.6	11.0	7.3	3.7	0.6	12.1	14.6	1.3	10.3	11.7	12.5	2.9	11.8	14.8	5.4	11.0	10.1
Testes	5.5	4.1	5.4	25.4	13.4	9.9	6.5	5.7	5.2	8.3	7.0	7.8	9.4	2.1	2.2	7.4	2.6	4.4	0.2	0.2	3.7

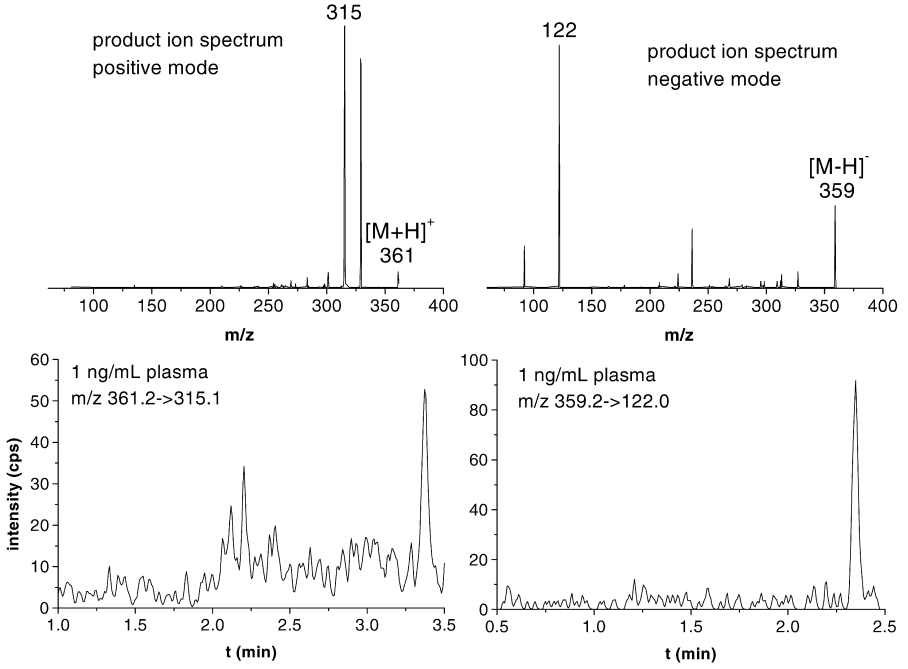


Fig. 5. Comparison of positive and negative SRM mode for nitrendipine: product ion spectra, 1 ng/ml plasma in positive mode at m/z 361.2→315.1 (injection of 100 μl) and negative mode at m/z 359.2→122.0 (injection of 10 μl).

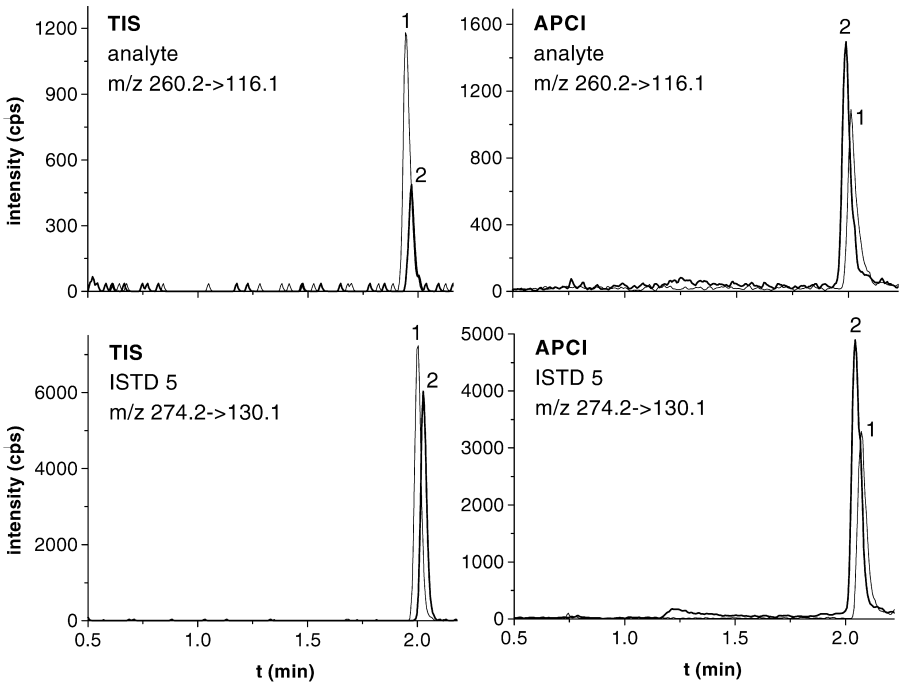


Fig. 6. TurboIonSpray (TIS) and APCI LC–MS–MS chromatograms of propranolol QC samples in 80 ng/ml 1:10 diluted gut (1) and 8 ng/ml undiluted gut samples (2) with corresponding internal standard peaks (ISTD 5).

comparable in either mode. Both possibilities were tested for nitrendipine during method development. Fig. 5 shows the product ion spectra for the ions at m/z 361 ($[M+H]^+$) and m/z 359 ($[M\text{-}H]^-$) and the LC–MS–MS chromatograms for nitrendipine in plasma at LOQ 1 ng/ml in positive and negative SRM mode. The response was better in negative than in positive mode. An injection volume of only 10 μl for negative SRM versus 100 μl in positive mode was required to achieve the 1 ng/ml LOQ. Therefore, study samples were analyzed for nitrendipine using negative SRM. To show the feasibility of single MS determinations, SIM experiments were performed for the analysis of benzodiazepines in different matrices (plasma, gut, liver, skin, testes, heart, muscle) and compared to the SRM mode. No interfering peaks were observed in blanks and spiked samples at 40 ng/ml (data not shown). The signal-to-noise in SIM mode was lower than in SRM mode leading to a higher LOQ of about 10 ng/ml. Nevertheless, it may be possible to perform the analyses using an LC–MS because the analyte concentrations exceeded 10 mg/ml in tissues. Atmospheric pressure chemical ionization (APCI) was not applied in the analysis of study samples due to lower sensitivity and peak tailing caused by the design of the API III$^+$ heated nebulizer probe. A comparison of APCI and TurboIonSpray was performed using propranolol as example. The sensitivity was lowered by a factor of 2–3 in APCI; therefore, the injection volume was increased by a factor of two. APCI did not suffer from matrix influences as strongly as TurboIonSpray. This could be demonstrated for the determination of propranolol in gut samples (Fig. 6). TurboIonSpray analysis resulted in decreased peak areas of analyte and ISTD 5 in undiluted versus diluted gut, while in APCI no decrease in peak areas was observed. The data are further discussed under matrix effects.

3.3. Off-line sample preparation

Homogenization of tissues was performed after addition of the threefold amount of water. Homogenization with organic solvents did not result in better recovery. Skin was homogenized with the ninefold amount to reduce viscosity. The plasma samples and tissue homogenates were diluted with the 2.5- to 3-fold volume of internal standard solution in ethanol. The threefold amount was preferred due to better accuracy of tissue samples. Ethanol was the best choice for protein precipitation regarding recovery of the analytes investigated and compactness of the precipitate. Nevertheless, felodipine samples were precipitated with a mixture of ethanol and acetonitrile 50:50 because the chromatographic peak shape was improved in this case. Samples containing analyte concentrations above the calibration range were diluted with water/internal standard solution. The accuracy was monitored through the analysis of diluted high QC samples.

3.4. Column-switching methods

3.4.1. Trapping procedure

The centrifuged samples were injected into a column-switching system for further clean-up. Table 2 presents the trapping conditions for each analyte. To avoid breakthrough on the trapping column, on-line dilution of the sample with aqueous solution was necessary using the pump P3 and a T-piece. Thus, an additional off-line step was omitted and higher recovery of less polar substances was achieved. Optimization of trapping program and gradient program on the analytical columns was carried out. The length of the trapping procedure was adapted to the size of the injection loop. An injection volume of only 10–40 μl into a 20- or 50-μl loop was sufficient for substances with good MS response, resulting in a short trapping time of 0.5 min due to the small rinsing volume needed. However, a prolonged trapping and washing time may improve the reproducibility in some cases, e.g. for Ro 24-6173, where a 1-min trapping was performed for better precision. Larger injection volumes, e.g. 150 μl for felodipine, required a longer sample application and rinsing time.

Flow rates for automated on-line dilution were optimized as well as the composition of solvents. Aqueous ammonium acetate containing small amounts of organic solvents and/or acetic acid served as solvent for trapping (loading, rinsing, dilution). A content of 2% acetonitrile in the solvent 1A was advantageous for matrix removal, better reproducibility, lower plasma-tissue differences and did not lead to breakthrough for most analytes. For nicardipine and Ro 24-6173, the differences in the

elution profiles of plasma and tissues were minimized when using 2% acetic acid in addition to acetonitrile in the trapping and dilution solvents, while rinsing without acid led to broader elution peaks, decreased reproducibility and lower accuracy of tissue QC samples. The other analytes showed better results regarding recovery and matrix effects if solvent 1A did not contain acid. A content of 10% MeOH or ACN in the trapping solvent 1A was well suited for felodipine to rinse off matrix components more effectively to reduce the differences between area ratios in plasma and tissues. Because of the strong retention of felodipine, partial breakthrough was observed only with an acetonitrile content above 15%. In contrast, propranolol showed a partial breakthrough with a content of 2% acetonitrile in the rinsing solvent 1A. Therefore, completely aqueous trapping using 15 m*M* ammonium acetate was performed.

Optimization of the relation between injection and dilution flow (pumps P1/P3) was carried out for each analyte to obtain the best signal intensity. For polar analytes, the dilution flow was four to six times higher than the injection flow. A high ratio of P3/P1 flow rates resulted not only in an improved recovery but also had a positive effect on the elution peak shape. It was of advantage to perform the sample injection with a comparatively low flow of 0.5–1 ml/min on P1 to obtain better recovery and reproducibility than using higher flow rates. The peak intensity of less polar analytes, such as felodipine, was not significantly influenced by a changed dilution flow.

Backflush rinse of the trapping column for 0.1 min before elution was carried out to remove particles and increase the lifetime of the column. After analyte elution and decoupling from the analytical column, the trapping column was rinsed with ethanol/water and pure ethanol to remove polar and less polar matrix compounds and to reduce carryover. The rinsing time of the trapping column was adapted to the chromatographic run time to minimize cycle times.

3.4.2. Trapping column selection

Various column materials and dimensions were investigated. All trapping columns were tested by analyzing spiked water, plasma, and some selected tissues (mostly brain) to assess plasma/tissue differences and reproducibility. Table 2 shows the trapping columns applied for the assays. Samples from the first two studies (determination of nicardipine and Ro 24-6173) were analyzed using a Nucleosil 100-5 C_{18} HD 4×8 mm, 5 μm column because it had been applied successfully in previous assays [25]. A narrow elution profile and only small differences between peak area ratios in plasma and tissues were achieved. However, the number of injections onto the Nucleosil C_{18} HD trapping column was limited by the increasing back-pressure and breakthrough after about 250 injections. The use of a smaller column volume (Nucleosil C_{18} 3×8 mm, 5 μm) led to narrower elution peaks and the possibility to save rinsing time, but the significant pressure increase after 30 injections made this column not useful for routine analysis. During the further development of methods an XTerra RP18 2.1×10 mm, 5 μm column was tested. A comparison of the XTerra with the Nucleosil C_{18} HD 4×8 mm resulted in good trapping behavior also for polar compounds and a narrower elution peak for the XTerra column. Sample application and elution of the analytes could be completed within a shorter time due to the smaller column volume. Furthermore, a significantly higher number of injections (up to 1000) was possible without pressure increase. Therefore, the XTerra trapping column was applied for the analysis of oxazepam, lorazepam, nitrendipine (negative SRM) and propranolol in rat plasma and tissue samples. Additional trapping columns were tested in case of a possible improvement of trapping. With the Oasis HLB 2.1×20 mm column (tested for Ro 24-6173) the pressure was significantly reduced due to the large particles of 25 μm, but this trapping column showed insufficient reproducibility even with variation of rinsing conditions, elution and gradient program and was not applied for routine work. The Symmetry C_{18} and XTerra MS C_{18} columns (2.1×10 mm, 3.5 and 5 μm) reduce the breakthrough for polar compounds, but no significant advantage compared to the XTerra RP18 2.1×10 mm column was achieved for our analytes. The Lichrospher RP Select B 4×4 mm, 5 μm has a lower capacity but was suited for less polar compounds and small injection volumes. The Kromasil RP18 3×8 mm, 5 μm column showed a similar trapping and elution be-

havior as the Nucleosil C_{18} HD column with no advantage compared to the XTerra RP18 column. More extensive method development was necessary for the determination of felodipine as well as for positive mode SRM analysis of nitrendipine in tissues due to matrix influences. Trapping column and method selection strategies for these compounds will be discussed in the following chapters.

3.4.2.1. Felodipine. Trapping parameters for felodipine were first optimized for the Nucleosil C_{18} HD 4×8 mm, 5 μm trapping column. Due to the relatively low MS response of the substance, the conditions had to be optimized for a larger injection volume and loop; 150 μl sample (and therefore, a relatively large volume of matrix) had to be injected to achieve the required LOQ of 1 ng/ml, leading to recovery differences between plasma and tissues. Furthermore, peak area decrease during repeated injections of brain was observed. After two to three runs with injection of water the trapping column was reconditioned, but extensive rinsing with organic and aqueous solvents in between injections did not result in the same effect. Other TCs were tested to overcome the problem. Lichrospher RP Select B 4×4 mm or Kromasil RP18 3×8 mm (5 μm) showed the same trapping and elution behavior as the Nucleosil column including the peak area decrease in tissues. The 25-μm particle Lichrospher RP18 ADS 4×25 mm, not suited for a variety of analytes because of a very flat elution profile, gave for felodipine reproducible peak areas of the substance in tissue samples and no decrease in recovery after several injections of brain. Therefore, this trapping column was used for further analyses.

3.4.2.2. Nitrendipine. The trapping column selection and method optimization for nitrendipine depended on the ionization mode. The injection of 100 μl sample was required to detect 1 ng/ml in positive mode SRM. The columns Nucleosil C_{18} HD and XTerra RP18 were not suited due to matrix effects. Considerable differences in recovery/matrix suppression were obtained for plasma and several tissues, especially gut and spleen which could not be compensated by the internal standard. Because trapping on the Lichrospher RP18 ADS 4×25 mm (25 μm) column was well suited to overcome plasma/tissue differences for felodipine, the same method was applied to nitrendipine with success. The required LOQ was reached in positive mode with no serious matrix suppression. In negative mode SRM, the injection volume could be reduced to only 10 μl due to the increased sensitivity, which led to a fast sample transfer and rinsing time. The XTerra RP18 2.1×10 mm, 5 μm column with its narrow elution profile and small dimensions was applied because less matrix effects occurred with the low sample volume and possibly also due to the negative ionization mode. Shorter washing was necessary leading to a reduced run time of 2.5 min compared to 3.5 min for analysis in positive mode. Data from Table 2 and Fig. 5 show the comparison of nitrendipine determination in positive and negative mode.

3.4.3. Elution from trapping column

The elution profile from the trapping column using different solvents was investigated with the goal to minimize the differences between peak area ratios in plasma and tissues. Trapping and analytical column were separated right after elution of the analytes to avoid the transfer of later eluting matrix compounds. Various mobile phase compositions for elution from the trapping column were tested to obtain good recovery and avoid elution of interferences, while the elution window of the substance should be minimized to save time. For elution of polar analytes (Ro 24-6173, propranolol, benzodiazepines), 100% of solvent 2A containing only 23% acetonitrile was sufficient. For less polar analytes (nicardipine, felodipine, nitrendipine), a higher organic solvent content was required for a narrow elution window. For that purpose, an appropriate content of solvent 2B was mixed with 2A. Table 2 summarizes the elution conditions for all analytes. The gradient program was usually started after isocratic elution of the substances to allow independent optimization of elution from trapping column and separation on the analytical column.

3.4.4. Mobile phases for gradient separation

After optimizing elution of the substances, the conditions on the analytical column were investigated to obtain good peak shape, sufficient sensitivity and short retention times. Fast gradient programs were applied (Table 2). Nicardipine, the first sub-

stance analyzed, was determined using mobile phase 2B* containing acetonitrile/methanol 35%/35%. Mobile phase 2B was changed for Ro 24-6173, felodipine and benzodiazepines to a higher organic solvent content (acetonitrile/methanol 45%/45%) to allow faster gradient separation and to provide the possibility to rinse off non-polar matrix components from the analytical column in between runs. For nitrendipine determinations in negative mode, solvent 2B was changed again to 5 m*M* ammonium acetate in acetonitrile/water 85:15 containing 0.5% acetic acid (2B**) due to shorter retention time (reduced by 0.4 min) and better sensitivity while maintaining the same peak shape and selectivity. It could be shown that a small content of acid in the mobile phase was of advantage for peak shape and even sensitivity despite the negative mode. Mobile phase $2B^+$ for propranolol gradient separation contained formic acid instead of acetic acid due to higher sensitivity. Another modification for propranolol was the reduced flow rate of 0.3 ml/min after 1.9 min.

3.4.5. Analytical column

Different analytical columns were used for the various analytes (Table 2). The XTerra MS C_{18} 2.1×50 mm, 3.5 μm was well suited for most analytes leading to the possibility to switch easily between the methods when required. A better peak shape was obtained for a variety of substances than with the SymmetryShield 2.1×50 mm, 3.5 μm column. Higher peaks than using short XTerra columns (2.1×10 or 20 mm, 3.5 or 2.5 μm) were observed. However, benzodiazepines were assayed using the 2.1×10 mm XTerra RP18. The SymmetryShield 2.1×50 mm analytical column was used for all felodipine experiments because it gave the same peak shape and sensitivity as the XTerra MS C_{18} 2.1×50 mm, 3.5 μm column but slightly shorter retention times leading to a run time of 3.8 min.

3.4.6. Application of short analytical column for benzodiazepines

To push the chromatography towards faster run times, the 2.1×10 mm XTerra RP18 3.5 μm was tested as analytical column for benzodiazepines and compared to the SymmetryShield 2.1×50 mm column. The gradient was optimized to achieve good peak shape on the short column. The trapping program had to be adapted to achieve the same run time as the chromatography. Re-equilibration of trapping column and analytical column had to be guaranteed. Whereas the gradient on the 50 mm AC started after elution of the substances from the trapping column, the gradient on the 10 mm AC was started 0.2 min before separation of TC and AC to obtain good peak shape and sensitivity. The analytes eluted within 2 min from injection using a gradient to 80% 2B in 0.8 min. It was sufficient to equilibrate the TC for 0.5 min with solvent 1A. Thus, the overall run time was only 2.2 min. The cycle time using the 50 mm AC was 3 min with a gradient from 0 to 100% 2B in 1 min. Initial experiments did not show significant differences of area ratios in plasma and tissues. The injection/dilution flow ratio was changed to 0.5/3 ml/min because of higher signal intensity in tissues. The analysis of two calibration sets and QC samples in plasma, lung, testes and kidney resulted in similar data for either column. Therefore, the XTerra RP18 2.1×10 mm AC was used for all further analyses. Robustness of the assay and acceptable accuracy and precision for oxazepam and lorazepam were demonstrated during the analysis of study samples.

3.4.7. Analysis of benzodiazepines without analytical column

The feasibility of elution directly from the trapping column into the mass spectrometer was demonstrated for benzodiazepines. The same trapping program as for the assay with analytical column was applied. The elution was carried out in forward flush mode in order to use the trapping column for a minimum separation. Isocratic elution was carried out with 40% mobile phase 2B. The retention time was 0.9 min from sample injection, while the trapping time was not adapted to this shorter chromatography. The accuracy at the LOQ of 1 ng/ml was 101.3% and the precision 6.2%. The accuracy of tissue QC samples was between 95.5 and 119%. Some carryover resulting from autosampler and column-switching system was observed. This approach may be suitable for routine analysis of higher analyte concentrations, but it caused reduced sensitivity, increased matrix effects and deteriorated peak shape, and was therefore not further applied.

3.5. Matrix effects

The relative fast trapping procedure and chromatographic separation led to questions about the negative influence of matrix effects on the analyte determination. To show signal suppression caused by matrix components, an infusion experiment [26] was carried out (set-up shown in Fig. 7, scheme at top portion). Continuous infusion was performed of oxazepam via an infusion pump and T-piece after the analytical column, and blank matrix was injected into the column-switching system. No signal decrease was obtained after injection of plasma compared to the injection of water (Fig. 7, bottom picture). Furthermore, brain, skin, muscle and testes did not cause a signal reduction (data not shown). A suppression of the oxazepam signal at the retention time of the analytes was observed with gut and liver (Fig. 7) and also with spleen (not presented). Smaller peak areas of analytes and ISTD 6 in these matrices compared to plasma confirmed the results. Matrix effects are not serious as long as the internal standard can correct their influence. In benzodiazepine analysis, the analytes and the internal standard were suppressed to the same extent leading to a good compensation via area ratios. Fig. 8 presents the LC–MS–MS chromatograms for oxazepam, lorazepam and ISTD 6 in testes and gut. As it can be seen, the areas of both ISTD 6 and analytes in gut were suppressed compared to testes by the same factor, leading to a good accuracy of QC samples. The internal standard did not compensate the matrix effect for propranolol analysis in gut samples. Only 50% accuracy was obtained for the quality control samples. Because the QC samples in all other tissues showed good accuracy, the method was not changed but the gut samples were diluted 1:10 to reduce the concentration of matrix components. Diluted QC samples possessed sufficient accuracy. Fig. 6 shows different suppression for the analyte and the internal standard in undiluted gut compared to diluted gut (same final concentration) using TurboIonSpray ionization. The signal for ISTD 5 was less decreased than the analyte signal. APCI-LC–MS–MS experiments were performed to determine whether the influence of matrix can be reduced in this way. Diluted and undiluted gut QC samples were compared. Fig. 6 demonstrates that signal suppression did not occur in APCI determinations. The internal standard and the analyte were influenced to the same extent, resulting in good accuracy of QC samples in undiluted gut. Matrix effects were much lower with the injection of small sample volumes (e.g. 10 μl for nicardipine analysis and negative mode determination of nitrendipine). Large injection volumes (e.g. 150 μl for felodipine) required a more effective trapping procedure. Matrix influences may be reduced when applying negative instead of positive mode ionization (e.g. for nitrendipine determination).

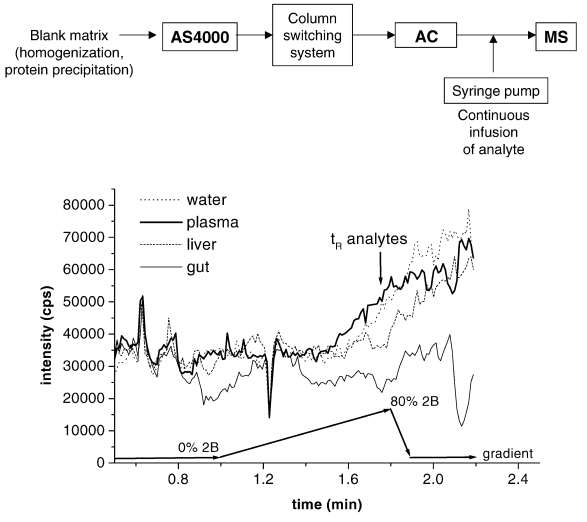


Fig. 7. Schematic set-up for suppression experiments (top) and influence of different matrices on oxazepam signal in SIM mode (bottom), continuous infusion of 1 ng/μl analyte after column, injection of blank matrix into column-switching system.

3.6. Assay performance

The selectivity of the assays was checked through the analysis of blank materials. No significant interfering peaks were observed in all matrices as demonstrated in the examples shown in Figs. 2 and 3. The limit of quantification (LOQ) was 1 ng/ml in plasma and 4 ng/ml in tissue with the exception of 10 ng/ml for skin due to the 10-fold dilution. The LOQ for lorazepam was 2.5 ng/ml in plasma due to the lower response. The linear calibration range reached from 1 to 500 ng/ml for Ro 24-6173, nitrendipine and propranolol. Calibration was linear up to 1000 ng/ml for the other analytes. Inter-assay precision and

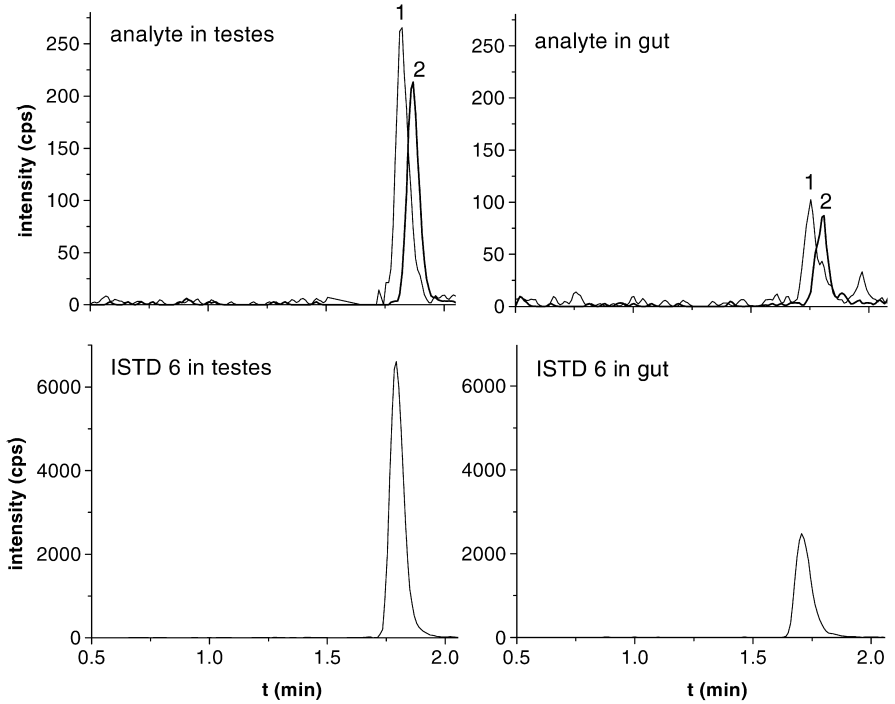


Fig. 8. LC–MS–MS chromatograms of benzodiazepines 4 ng/ml QC samples: oxazepam (1) at m/z 287.1→ 241.1, lorazepam at m/z 321.1→275.1 (2) and the corresponding internal standard ISTD 6 at m/z 305.1→259.1 in testes and gut.

accuracy are shown in Table 3. The accuracy deviated by less than 15% from the nominal values. The precision at all levels was better than 15% RSD. The intra-assay accuracy and precision derived from analysis of quality control samples is shown in Tables 4 and 5. The accuracy of QC samples in plasma and tissues was in the range of 89–116% for nicardipine, 80–120% for Ro 24-6173, 86–115% for felodipine, 84–117% for oxazepam, 85–130% for lorazepam, 81–118% for nitrendipine and 86–118% for propranolol. The precision resulting from repeated analysis of QC samples was in the range of 0.5–15% for nicardipine, 2–20% for Ro 24-6173, 0.5–20% for felodipine, 1.5–15% for oxazepam, 2–16% for lorazepam, 0.4–13.8% for nitrendipine and 0.2–15.4% for propranolol. Stability of the compounds was checked by comparing freshly spiked QC samples with the calibration samples stored at −20 °C. No instability problems were observed during the assay period of 2–4 weeks per compound. The study samples were stored at −80 °C to avoid possible degradation during a longer period of time. The methods were applied to about 100 plasma and 88 tissue samples per substance. The analyte concentrations ranged from near LOQ values up to 10 times higher than the calibration range (dilution was performed). The concentration values obtained after duplicate analysis of rat tissues deviated by less than 15% (in most cases less than 10%).

4. Conclusions

We have shown that column-switching LC–MS–MS methods can be used successfully for the determination of a wide variety of pharmaceutical compounds in plasma and tissues. Robust assays providing data with a good quality of precision and accuracy and sufficient limits of quantification were developed and applied for the analysis of real study samples. Laborious off-line sample extraction was not necessary. The same HPLC set-up was used for all analytes with minor variations. An optimized

trapping served as a good clean-up procedure which reduced interfering matrix components and led to similar recoveries from all materials. Thus, it was possible to calculate sample concentrations from calibration standards in human plasma and prepare only the quality control samples in animal tissues. Although the structural analogs used as internal standards were well-suited to correct system variability and matrix influences, isotopically-labeled compounds would be of advantage for better compensation of matrix effects. An increase in sample throughput is restricted due to the time-consuming, manual homogenization of tissues. Nevertheless, there may be the potential for faster analysis in shortening the trapping cycle and chromatographic run times. In summary, column-switching LC–MS–MS is an elegant, rapid, fully automated, robust method for complex samples and will be further used as a generic approach to determine drug candidates in tissues.

References

[1] K. Krishnan, M.E. Andersen, Physiologically based pharmacokinetic modeling in toxicology, in: W. Hayes (Ed.), Principles and Methods of Toxicology, 3rd ed., Raven Press, New York, 1994, p. 149.
[2] P. Poulin, F.-P. Theil, J. Pharm. Sci. 89 (2000) 16.
[3] W.A. Korfmacher, K.A. Cox, M.S. Bryant, J. Veals, K. Ng, R. Watkins, C.C. Lin, Drug Discov. Today 2 (1997) 532.
[4] J. Henion, E. Brewer, G. Rule, Anal. Chem. 70 (1998) 650A.
[5] W.M.A. Niessen, J. Chromatogr. A 856 (1999) 179.
[6] T. Inoue, K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 744 (2000) 213.
[7] T. Oguma, H. Toyoda, T. Toida, T. Imanari, J. Chromatogr. B 754 (2001) 153.
[8] S. Ohmori, T. Hayashi, M. Kawase, S. Saito, Y. Morimoto, J. Chromatogr. B 740 (2000) 253.
[9] D. Fluchard, S. Kiebooms, M. Dubois, P. Delahaut, J. Chromatogr. B 744 (2000) 139.
[10] Y. Ito, Y. Ikai, H. Oka, H. Matsumoto, Y. Miyazaki, K. Takeba, H. Nagase, J. Chromatogr. A 911 (2001) 217.
[11] M. Dubois, D. Fluchard, E. Sior, P. Delahaut, J. Chromatogr. B 753 (2001) 189.
[12] M. Cherlet, S. De Baere, S. Croubels, P. De Backer, J. Chromatogr. B 742 (2000) 283.
[13] G.-F. Pang, Y.-Z. Cao, C.-L. Fan, J.-J. Zhang, X.-M. Li, C. Wang, J. Chromatogr. A 882 (2000) 85.
[14] K. Srinivasan, P. Wang, A.T. Eley, C.A. White, M.G. Bartlett, J. Chromatogr. B 745 (2000) 287.
[15] G.Y. Eng, R.J. Maxwell, E. Cohen, E.G. Piotrowski, W. Fiddler, J. Chromatogr. A 799 (1998) 349.
[16] R. Draisci, C. Marchiafava, L. Palleschi, P. Cammarata, S. Cavalli, J. Chromatogr. B 753 (2001) 217.
[17] K. Schmeer, T. Sauter, J. Schmid, J. Chromatogr. A 777 (1997) 67.
[18] V.C.X. Gao, W.C. Luo, Q. Ye, M. Thoolen, J. Chromatogr. A 828 (1998) 141.
[19] N.G. Knebel, S. Grieb, S. Leisenheimer, M. Locher, J. Chromatogr. B 748 (2000) 97.
[20] U. Christians, W. Jacobsen, N. Serkova, L.Z. Benet, C. Vidal, K.-F. Sewing, M.P. Manns, G.I. Kirchner, J. Chromatogr. B 748 (2000) 41.
[21] U. Timm, H. Birnböck, R. Erdin, G. Hopfgartner, R. Zumbrunnen, J. Pharm. Biomed. Anal. 21 (1999) 151.
[22] C. Hummert, B. Luckas, H. Siebenlist, J. Chromatogr. B 668 (1995) 53.
[23] R. Wyss, F. Bucheli, W. Philipp, J. Chromatogr. A 797 (1998) 211.
[24] R. Wyss, F. Bucheli, W. Philipp, J. Chromatogr. A 870 (2000) 189.
[25] K. Heinig, F. Bucheli, Determination of a pharmaceutical drug and its metabolites by column-switching LC–MS–MS with TurboIonSpray and atmospheric pressure chemical ionization, poster at 17th Montreux Symposium on LC–MS.
[26] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175.