Comparison of turbulent-flow chromatography with automated solid-phase extraction in 96-well plates and liquid–liquid extraction used as plasma sample preparation techniques for liquid chromatography–tandem mass spectrometry

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Abstract

Turbulent flow chromatography (TFC) combined with the high selectivity and sensitivity of tandem mass spectrometry (MS–MS) is a new technique for the fast direct analysis of drugs from crude plasma. TFC in the 96-well plate format reduces significantly the time required for sample clean-up in the laboratory. For example, for 100 samples the workload for a technician is reduced from about 8 h by a manual liquid–liquid extraction (LLE) assay to about 1 h in the case of TFC. Sample clean-up and analysis are performed on-line on the same column. Similar chromatographic performance and validation results were achieved using HTLC Turbo-C_{18} columns (Cohesive Technologies) and Oasis HLB extraction columns (Waters). One 96-well plate with 96 plasma samples is analyzed within 5.25 h, corresponding to 3.3 min per sample. Compared to this LLE and analysis of 96 samples takes about 16 h. Two structurally different and highly protein bound compounds, drug A and drug B, were analyzed under identical TFC conditions and the assays were fully validated for the application to toxicokinetics studies (compliant with Good Laboratory Practices – GLP). The limit of quantitation was 1.00 µg/l and the linear working range covered three orders of magnitude for both drugs. In the case of drug A the quality of analysis by TFC was similar to the reference LLE assay and slightly better than automated solid-phase extraction in 96-well plates. The accuracy was −3.1 to 6.7% and the precision was 3.1 to 6.8% in the case of drug A determined for dog plasma by TFC–MS–MS. For drug B the accuracy was −3.7 to 3.5% and the precision was 1.6 to 5.4% for rat plasma, which is even slightly better than what was achieved with the validated protein precipitation assay. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Turbulent flow chromatography; Sample preparation; Validation; Pharmaceutical analysis

1. Introduction

Liquid chromatography interfaced to tandem mass spectrometry (LC–MS–MS) using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) in its high-flow version (TurboIonspray) has led to a breakthrough in sensitivity, selectivity and speed of bioanalysis in the pharmaceutical industry. Due to the high selectivity of MS–MS, sample run times of less than 5 min (mostly 2 to 3 min) are typically achieved which gives rise to sample throughputs of 150–200 samples during overnight runs. Sample preparation became clearly the rate-limiting step in bioanalysis. In pharmacokinetics plasma samples are prepared using liquid–liquid extraction (LLE), solid-phase extraction (SPE) (manually or on robotics), protein precipitation or different approaches for direct injection of

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crude plasma onto suitable sample extraction columns by column switching. For the latter approach the LiChrospher RP-18 ADS column (Merck) had been used in our laboratory for the determination of Azlocillin and its metabolites [1]. The analysis time was too long (6 min per sample) since 4 min were required to get rid of the plasma constituents followed by 2 min analysis on an analytical column.

The analysis time can be significantly reduced when chromatography is performed under turbulent flow conditions in open tubular columns [2].

Turbulent flow chromatography (TFC) in packed columns was recently introduced by Quinn and Takarewski from Cohesive Technologies (Franklin, MA, USA) in 1997 [3] as a fast method for direct injection of crude plasma onto special columns packed with 50 μm spherical porous particles. These columns serve as extraction and analytical column at the same time, total analysis time is about 2 min with high flow-rates of e.g., 4 to 6 ml/min on a 1 mm I.D. column. While such high flow-rates are far outside the usual optimum conditions (reduced plate height, h, about 2) for laminar flow conditions, a second optimum of the Van Deemter curve was found [3] under turbulent flow conditions. Thus the chromatographic efficiency with turbulent flow at high flow-rates is similar to laminar flow at the commonly used much lower flow-rates. Under turbulent flow conditions a fast and efficient separation of the small drug from the large plasma proteins is thus achieved even in case of 99% protein bound drugs (see below). Proteins are eluted as a sharp peak within 0.5 min using a pure water or buffer mobile phase, meanwhile the drug is retained on e.g., C_2, C_8, C_{18} or phenyl chains inside the pores of the particles. The high flow-rate and the plug profile solvent front allow for very steep changes in the mobile phase composition from 100% water to e.g., 90% acetonitrile within typically 5 s which causes the drug(s) and internal standard(s) to elute in a sharp peak.

The suitability of TFC–MS–MS for the analysis of plasma samples was recently reported by Ayrton et al. [4]. They used a standard HP1090 liquid chromatograph for TFC and showed a brief validation for an isoquinoline drug. Other high-throughput sample preparation techniques are based on automatized SPE in the 96- or 384-well microplate format using pipetting robots [5] followed by off-line per-

Table 1
Brief physiochemical characterization of drugs A and B

	Drug A	Drug B
Salt (form)	No (Betaine)	HCl
Water solubility	Insoluble ($<10^{-6}$ g/l)	35 g/l
Protein binding	99.8%	94–95%
f_u	0.2%	5–6%
Log P (octanol–water)	6.3	2.9

formed LC–MS–MS analysis of the extracts. We applied this technique to drug A before the introduction of TFC to our laboratory. The control of the vacuum and the evaporation of the extracts were critical and limiting factors.

This report describes fully validated assays which had been applied to toxicokinetics studies [Good Laboratory Practices (GLP)] with two of our development compounds, drug A and drug B which are highly protein bound. Some physiochemical parameters of drugs A and B are given in Table 1. The structures cannot be disclosed for commercial reasons.

2. Experimental

2.1. Chemicals

Drugs A and B are development compounds of Bayer (Leverkusen, Germany).

Ammonium acetate was supplied by Merck (Darmstadt, Germany), acetonitrile (gradient grade), formic acid (analytical-reagent grade) and water (gradient grade) were obtained from Riedel-de Haen (Seelze, Germany).

2.2. Historic assays before the introduction of TFC

Since the main focus of this paper lies on TFC the formerly used techniques are not outlined in detail here. Before the introduction of TFC drugs A and B were analyzed in our laboratory using different (validated) approaches for sample preparation. In case of drug A two assays were in use before: (1) manual LLE of 0.2 ml plasma using a mixture of ethyl acetate–*n*-heptane (70:30, v/v) followed by reconstitution of the dry extract in 0.2 ml mobile

phase yielding a limit of quantitation (LOQ) of 0.100 µg/l and (2) automated SPE on a Packard Multiprobe 104 liquid handler (Packard, Meriden, CT, USA) using 96-well extraction plates filled with Waters Oasis (30 mg) or Whatman-C_{18} (100 mg). The extract was not evaporated giving an end volume of 1 ml and a LOQ of 1.00 µg/l. One 96-well plate was processed within 60 min. The general procedure of 96-well plate SPE on a Packard Multiprobe liquid handler was earlier described in Ref. [5]. Sample analysis was performed by LC–MS–MS using either APCI or TurboIonspray.

For drug B a simple manual protein precipitation out of 0.2 ml plasma with 1.00 ml acetonitrile was done followed by evaporation of the supernatant and reconstitution in 0.300 ml mobile phase. Analysis was performed by TurboIonspray-MS–MS with an LOQ of 0.500 µg/l.

2.3. Preparation of stock and working solutions of the analytes and internal standards

Two stock solutions denoted A and B were prepared for drug A by dissolving duplicate weighings of 10 mg of the authentic analyte reference standard in 10 ml of acetonitrile. Dilutions of the stock were made with acetonitrile in order to yield working solutions which were used for the preparation of calibration samples and quality control samples. The stock solution of the internal standard [$^2H_2^{15}N$]drug A was prepared by dissolving 10 mg in 10 ml acetonitrile. This solution was further diluted in acetonitrile to working solutions which were used to prepare an internal standard (I.S.) spiking plasma pool. The concentration of the I.S. in the spiking plasma pool was 300 µg/l.

Two stock solutions denoted A and B were prepared for drug B by dissolving duplicate weighings of 10.7 mg of the authentic analyte reference standard (hydrochloride) in 10 ml of a mixture of methanol–water (80:20). Dilutions of the stock were made with the same mixture in order to yield working solutions which were used for the preparation of calibration samples and quality control samples. The stock solution of the internal standard [$^2H_5$]drug B was prepared by dissolving 10 mg in 10 ml of methanol–water (80:20). This solution was further diluted in the same mixture to working

solutions which were used to prepare an I.S. spiking plasma pool.

All stock and working solutions were stored in the refrigerator at about 5°C for no longer than eight weeks. The I.S. spiking plasma pools were freshly prepared on each analysis day.

2.4. Preparation of calibration and validation samples and validation

Calibration samples were prepared freshly in duplicates always on the day of sample analysis by spiking control rat or dog plasma with 10 µl of working solutions containing the analyte in appropriate concentration. In case of drug A the concentrations of the calibration samples were 1.00, 2.00, 10.0, 100, 1000 and 2500 µg/l while for drug B samples were prepared at 0.500, 1.00, 2.50, 5.00, 25.0, 50.0, 250, 500, 1000 and 2500 µg/l.

For pre-study validation two independent sets of validation samples were prepared on two days by spiking control rat or dog plasma, each set consisting of four concentration levels with six replicates at each level. For drug A the four levels were 5.00, 50.0, 500 and 1500 µg/l and for drug B they were 2.00, 10.0, 100 and 1000 µg/l. The samples were run independently on two validation days together with calibration samples in duplicates and four blanks.

In case of drug A one set of validation samples was run twice on the same day: the first run was performed on the HTLC Turbo-C_{18} column, the second run on the Waters Oasis HLB column.

2.5. Cross validation

Since TFC is a new technique and chromatographic retention times are very short the assay was checked for its selectivity regarding possible interferences from drug metabolites, drug formulation, sample matrix and other sources inherent to real study samples. Therefore study samples which had been analyzed previously using the fully validated historic reference assays were reanalyzed by TFC. For example in case of drug A 42 study samples obtained from three Beagle dogs, taken at time points 5, 10, 15, 20, 30, 45 min, 1, 1.5, 2, 3, 5, 7, 9 and 24 h after a single oral administration of 0.3

mg/kg which were originally analyzed by the reference LLE assay were re-analyzed by TFC–MS–MS. These study samples and the corresponding quality control samples were stored for four months at $-20°C$ between the first analysis and the re-analysis by TFC.

2.6. Sample preparation for TFC

Sample preparation has been minimized to centrifugation, I.S. addition and aliquotation into a 96-well plate (or HPLC vial). Centrifugation is essential in order to protect the column from particles which might cause early plugging.

Large plasma volumes like e.g., plasma pools used for the preparation of calibration and validation samples were centrifuged in a Beckman centrifuge type GS-6R at 3700 g for 10 min. Study samples and quality control samples (typically stored in Eppendorf vessels) were centrifuged in an Eppendorf 5415 centrifuge at 17 500 g for 10 min. In the case of drug A, after centrifugation of the plasma an aliquot (150 μl) was transferred to a 96-well plate and mixed with a 20-μl aliquot of the I.S. spiking plasma pool (resulting I.S. concentration in the sample 35.3 μg/l).

In the case of drug B, 0.100 ml plasma was mixed with 50 μl of the I.S. spiking plasma pool (resulting I.S. concentration in sample 1667 μg/l).

2.7. Waters Oasis HLB column ruggedness test

One hundred and sixty eight injections with an injection volume of 25 μl of rat plasma, consisting of six blanks and plasma samples spiked at 1.00 μg/l (LOQ) (118 samples) and 10.0 μg/l (44 samples) with drug A and with the stable-isotope-labeled drug as I.S. were made onto a Waters Oasis HLB column. The peak height of the analyte and of the I.S. was evaluated regarding the relative standard deviation (RSD).

The ruggedness of the HTLC Turbo-C_{18} columns was proven during the numerous validation runs with both drugs.

2.8. Instrumentation and chromatographic conditions for TFC

TFC was performed using a 2300 HTLC System (Cohesive Technologies) which is a modified HP 1100 modular liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) consisting of an isocratic pump for clean-up and flush of liquid lines, a binary pump for elution of retained analytes and a valve switching module with two Rheodyne six-port valves. The autosampler was a CTC HTS PAL (CTC Analytics, Zingen, Switzerland) equipped with a 100-μl injection syringe. The 2300 HTLC system was controlled by the 2300 HTLC V 1.4.1 software from Cohesive Technologies.

Either the 50×1.0 mm HTLC Turbo-C_{18} columns (Cohesive Technologies) packed with 50 μm porous particles or the 50×1.0 mm Waters Oasis HLB extraction columns (Waters, Milford, MA, USA) filled with 30 μm particles were used. These columns serve as extraction and analytical column at the same time, hence no other (analytical) column is needed. Identical chromatographic conditions were applied to both types of columns and to all compounds reported here. Furthermore in the meantime these conditions were applied after minor modifications to other drugs and drug candidates hence they appear to be generic.

In the first load and clean-up step (duration 60 s) 20 μl of the crude plasma sample were injected onto the column, the mobile phase delivered by the isocratic pump at 4.0 ml/min was buffer (2 mM aqueous ammonium acetate, pH 6.8). The proteins were flushed to the waste while the small molecules were retained on the C_{18} chains inside the porous particles. During this step the binary pump delivered acetonitrile–2 mM ammonium acetate (pH 6.8; 10:90) at a flow-rate of 1.5 ml/min to the mass spectrometer. In the second step (duration 30 s) the valves were switched and the isocratic pump flushed the tubings and valves with buffer at a flow-rate of 6.0 ml/min to waste, while elution of the retained analytes (and I.S.) was performed in forward-flush with the binary pump at a flow-rate of 1.5 ml/min by increasing the acetonitrile content in the mobile phase (buffer) within 5 s from zero to 95% which was held for 25 s. In the third flush step the valves were switched back to the initial position and the isocratic pump was re-connected to the column (forward) flushing the column and tubings at 4.0 ml/min with pure acetonitrile for 30 s while the binary pump was delivering mobile phase (acetonitrile–2 mM ammonium acetate, pH 6.8,

10:90) to the mass spectrometer. In the last step (duration 30 s) a re-equilibration of the column was performed with buffer at a flow-rate of 4.0 ml/min, while the binary pump was instantly pumping mobile phase to the mass spectrometer like in step 3. Thus the whole run time was 2.5 min per sample.

2.9. Mass spectrometry and data evaluation

Samples were analyzed by MS–MS using a Perkin-Elmer Sciex API 365 triple quadrupole tandem mass spectrometer (Concord, Canada). The liquid flow from the TFC column was directed either directly to the APCI interface (analysis of drug A) or after a 1:5 split to the TurboIonSpray interface (analysis of drug B). The mass spectrometer was operated in positive-ion multiple reaction monitoring (MRM) mode with key parameter settings given in Table 2.

Nitrogen 5.0 grade was used for the auxiliary gas, the nebulizer gas, the curtain gas and the collision gas. The required sensitivity was achieved by lens voltage tuning and by additionally reducing mass resolution of the quadrupoles to 1.0 u (Q_1) and 1.5 u (Q_3) peak width at half height. The API 365 was equipped with a so called ''Autobox'' (made to our order by Mr. Möller from Perkin-Elmer, Langen, Germany) with digital readouts for the ionization current (in μA), the interface temperature and with a solenoid switching valve for gas 2 (auxiliary). Two Apple MacIntosh Power PC 8500 computers were used, one for data acquisition and one for data processing. The peaks in the MRM traces were integrated with the PE-Sciex MacQuan processing software version 1.5. Calibration and concentration calculations were done by the laboratory-written software [6]. The calibration curves were calculated from the peak area ratios of drug A and drug B to their corresponding internal standards using a $1/y^2$-weighted linear least-squares regression model.

3. Results and discussion

3.1. Mass spectra and chromatograms of drug A and the I.S.

The pseudo molecular ion $[M+H]^+$ of the analyte drug A was recorded at m/z 532.3. After collisionally induced dissociation (CID) of $[M+H]^+$ the product ion spectrum shows two strong fragment ions at m/z 299.2 and 209.2. These two key fragment ions were used to monitor drug A in MRM mode. Since the signal-to-noise ratio in plasma samples was better in favor of the ion at m/z 299 this ion was used for quantitation (quantifier ion) whereas the ion at m/z 209 was used as a qualifier ion for confirmation. The intensity ratio of the two MRM signals at m/z 532.3/299.1 and at m/z 532.3/209.1 is usually about 1:1 to 1:0.8 in pure standards as well as in spiked plasma samples or real samples, respectively, as seen in Fig. 1. In cases where the quantifier ion at m/z 299 was superimposed by chemical noise the quantitation was done using the ion at m/z 209.

An I.S. is usually required for precise and accurate quantitative determinations in bioanalysis. It should

Table 2
Key parameters for the analysis of drugs A and B by LC–MS–MS in positive-ion MRM mode on a PE Sciex API 365 instrument

| Analyte	Drug A	Drug B
Interface	APCI	TurboIonspray
Temperature	500°C	300°C
Ionization current/voltage	3 μA	4800 V
Orifice potential	48 V	44 V
Ring potential	270 V	220 V
Collision energy	44.5 eV	51 eV
MRM quantifier ions Q_1/Q_3	m/z 532.3/299.1	m/z 489.3/168.8
Dwell time	700 ms	500 ms
MRM qualifier ions Q_1/Q_3	m/z 532.3/209.1	m/z 489.3/312.1
Dwell time	200 ms	500 ms
MRM ions for I.S. Q_1/Q_3	m/z 535.3/209.1	m/z 494.4/168.8
Dwell time	100 ms	200 ms
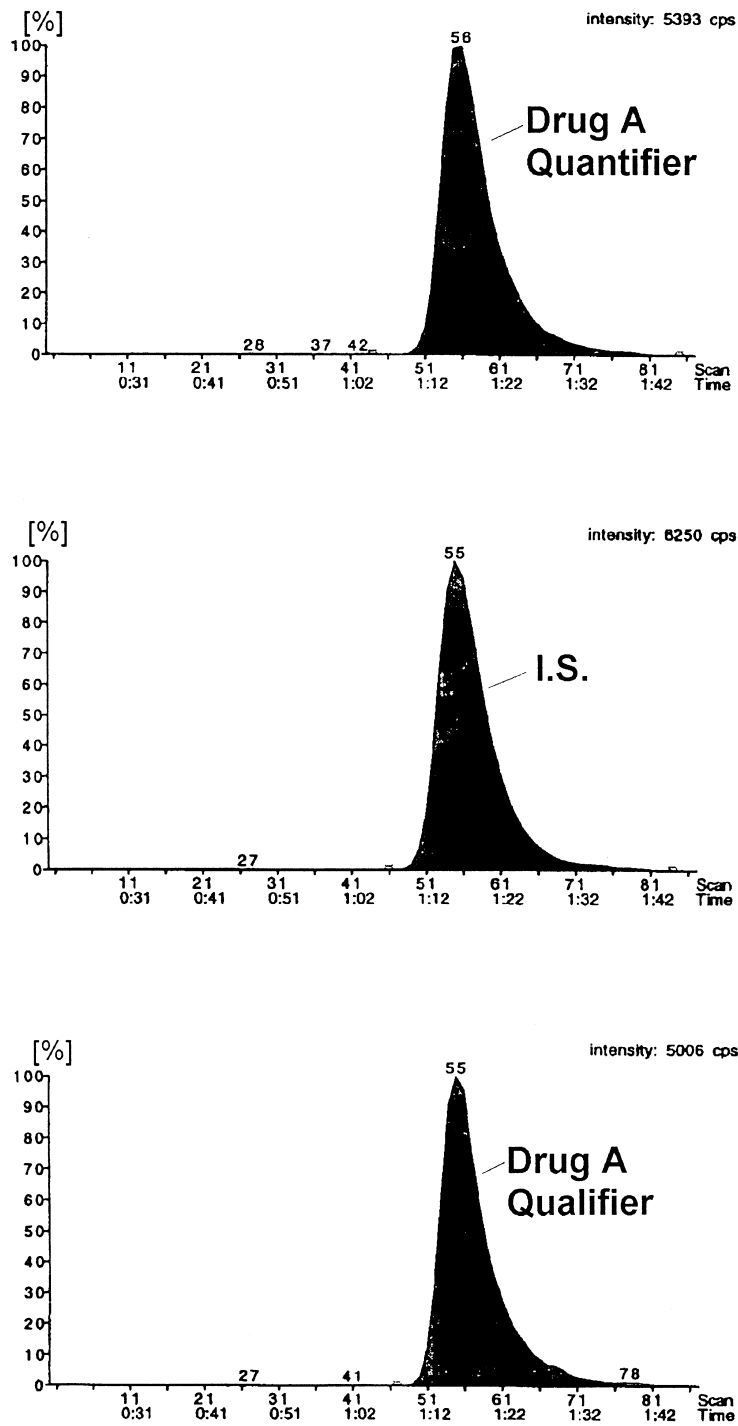
Fig. 1. Drug A in a toxicity study sample 4 h after oral administration of 80 mg/kg to rats. Plasma concentration by TFC: 56.5 μg/l (reference assay: 58.5 μg/l). Monitored transitions: m/z 532.3→299.1 (drug A, quantifier), m/z 535.3→299.1 (I.S.) and m/z 532.3→209.1 (drug A, qualifier). Time scale in min.
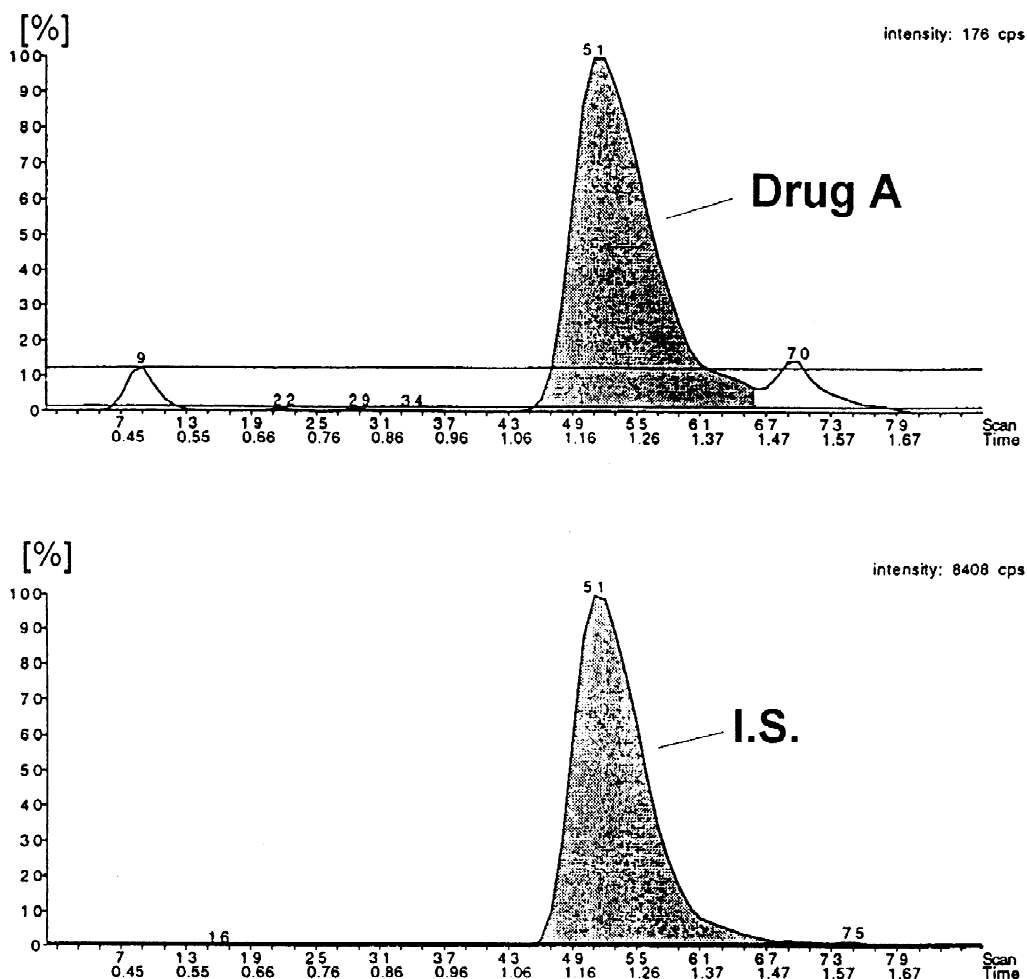
Fig. 2. Drug A: TFC–MS–MS chromatogram from the validation in dog plasma. Calibration sample at 1.00 μg/l (LOQ), HTLC Turbo-C$_{18}$ column. Monitored transitions: m/z 532.3→299.1 (drug A) and m/z 535.3→299.1 (I.S.). Time scale in min.

match the structure of the analyte as closely as possible. The ideal I.S. used in mass spectrometric quantitation either by LC–MS or GC–MS is the stable-isotope-labeled analyte. Amongst others the most important reason to use the isotope-labeled analyte is its coelution with the analyte thus compensating for ion suppression effects which are a common observation in LC–ESI–MS–MS. Two-fold deuterium- and in one position ^{15}N-labeled drug A was used as I.S. ($[^2H_2^{15}N]$drug A). The fragmentation pattern is similar to that of the analyte and therefore the reaction m/z 535.3/299.1 was used to monitor the I.S.

Quantitation was performed using the peak area (or height) ratios of drug A and the coeluting I.S. Typical chromatograms of a calibration sample at the LOQ (1.00 μg/l), and of a real study sample are shown in Figs. 1 and 2. The peak area (or height) ratios of drug A to the I.S. were linear from 1.00 (LOQ) to 2500 μg/l out of rat, dog and mouse plasma.

3.2. Mass spectra and chromatograms of drug B and the I.S.

The pseudo molecular ion $[M+H]^+$ of the analyte drug B was recorded at m/z 489.3. After CID of $[M+H]^+$ the product ion spectrum shows two strong

fragment ions at m/z 312.1 and m/z 168.8, respectively. These two key fragment ions were used to monitor drug B in the MRM mode. Since the signal-to-noise ratio in plasma samples was better in favor of the ion at m/z 312.1 this ion was mainly used for quantitation whereas the ion at m/z 168.8 was used as a qualifier ion for confirmation.

Five-fold deuterium-labeled drug B was used as I.S. The fragmentation pattern is similar to that of the analyte and the reaction m/z 494.4/168.8 was used to monitor the I.S.

Quantitation is performed using the peak area (or height) ratios of drug B and the coeluting I.S. A typical chromatogram of a calibration sample at the LOQ (1.00 μg/l) is shown in Fig. 3. The peak area (or height) ratios of drug B to the I.S. were linear from 1.00 (LOQ) to 2500 μg/l out of rat plasma.

3.3. Drug A: Validation results

The validation results are given in Tables 3–6. Rat, mouse and dog plasma samples were analyzed on the HTLC Turbo-C_{18} column (Tables 3–5). The dog plasma samples were additionally run on the Oasis HLB column as well (Table 6). Inter-assay accuracy of rat plasma was −1.2 to 11.0% and inter-assay precision was 2.1 to 8.2% (two validation days, three concentrations). In the case of mouse plasma accuracy was −5.1 to 4.5% and precision was 4.0 to 8.7% (one validation day, four concentrations). For dog plasma, inter-assay accuracy was −3.1 to 6.7% and inter-assay precision was 3.1 to 6.8% (two validation days, four concentrations) for the HTLC Turbo-C_{18} column. In case of the Oasis HLB column the results were similar (one validation day, four concentrations), intra-assay accuracy was −5.2 to 3.8% and precision 2.8 to 4.6%. Peak shape and response were similar for both types of column, the slope of the calibration curve was 0.016 with the HTLC Turbo-C_{18} column and 0.012/0.015 during the cross validation/validation using the Oasis HLB column (dog plasma in each case).

3.4. Comparison of TFC with automated 96-well SPE and manual LLE for drug A

Comparing the three different techniques for dog plasma one has to keep in mind that different plasma

volumes were used, consequently the LOQ was different. While in TFC only 25 μl of crude plasma was injected yielding an LOQ of 1.00 μg/l, in automated 96-well plate SPE and in LLE 0.2 ml plasma was worked up. In LLE no dilution was applied to the sample since 0.2 ml plasma were extracted into 0.2 ml sample solvent, while in SPE a dilution to 1 ml solvent was done, consequently the LOQ was 0.100 μg/l by LLE and 1.00 μg/l by SPE.

Regarding accuracy the best results were achieved by LLE (−3.7 to 4.4%), closely followed by TFC (−3.1 to 6.7%) and automated SPE (−4.1 to 10.6%). In terms of precision TFC and LLE were similar (3.1 to 6.8% and 3.1 to 5.1%) and automated SPE showed a precision of 5.1 to 5.5%. Therefore TFC compares well with LLE while automated SPE demonstrated slightly lower accuracy but still better than 15%.

The key advantage of TFC is clearly the significant reduction of working time needed in the laboratory for sample preparation and the reduction of total run time per sample. By the example of drug A, in the case of LLE 8 h were needed for sample preparation including labeling of extraction tubes and vials, whereas for TFC and automated SPE (both in 96-well plates) only 1 h was needed (without evaporation of extract in SPE). Automated SPE requires about 1 h to process a 96-well plate where only some minor intervention by the operator was needed (change of the extraction plate for the collection plate, supervision of vacuum). In total the whole duration of one batch (96 samples) consisting of sample preparation and analysis takes 5.25 h (TFC), 9 h (automated SPE) and 16 h (LLE). Thus the most efficient technique is TFC which has the additional advantage that it is on-line linked to the MS–MS analyzer hence saving additional time for manual sample transfer from the sample preparation laboratory to the MS laboratory.

3.5. Drug A: Cross validation results from real study samples

The re-analysis of 42 real study samples (dog, drug A, 0.3 mg/kg single oral dose) by TFC–MS–MS revealed very good agreement between the plasma concentrations measured by TFC–MS–MS and those measured earlier with the reference LLE–
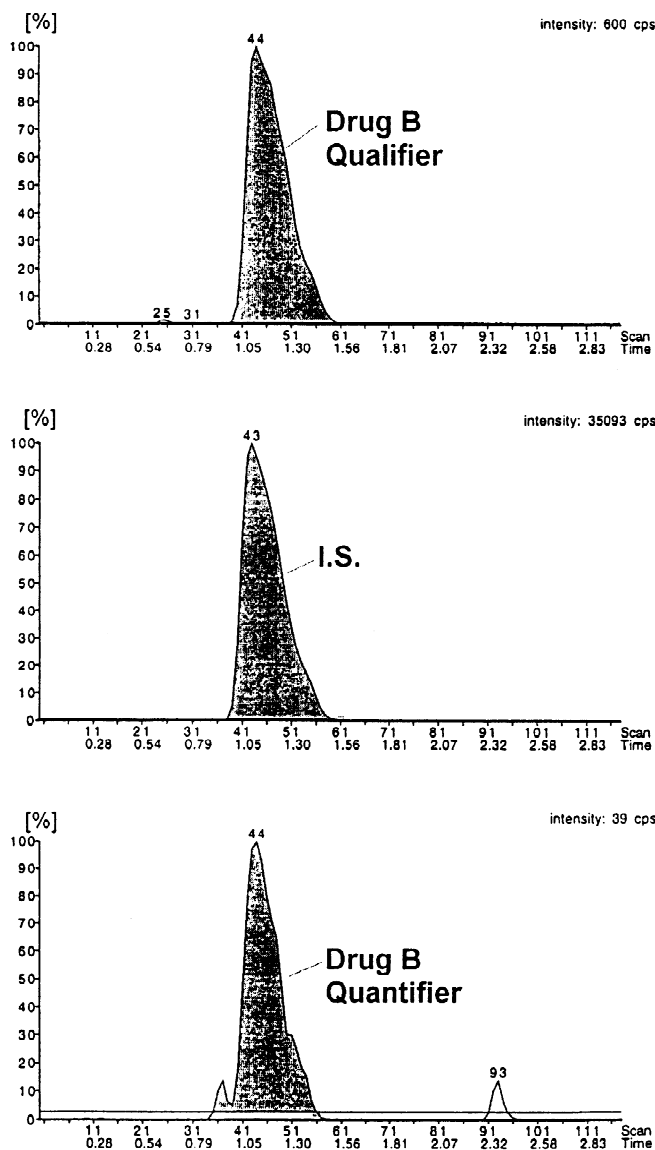
Fig. 3. Drug B: TFC–MS–MS chromatogram from the validation in rat plasma. Calibration sample at 1.00 μg/l (LOQ), HTLC Turbo-C$_{18}$ column. Monitored transitions: m/z 489.3→312.1 (drug B, qualifier), m/z 494.4→168.8 (I.S.) and m/z 489.3→168.8 (drug B, quantifier). Time scale in min.

LC–MS–MS assay (reference concentrations). The deviations of the data obtained by TFC–MS–MS from the reference concentrations were between −17.8 and 15.5% (Fig. 4). The mean of the deviations of 42 values was −1.84% with a RSD of 5.14% only. A similar cross validation was per-formed with 20 rat study samples. The deviations of the measured concentrations by TFC–MS–MS from the reference concentrations were between −4.89 and 15.4% (mean 7.77%). A chromatogram of a plasma sample obtained during a toxicity study in rats is shown in Fig. 1. The measured plasma

Table 3
Drug A: Validation of TFC assay on a HTLC Turbo-C_{18} column by injection of 25 μl crude rat plasma

	Concentration (μg/l)			
	5.00	50.0	500	1.00 (LOQ)
n	12	12	12	8
Student *t* value, *p*=95%	2.20	2.20	2.20	2.37
Mean (μg/l)	5.55	52.4	494	1.00
SD (μg/l)	0.187	4.27	10.5	0.025
Precision (%)	3.4	8.2	2.1	2.5
Accuracy (%)	11.0	4.7	−1.2	0.4
Upper limit of CI[a] (μg/l)	5.67	55.1	500	1.03
Lower limit of CI[a] (μg/l)	5.43	49.7	487	0.983

[a] CI=Confidence interval.

Table 5
Drug A: Validation of TFC assay on a HTLC Turbo-C_{18} column by injection of 25 μl crude dog plasma

	Concentration (μg/l)			
	5.00	50.0	500	1.500
n	12	12	12	12
Student *t* value, *p*=95%	2.20	2.20	2.20	2.20
Mean (μg/l)	4.84	51.8	533	1456
SD (μg/l)	0.329	2.52	16.6	97.9
Precision (%)	6.8	4.9	3.1	6.7
Accuracy (%)	−3.1	3.7	6.7	−2.9
Upper limit of CI[a] (μg/l)	5.05	53.5	544	1518
Lower limit of CI[a] (μg/l)	4.63	50.2	523	1394

[a] CI=Confidence interval.

concentration in this sample was 56.5 μg/l (TFC–MS–MS) or 58.5 μg/l (LLE–LC–MS–MS), respectively.

The good agreement between the concentrations measured by the two independent assays clearly demonstrates that there were no interferences from drug metabolites, drug formulation or endogenous plasma constituents, therefore the TFC–MS–MS assay is selective.

3.6. Drug B: Validation results

Drug B, although structurally completely different from drug A, was run under the TFC conditions of drug A using the HTLC Turbo-C_{18} column as well as the Oasis HLB column. The results are given in

Table 6
Drug A: Combined of results obtained on a HTLC Turbo-C_{18} column and on a Oasis HLB column by injection of 25 μl crude dog plasma

	Concentration (μg/l)			
	5.00	50.0	500	1.500
n	11	12	12	12
Student *t* value, *p*=95%	2.23	2.20	2.20	2.20
Mean (μg/l)	4.85	50.3	524	1458
SD (μg/l)	0.237	1.94	16.3	46.4
Precision (%)	4.9	3.9	3.1	3.2
Accuracy (%)	−3.1	0.5	4.8	−2.8
Upper limit of CI[a] (μg/l)	5.00	51.5	534	1487
Lower limit of CI[a] (μg/l)	4.68	49.0	513	1428

[a] CI=Confidence interval.

Table 4
Drug A: Validation of TFC assay on a HTLC Turbo-C_{18} column by injection of 25 μl crude mouse plasma

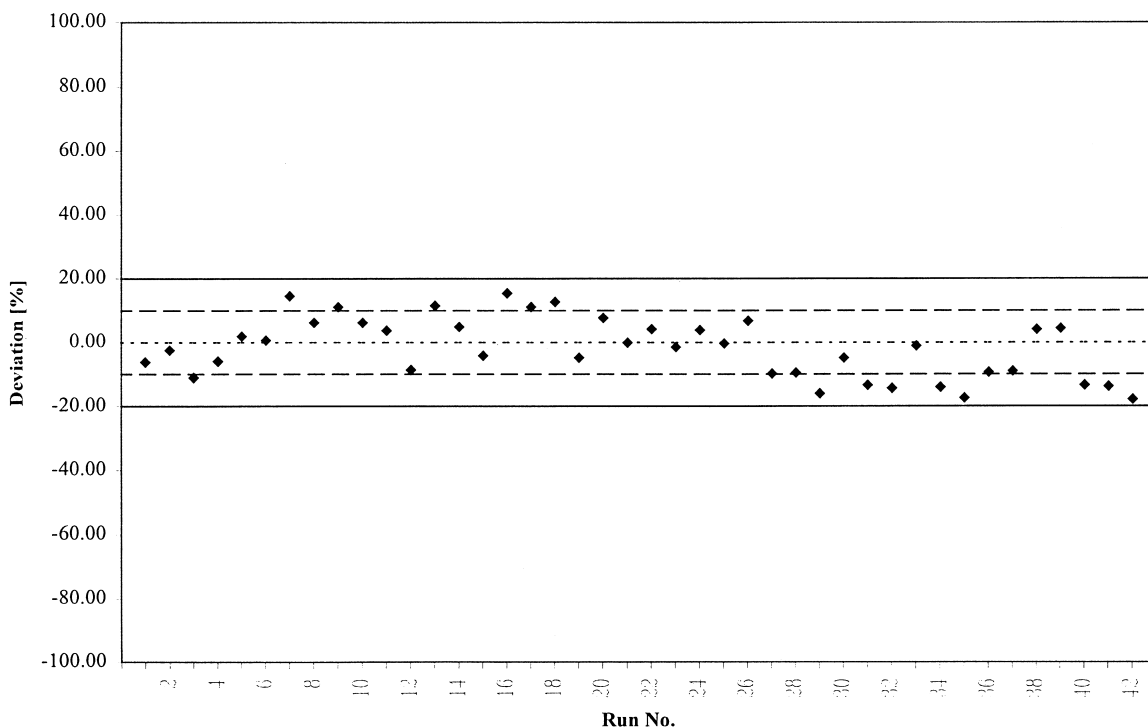|	Concentration (μg/l)				
	5.00	50.0	500	1.500	1.0 (LOQ)
n	12	12	12	12	16
Student *t* value, *p*=95%	2.20	2.20	2.20	2.20	2.13
Mean (μg/l)	5.05	51.4	522	1437	0.957
SD (μg/l)	0.433	4.25	21.4	49.0	0.0712
Precision (%)	8.6	8.3	4.1	3.4	7.4
Accuracy (%)	1.0	2.8	4.4	−4.2	−4.3
Upper limit of CI[a] (μg/l)	5.33	54.1	536	1468	0.995
Lower limit of CI[a] (μg/l)	4.78	48.7	508	1406	0.919

[a] CI=Confidence interval.

Fig. 4. Drug A: Cross Validation TFC versus LLE reference method with study samples after oral administration of 0.3 mg/kg to dogs. Deviation of concentration measured by TFC from results by LLE.

Tables 7 and 8. The results at four concentrations from crude rat plasma were similar for both types of columns except with poorer precision and accuracy for the Oasis near the LOQ (1.00 µg/l): accuracy 3.7 to 6.7%, near the LOQ −15.9% (Oasis) and −3.7 to 3.5% (Turbo-C_{18}), precision 0.8 to 9.1%, near the LOQ 20.6% (Oasis) and 1.6 to 5.4% (Turbo-C_{18}). A comparison of the TFC results with the reference assay shows good agreement between TFC and protein precipitation (Table 9). Accuracy and preci-

Table 7
Drug B: Validation of TFC assay on a HTLC Turbo-C_{18} column by injection of 25 µl crude rat plasma

	Concentration (µg/l)			
	2.00	10.0	100	1000
n	3	6	6	6
Student *t* value, *p*=95%	4.30	2.57	2.57	2.57
Mean (µg/l)	2.07	10.3	98.2	963
SD (µg/l)	0.111	0.323	1.93	14.9
Precision (%)	5.4	3.1	2.0	1.6
Accuracy (%)	3.5	2.9	−1.8	−3.7
Upper limit of CI[a] (µg/l)	2.35	10.6	100	978
Lower limit of CI[a] (µg/l)	1.79	9.95	96.1	947

[a] CI=Confidence interval.

Table 8
Drug B: Validation of TFC assay on a Oasis HLB column by injection of 25 µl crude rat plasma

	Concentration (µg/l)			
	2.00	10.0	100	1000
n	4	6	6	6
Student *t* value, *p*=95%	3.18	2.57	2.57	2.57
Mean (µg/l)	1.68	10.6	107	1037
SD (µg/l)	0.347	0.962	2.34	8.73
Precision (%)	20.6	9.1	2.2	0.8
Accuracy (%)	−15.9	6.3	6.7	3.7
Upper limit of CI[a] (µg/l)	2.23	11.6	109	1046
Lower limit of CI[a] (µg/l)	1.13	9.62	104	1028

[a] CI=Confidence interval.

Table 9
Drug B: Comparison of validation data – TFC assay versus manual protein precipitation

Nominal concentration (μg/l)	Drug B	Turbulent flow chromatography		Reference, Protein precipitation
		HTLC C$_{18}$ column	Oasis column	
2.00	Replicates (n)	3	4	8
	Precision (%)	5.4	20.6	10.5
	Accuracy (%)	3.5	−15.9	6.9
10.0	Replicates (n)	6	6	12
	Precision (%)	3.1	9.1	8.6
	Accuracy (%)	2.9	6.3	5.5
100	Replicates (n)	6	6	12
	Precision (%)	2.0	2.2	3.6
	Accuracy (%)	−1.8	6.7	2.8
1000	Replicates (n)	6	6	12
	Precision (%)	1.6	0.8	3.9
	Accuracy (%)	−3.7	3.7	0.0
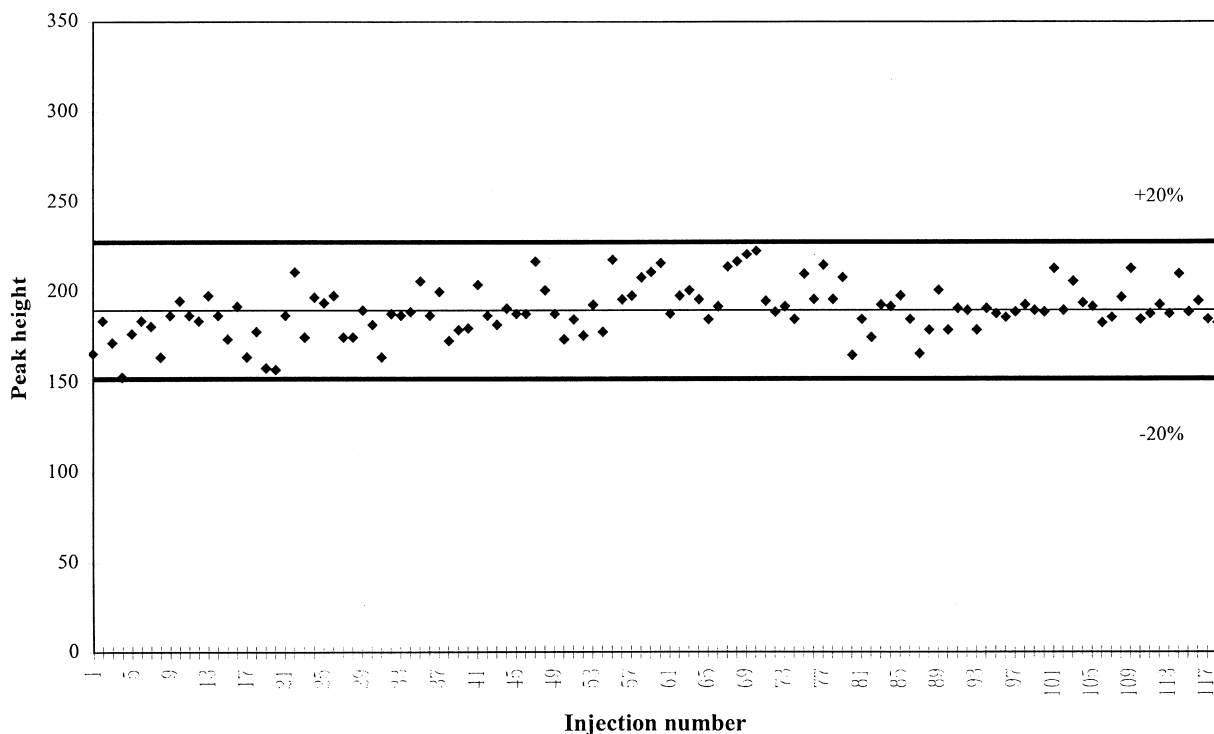
Fig. 5. Ruggedness test on Oasis column (118 injections). Peak height of drug A (1.00 μg/l) in dog plasma analyzed by TFC–MS–MS.

sion were better than 10% except near the LOQ with the Oasis column, where accuracy was −15.9% and precision 20.6%.

3.7. Ruggedness and life-time of columns

A ruggedness test was performed with drug A on a Oasis HLB column (Fig. 5).

The ruggedness test with drug A demonstrated a low variability of the peak areas and heights. The peak height of drug A showed a RSD of 7.3% from 118 injections at 1.00 μg/l (LOQ) and a RSD of 9.0% from 44 injections at 10.0 μg/l. The peak height of the I.S. had a RSD of 8.5 and 9.5% calculated from the 1.00 μg/l and the 10.0 μg/l samples (in total 162 injections), respectively.

The columns lasted for about 400 to 600 injections of 25 μl of undiluted crude plasma.

4. Conclusions

TFC is a pioneering technique for the direct analysis of even highly protein bound drugs from crude plasma. TFC eliminates the need for time-consuming sample clean-up in the laboratory, instead of this one 96-well plate with 96 plasma samples is analyzed by TFC–MS–MS within 5.25 h, corresponding to 3.3 min per sample (which can be shortened by reduction of the autosampler duty cycle). Two structurally completely different compounds were analyzed under identical TFC conditions and one assay was fully validated for the application to GLP studies. With TFC even better validation results were achieved than with automated 96-well SPE and manual LLE.

The sensitivity of the assays with an LOQ of 1.00 μg/l and the linear working range of three- up to four-orders of magnitude fully met the demands of preclinical pharmacokinetic and toxicokinetic studies. The quality of analysis by TFC measured in terms of accuracy and precision compares well with the reference LLE assay. Similar chromatographic performance and validation results were achieved using original Turboflow columns (Cohesive Technologies) and the Oasis columns (Waters). TFC–MS–MS proved to be rugged and selective.

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