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Ultra-fast quantitative bioanalysis of a pharmaceutical compound using liquid chromatography-tandem mass spectrometry

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Abstract

This paper describes the ultra-fast determination of a pharmaceutical compound using TurboIonSpray™ LC-MS-MS on an API 4000 mass spectrometer. Sample preparation consisted of plasma protein precipitation, centrifugation and dilution of the supernatant. The use of small analytical column dimensions (2.1 mm × 10 mm) and high eluent flow rates (up to 2.2 ml/min) in isocratic mode led to a retention time of 9 s. A sample-to-sample cycle time of only 10 s was achieved by coupling two autosamplers. Partial separation of the drug and its main metabolite could be obtained. The d5-labeled drug used as internal standard compensated for matrix suppression effects. The assay was linear in the concentration range 1–1000 ng/ml, using standards prepared in human plasma. Inter-assay accuracy and precision were 98.5 and 6.2%, respectively. Mean intra-assay accuracy and precision calculated from quality control (QC) samples in human, rat and dog plasma at 3, 30 and 800 ng/ml were 100.8 and 3.8%, respectively. The ultra-fast LC-MS-MS method was successfully cross-validated against a commonly used column-switching LC-MS-MS assay with 2.3 min run time by analyzing real study samples.

Keyword: Ultra-fast quantitative bioanalysis

1. Introduction

In the pharmaceutical industry, it is important to reduce the time for drug discovery and development. Therefore, there is a great need for fast analytical methods to support pre-clinical and clinical studies. The quantification of drugs at low concentrations in complex matrices (plasma, urine, tissues) in the presence of metabolites and endogenous components demands high selectivity and sensitivity. Liquid chromatography-tandem mass spectrometry is an efficient analysis tool providing low detection limits, reduced influence of interferences and the possibility for shorter run times. During the last few years high-throughput techniques have emerged, including fast, automated sample handling and preparation as well as data analysis and interpretation [1,2]. To improve the sample throughput in general, two approaches are possible: serial and parallel. Parallel approaches are realized by coupling several columns (and also sprayers) while using "conventional" LC run times. The serial mode speeds up the chromatography

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through the use of small column dimensions and significantly increased eluent flow rates. Short columns, large particle sizes or an increased column temperature can reduce the column backpressure normally resulting from such high solvent flows. Capillary LC at 130 μl/min achieved on a 0.18 mm × 50 mm Oasis HLB column with 30 μm particles combined good sensitivity with higher speed and low sample/solvent consumption [3]. Monolithic columns are well suited for high-speed separations in bioanalysis because the low backpressure enables high flow rates of up to 6 ml/min on 4.6 mm × 50 mm analytical columns while still providing sufficient chromatographic resolution [4]. Although fast gradient analysis was reported (e.g. eight beta-blockers within 65 s on a 4.6 mm × 30 mm C18 column at 2 ml/min [5]), isocratic conditions are more suited to achieve short run times. Dextromethorphan was determined in dog plasma with 0.6 min retention time and 1 min entire LC-MS-MS run time using a Symmetry C8 2.1 mm × 50 mm column enabling the analysis of more than 1000 samples within 19 h [6]. The separation of mixtures of five benzodiazepines or tricyclic amines was achieved in 15–18 s on a 2.1 mm × 15 mm C18 column at 1.9 ml/min [7,8]. Separation of idoxifene and its main metabolite was achieved within 10 s on a Phenomenex Luna C18 1 mm × 30 mm at 0.7 ml/min applying a temperature of 70 °C to reduce the backpressure [9]. However, the cycle time of conventional autosamplers (usually at least 1 min) is not short enough to keep up with these fast separations. Multiple probe liquid handlers, such as the eight-port Gilson 215 used to provide a 23 s cycle time for the idoxifene assay [9], are not always available. Coupling four autosamplers to one analytical column and one MS, the run time for five benzodiazepines in human urine was reduced to 30 s per sample enabling the analysis of more than 1000 samples within 12 h [10]. Estrogen receptor modulators were determined in human plasma using the same setup to achieve a throughput of 2000 samples in 24 h [11].

A reduction of the overall analysis time is achieved by implementing automated parallel off-line sample pretreatment in the 96- or 384-well format (protein precipitation [5], liquid–liquid extraction [9–11] and SPE [2,12,13]). The possibility to directly inject biological materials into turbulent flow systems [14] eliminates time-consuming sample preparation. Automated on-line SPE-LC-MS-MS using commercially available devices (“Prospekt” [15]) or in-house assembled column-switching systems with single or dual extraction columns [16,17] can provide efficient clean-up and short run times.

This paper presents the development and validation of an ultra-fast LC-MS-MS method for the determination of a drug candidate in plasma. Coupling two autosamplers to one short, narrow-bore analytical column, a sample-to-sample cycle time of 10 s was achieved. The results from analysis of real study samples were compared with a conventional column-switching HPLC-MS-MS assay.

2. Experimental

2.1. Chemicals and materials

The drug, its phenol metabolite and the d5-labeled drug used as internal standard (ISTD) were obtained from F. Hoffmann-La Roche (Basle, Switzerland). The purity of the substances were 99.4% for the drug, 98% for the metabolite and 99% for the ISTD. HPLC-grade acetonitrile (ACN) was purchased from Rathburn (Walkerburn, Scotland). Methanol (MeOH), ethanol (EtOH), acetic acid (HOAc), formic acid and ammonium acetate (NH_4OAc) pro analysi were obtained from Merck (Darmstadt, Germany). All solutions were prepared using in-house generated doubly distilled water. Human control plasma was purchased from a blood bank (Blutspendezentrum SRK Beider Basel). Rat and dog plasma was obtained from animals used for pharmacokinetic and toxicokinetic experiments in our facility.

2.2. Solutions and standards

Stock solutions of internal standard and analytes (500 μg/ml) were prepared in ethanol. Internal standard working solution (100 ng/ml) to use as protein precipitation solvent for plasma samples was prepared by diluting the stock solution with ethanol. Internal standard working solution and doubly distilled water were mixed 3:1 to obtain the internal standard dilution solution for diluting samples with high analyte concentrations. The analyte stock solution (different weighing for calibration standards and quality

control samples) was diluted with ethanol/water 70:30 to obtain spiking solutions. Calibration standards in the range 1–1000 ng/ml were prepared by spiking human control plasma (volume of spiking solution ≤2% of matrix volume). Quality control (QC) samples at low (3 ng/ml), medium (30 ng/ml) and high (800 ng/ml) levels were prepared by spiking the matrix to be assayed (drug-free rat, dog and human plasma). Both calibration standards and QC samples were divided into aliquots of 200 μl in 1.5 ml Eppendorf vials and stored at −20 °C until use.

2.3. Sample preparation (off-line)

Study samples were thawed at room temperature together with calibration standards and QC samples. To aliquots of 200 μl (or less) plasma the three-fold volume of internal standard working solution was added. After short mixing, the samples were stored for 5–10 min in the deep freezer at approximately −20 °C to achieve optimal protein precipitation. The samples were centrifuged for 5 min at 15,000 rpm (ca. 18,000 × *g*) at 10 °C in a Heraeus Sepatech Megafuge 2.0 R. If the expected sample concentration exceeded the calibration range, the supernatant was diluted with internal standard dilution solution after centrifugation. High QC samples were treated the same way to provide dilution QC samples. A 10-μl aliquot of the supernatant was injected into the column-switching system. The ultra-fast LC-MS-MS method required dilution with two-fold volume of water to reduce the elution strength before injecting 10 μl directly onto the analytical column.

2.4. HPLC system for fast LC-MS-MS

The LC system consisted of a L-6200A pump and a L-6000A pump (both Merck-Hitachi, Tokyo, Japan) operating in high-pressure gradient mode using a 75-μl dynamic mixer (Labsource, Reinach, Switzerland), two autosamplers (AS 1 and AS 2, both Merck AS 4000), a solvent degassing unit SDU 2003 and a switching valve high speed valve 7000E (Labsource) for coupling the two autosamplers to one analytical column (Fig. 1, top). Several analytical columns, such as XTerra MS^{TM} C18 (2.1 mm × 30 mm or 2.1 mm × 10 mm, 3.5 μm particle size and 2.1 mm × 20 mm, 2.5 μm particles) and XTerra^{TM} RP18 (2.1 mm × 10 mm, 5 μm particles) from Waters (Milford, MA, USA), were tested under isocratic and gradient conditions. The analytical columns were protected using a C18 2 mm × 4 mm SecurityGuard^{TM} cartridge (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water/ACN/formic acid. Flow rates between 1 and 2.2 ml/min were applied.

2.5. Column-switching HPLC system

An in-house assembled system (Merck-Hitachi, Tokyo, Japan), as shown in Fig. 1 (bottom), was applied for column-switching LC-MS-MS. The L-6200 Intelligent Pump (P1) was used for trapping and washing, while the pump L-6000 (P3) served for on-line dilution of the injection solution. For gradient separation on the analytical column, pumps L-6200A (P2A) and L-6000A (P2B) operated in high-pressure mode using a 75-μl dynamic mixer (Labsource). The autosampler was a Merck AS 4000. The solvent degassing unit SDU 2003 and the two switching valves (high speed valve 7000E) were from Labsource. As trapping column (TC), the XTerra^{TM} RP18 (2.1 mm × 10 mm, 5 μm particle size) from Waters was applied. The analytical column (AC) was a XTerra MS^{TM} C18 (2.1 mm × 50 mm, 3.5 μm particles) from Waters, protected by a C18 2 mm × 4 mm SecurityGuard^{TM} cartridge (Phenomenex). Mobile phases were 20 mM NH_4OAc/ACN 98:2 (1A) for on-line dilution, trapping and rinsing, water/EtOH 20:80 (1B) and pure ethanol (1C) for washing of the trapping column in between injections. Mobile phase compositions for elution from the trapping column and separation on the analytical column were water/ACN 77:23 containing 15 mM NH_4OAc and 1% acetic acid (2A) and water/ACN/MeOH 10:45:45 containing 5 mM NH_4OAc and 1% acetic acid (2B). The sample is transferred to the TC within 0.3 min with mobile phase 1A using pumps P1 and P3 at 1 and 2 ml/min, respectively, to reduce the elution strength of the injection solution by on-line dilution. After that, the TC is rinsed with 1A (P1) at 3 ml/min for 0.2 min. Valve V1 is then switched to backward flow, and the TC is rinsed for 0.1 min. By switching V2, the trapping column is coupled with the analytical column, and the analyte is transferred with mobile phase 2A (pump P2) at 0.4 ml/min within 0.6 min. During this time, the capillaries are rinsed with 1B. After

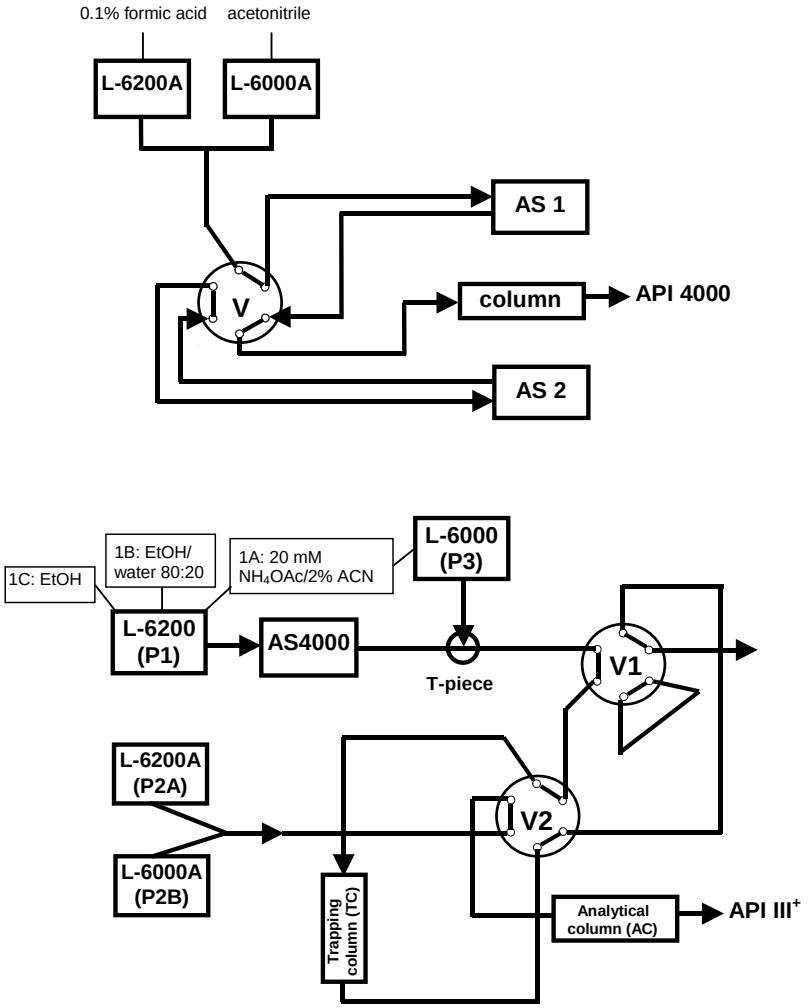

Fig. 1. Schemes of ultra-fast dual autosampler setup (top) and column-switching LC-MS-MS setup (bottom).

switching V2 to separate TC and AC, the gradient on P2 is started to elute the analyte from the AC (2A to 2B within 0.5 min at 0.35 ml/min). The trapping column is rinsed at 3 ml/min with 1B (0.2 min), 1C (0.2 min) and finally, 1A for re-equilibration (0.6 min). V1 is switched to forward flow 0.1 min before the program is finished. The sample-to-sample cycle time is 2.3 min.

2.6. Mass spectrometer

The API 4000 (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) was used for the ultra-fast LC-MS-MS method, the API III$^+$ triple quadrupole mass spectrometer from PE Sciex for the column-switching LC-MS-MS assay. TurboIonSpray^{TM} in positive ion selected reaction monitoring mode (SRM) was applied as ionization technique. Nitrogen served as nebulizer, auxiliary and curtain gas, argon was used as collision gas in the API III$^+$, nitrogen as collision gas in the API 4000. Gas flow rates, temperatures, ionization voltages and collision energies were optimized for the compounds by infusion of 0.1 ng/μl (API 4000) or 1 ng/μl (API III$^+$) standard solutions of the analytes in MeOH/water/acetic acid 50:50:1 at 20 μl/min and by flow injection analysis at

the LC flow rate. The drug was monitored at a transition of *m/z* 259 → 242 and the metabolite at *m/z* 231 → 214. The MS-MS transition for the internal standard was *m/z* 264 → 247. Data acquisition was performed with dwell times of 50 ms (API 4000) or 120 ms (API III$^+$). The quadruples 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 using a mixture of quaternary ammonium salts.

2.7. 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. Seven calibration standards were prepared. Validation of the lower limit of quantification (LLOQ) was performed by analyzing five replicates and calculating the values for precision and accuracy against one calibration curve. Furthermore, duplicate sets of calibration standards were analyzed on 3 days (1 as calibration and 2 as quality control) to obtain data on inter-assay precision and accuracy. Quality control samples, prepared in plasma at three levels, were analyzed ($n = 5$) and calculated with one set of calibration standards to obtain data on intra-assay precision and accuracy. Recovery was determined as a combined value of extraction recovery and ionization suppression by comparing peak areas of analyte in spiked water (100% value) and spiked human, dog and rat plasma at 3, 30 and 800 ng/ml (five replicates). Stability investigations (24 h at room temperature, 3 and 6 months at −20 °C, freeze–thaw stability and autosampler stability) were performed using the column-switching method and are not further discussed in this paper. No degradation of the substance was observed in all experiments.

3. Results and discussion

Our goal was to demonstrate the possibility of pushing the chromatography for the LC-MS-MS analysis of a drug candidate in biological samples towards retention times below 15 s. The mass spectrometer API 4000 with its new Turbo-V ion source is well suited to tolerate high eluent flow rates and “dirty” matrices. Furthermore, very good sensitivity is obtained on this instrument. Thus, we selected it for our ultra-fast analysis approach. Good MS response was obtained for our drug in positive mode ionspray ionization due to an amino group in the molecule structure. The loss of 17 mass units (NH_3) was monitored in MS-MS. Although this is not a specific fragmentation, no interfering influence was observed in all matrices investigated.

Short analytical columns used with high eluent flow rates are required for very short run times. The XTerra^{TM} RP18 or MS C18 materials were found to be endurable and provided good retention behavior and peak shape for a variety of drugs in previous experiments. Columns of 30, 20 and 10 mm length could provide fast determination of our compound with retention times less than 20 s. Fast gradient analysis was possible using 2.1 mm × 20 mm XTerra MS^{TM} C18 column (2.5 μm particle size) with a step gradient from 0.2% formic acid/ACN 90:10 to 10:90 within 0.1 min at 1 ml/min with a retention time of 15 s ($k' = 0.8$, $t_0 = 9$ s). Re-equilibration of the analytical column with the initial mobile phase was necessary, leading to an overall run time of at least 40–50 s. Higher speed was obtained with isocratic elution, leading to a retention time of less than 15 s ($k' = 0.3$, $t_0 = 9$ s) with no need for equilibration. Therefore, the gradient approach was not investigated further. However, the possibility for gradient runs in less than a minute can be provided, with the potential advantage of better sensitivity (factor two higher signal-to-noise) and less matrix suppression effects (1.5-fold lower) than obtained with isocratic elution.

High back-pressure on 20 mm column limited a further increase of speed by increased flow rate, whereas flow rates of more than 2 ml/min could be applied on the 2.1 mm × 10 mm XTerra^{TM} RP18 column. Using mobile phase compositions with 25–30% acetonitrile and flow rates of 1.8–2.2 ml/min, the retention time of the drug was decreased to about 6 s ($k' = 1$, $t_0 = 3$ s). Fig. 2 shows representative SRM-LC-MS-MS chromatograms for blank plasma (A), for the drug at LLOQ of 1 ng/ml (B) and for the corresponding internal standard (C) in human plasma. No interferences at the selected transitions for analyte and internal standard were observed in all blank matrices investigated (plasma from different human individuals as well as from dogs and rats). Sample workup

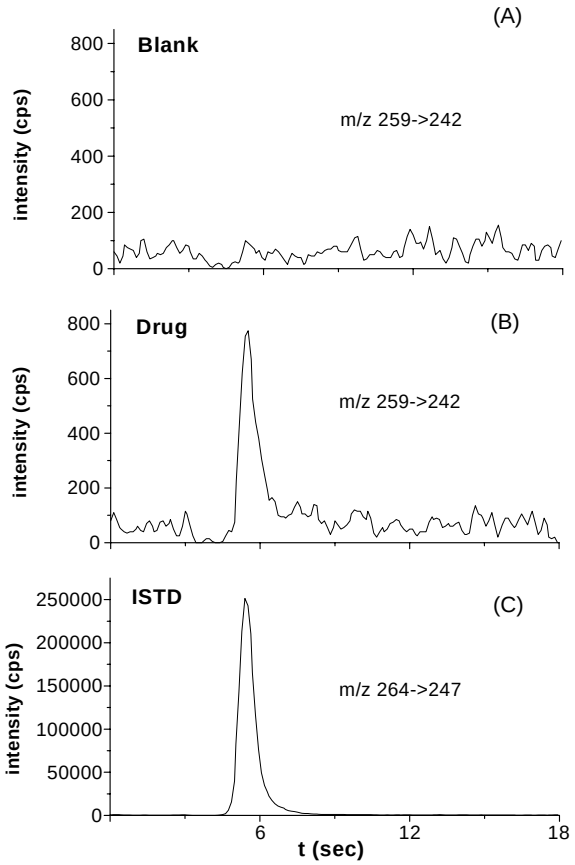

Fig. 2. SRM-LC-MS-MS chromatograms of (A) blank plasma at *m/z* 259 → 242, (B) drug spiked to plasma at LLOQ 1 ng/ml (*m/z* 259 → 242), and (C) internal standard (*m/z* 264 → 247); column: 2.1 mm × 10 mm 5 μm XTerra™ RP18; mobile phase: 25% acetonitrile in 0.1% formic acid; flow rate: 2 ml/min.

involved only protein precipitation of plasma with the three-fold volume of ethanol containing the internal standard (d5-drug), centrifugation and dilution of the supernatant with the two-fold volume of water to reduce the elution strength. The required LLOQ with a signal-to-noise ratio of approximately 10 was achieved by injecting only 10 μl of the sample solution.

With the very short retention time of the analyte, the autosampler became the bottleneck for increased throughput. A 20 s cycle from injection to injection was the fastest sample turnaround time that could be obtained using the Merck AS 4000, optimizing all steps of the autosampler program (sample uptake, injection and washing). Only one inside needle wash and loop rinse with 500 μl solvent was programmed. Carryover was minimized by using 1% formic acid in ethanol as rinsing solvent. The first blank plasma injected after the highest standard (2500 ng/ml) gave a peak corresponding to 0.5 area% of the previous injection, while the second blank gave a completely clean chromatogram. With the coupling of two autosamplers, it should be possible to achieve a run time of 10 s. Both autosamplers (AS) were connected to one analytical column via a switching valve. Fig. 1 (top) shows the setup. The AS 1, AS 2 and valve were controlled by the L-6200A HPLC pump, which got a busy signal from the mass spectrometer. When the MS was ready for data acquisition, the busy signal was turned off and the pump gave a start signal to AS 1, which had already a sample in the loop and was waiting to inject. MS data acquisition was then started by a signal from AS 1 to the MS. In the meantime, AS 2 loaded a sample and waited for the signal from pump to inject. The valve was switched by a signal from the pump to connect either AS 1 or 2 to the analytical column. It was important that the connecting capillaries from both autosamplers to the switching valve possessed the same dead volume. Otherwise, retention time differences between the two runs occurred. No delay time resulted from the MS data acquisition because the MS did not control the HPLC pump and autosamplers. The acquisition time was 9 s, while the real time between injections was 10 s. Examples for SRM-LC-MS-MS chromatograms of rat plasma samples from a pharmacokinetic study analyzed using the dual autosampler setup are presented in Fig. 3.

The simple sample preparation and fast chromatography led to questions about matrix influences on the analyte determination. Considerable matrix suppression effects were observed, partly due to the minimum sample clean-up (protein precipitation and dilution only) and partly due to the short retention time (elution near interfering plasma components). The mean peak area of the analyte in plasma compared to the peak area in water was only 30.1 ± 3.9%. This value corresponds to matrix suppression because the recovery (determined by comparing post-extraction spikes with samples spiked before protein precipitation) was nearly 100%. The area ratios (analyte/internal standard) in plasma compared to area ratios in water were 95.4 ± 3.8% in average because the internal standard (five-fold deuterated drug) compensated the matrix

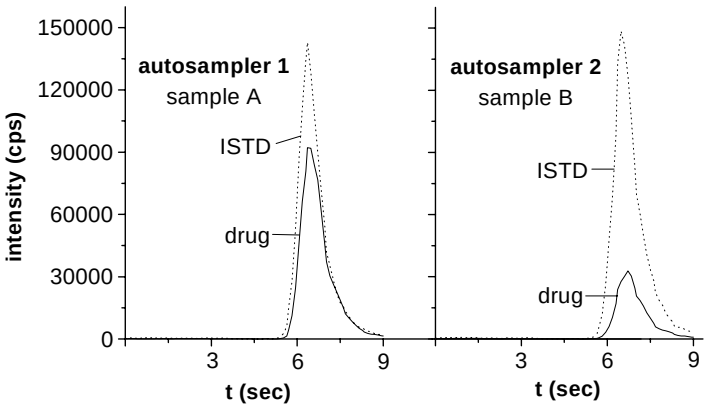

Fig. 3. SRM-LC-MS-MS chromatograms of drug (*m*/*z* 259 → 242) and ISTD (*m*/*z* 264 → 247) in two rat plasma samples analyzed using the dual autosampler setup; column: 2.1 mm × 10 mm 3.5 μm XTerra^{TM} RP18 5 μm; mobile phase: 25% acetonitrile in 0.1% formic acid; flow rate: 2 ml/min.

influence. As long as the required limit of quantification can be achieved, and the accuracy and precision data meet the acceptance criteria, no actions are necessary to reduce the matrix effects.

Even with the fast chromatography some selectivity could be achieved. A more polar main metabolite (phenol resulting from de-ethylation) could be partially separated from the drug (Fig. 4). Chromatographic resolution was increased when using a lower content of organic solvent ($k'_{[drug]} = 1.2$, $k'_{[metabolite]} = 0.7$, $R = 0.3$ for 30% acetonitrile; $k'_{[drug]} = 1.4$, $k'_{[metabolite]} = 1$, $R = 0.4$ for 25% acetonitrile; and $k'_{[drug]} = 2$, $k'_{[metabolite]} = 1.2$, $R = 0.7$ for 20% acetonitrile). The eluent flow rate was adapted to still allow an elution time below 10 s. Matrix suppression was higher for the metabolite than the drug because interference with early eluting matrix compounds due to the shorter retention time was more likely. Therefore, sensitivity for the metabolite with the ultra-fast method was lower than for the parent drug. We did not perform further investigations regarding metabolite analysis because the goal was to develop a method for the parent drug only. However, we believe that quantification of the metabolite may be possible with the ultra-fast assay, particularly when using an isotopically labeled internal standard

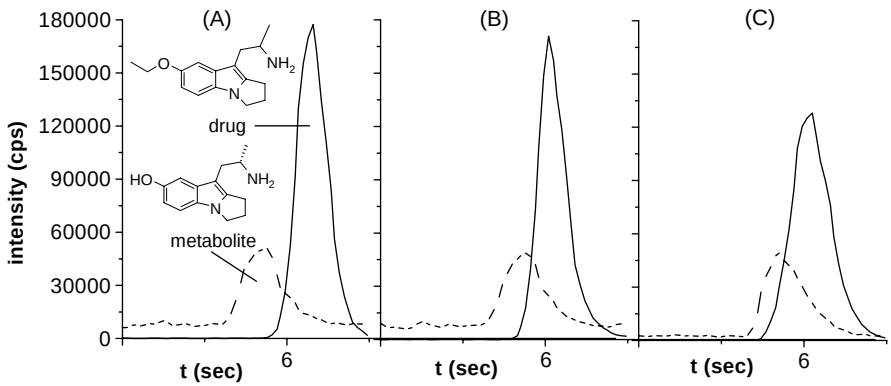

Fig. 4. Separation of drug and phenol metabolite using the dual autosampler setup with mobile phase containing (A) 20%, (B) 25% and (C) 30% acetonitrile in 0.1% formic acid; flow rates: 2.2, 2 and 1.8 ml/min, respectively; column: 2.1 mm × 10 mm XTerra^{TM} RP18 5 μm; selected reaction ion current profiles at *m*/*z* 259 → 242 (drug) and *m*/*z* 231 → 214 (metabolite).

Table 1
Inter-assay accuracy and precision (%) of ultra-fast LC-MS-MS assay ($n = 5$)[a]

QC	Human plasma		Rat plasma		Dog plasma	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
Low[b]	95.6	6.7	100.4	7.3	102.5	8.5
Mid[b]	95.3	7.7	106.9	5.8	94.9	4.4
High[b]	89.9	4.5	100.2	7.3	101.2	3.8

[a] For human plasma, $n = 4$.
[b] Low/mid/high QC: human 2.5/50/1000 ng/ml, rat and dog 3/30/800 ng/ml.

for compensation of matrix influences. The use of a structural homologue is possible but it may not experience the same chromatographic and ionization conditions as the analyte and is therefore not an ideal internal standard. Therefore, the ultra-fast method is more suited for drug development, where stable isotope-labeled drugs are available, than for the drug discovery field.

To perform method validation and sample analysis, calibration standards were prepared in human plasma to cover the range of 1–1000 ng/ml. Accuracy and precision at the LLOQ of 1 ng/ml were 106.2 and 4.5%, respectively (mean of five determinations, calculated against one calibration curve). Quality control (QC) samples were prepared in human, dog and rat plasma. Data on inter-assay validation are presented in Table 1. Inter-assay mean accuracy and precision were 98.5 and 6.2%, respectively. Intra-assay mean accuracy and precision were 100.8 and 3.8%, respectively (see Table 2). Values for precision and deviations from accuracy were far below 15% for each species at all QC levels.

Plasma samples from pharmacokinetic and toxicokinetic studies (60 dog and rat samples) were used for cross-validation of the ultra-fast assay against a validated column-switching method. The sample clean-up via a trapping column was advantageous for complex matrices (tissues) compared to direct injection onto the analytical column, even with low injection volumes. The experimental setup for column-switching LC-MS-MS is shown in Fig. 1 (bottom). The automated on-line dilution of the sample solution saved one additional off-line sample preparation step. Backflush rinsing of the trapping column and backflush elution with optimized elution time was useful to remove matrix components more efficiently. A comparatively short overall run time of 2.3 min was achieved. Fig. 5 shows the selected ion

Table 2
Intra-assay accuracy and precision (%) of ultra-fast LC-MS-MS assay ($n = 5$)

Concentration (ng/ml)	Human plasma		Rat plasma		Dog plasma	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
3	102.8	5.9	103.4	2.8	100.5	2.5
30	98.4	5.6	103.4	1.8	105.6	2.3
800	97.2	9.7	96.2	2.2	99.4	1.7

Table 3
Inter-assay accuracy and precision (%) of column-switching LC-MS-MS assay ($n = 5$)

Concentration (ng/ml)	Human plasma		Rat plasma		Dog plasma	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
1	101.1	5.7	99.6	7.5	106.1	8.3
30	95.4	2.3	97.8	2.9	96.1	9.8
1000	87.3	2.0	91.5	9.6	91.5	4.0

Table 4
Intra-assay accuracy and precision (%) of column-switching LC-MS-MS assay ($n = 5$)

Concentration (ng/ml)	Human plasma		Rat plasma		Dog plasma	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
3	97.6	9.2	96.7	9.8	96.6	3.8
30	99.2	5.3	97.4	1.3	96.3	4.1
800	93.0	2.7	94.6	1.9	92.6	2.0

chromatograms obtained for a blank human plasma (A), for a calibration standard at the LLOQ of 1 ng/ml (B) and for the internal standard in the LLOQ sample (C). Calibration samples were prepared in human plasma, while QC samples were prepared in the matrix to be assayed. The method was successfully applied for the routine determination of the drug in plasma, urine and tissues. Tables 3 and 4 show inter- and intra-assay validation data for plasma from different species. The recovery (including matrix suppression) of this method was near 100% due to an efficient clean-up during the trapping process. According to our guidelines for cross-validation, less than 15% of the unknown samples are allowed to deviate by more than 15% from the concentration values obtained with the reference assay. The cross-validation of the ultra-fast assay was successful, with only 8% of the samples deviating by more than 15% from the results obtained with column-switching LC-MS-MS. Larger deviations between the methods occurred only with sample concentrations near the LLOQ. The average bias was +3.4%. These data show that the ultra-fast assay can provide reliable results without relevant interference from endogenous matrix compounds or metabolites.

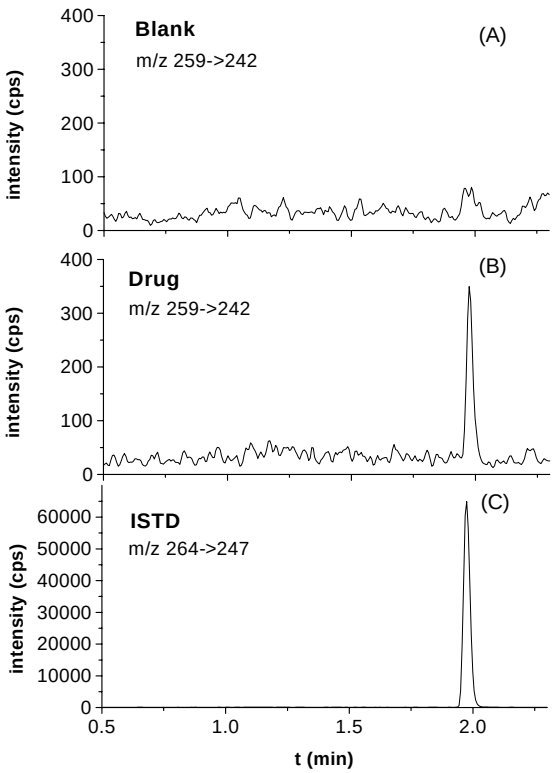

Fig. 5. Column-switching SRM-LC-MS-MS chromatograms of (A) blank plasma at *m/z* 259 → 242, (B) drug spiked to plasma at LLOQ 1 ng/ml (*m/z* 259 → 242), and (C) internal standard (*m/z* 264 → 247); for conditions, see Table 1.

4. Conclusions

An ultra-fast LC-MS-MS method was developed and validated for the determination of a pharmaceutical drug in plasma. The TurboIonSpray™ source on the API 4000 mass spectrometer was well suited to accommodate the high eluent flow rates used. Isocratic separation on a small analytical column (XTerra™ RP18, 2.1 mm × 10 mm, 5 μm particle size) provided retention time of as low as 6 s. Coupling two autosamplers to one analytical column resulted in an injection cycle of 10 s. The partial separation of the drug and its main metabolite demonstrated that some selectivity could be achieved even with this fast chromatography. Matrix suppression effects were compensated by the deuterium labeled drug used as internal standard. The sensitivity of the assay was sufficient to measure plasma profiles in samples from PK and toxicology studies. Calibration was linear in the investigated range of 1–1000 ng/ml. Good accuracy and precision were achieved for quality control

samples prepared in human, rat and dog plasma. The cross-validation results from rat and dog study samples showed that the ultra-fast LC-MS-MS assay is equivalent to a usually applied, validated and robust method. The run time of 6 h for a typical tray of 150 samples using a common column-switching method can be shortened to about 30 min using the ultra-fast method. Rapid analysis does not require a lot of effort. The current mass spectrometers are sensitive and can accommodate high solvent flow rates. Fast autoinjectors are available, or common autosamplers can be coupled. However, matrix suppression, interferences and carryover have to be addressed. The value of such short analysis time is the possibility to provide results for dose range finding studies very rapidly. Thus, bioanalytics is not the "bottleneck" in the drug development process. "On-line" analytics is important for ascending dose studies to know drug exposures immediately. Using ultra-fast analytical assays it is even possible to analyze all samples from an entire clinical study in 1 day to save resources. Furthermore, the use of the mass spectrometer is maximized. An efficient off-line sample preparation, such as automated SPE or liquid–liquid extraction in the 96-well format, may be suited to reduce matrix effects and to increase the robustness of the ultra-fast LC-MS-MS method for routine use. The potential of the technique will be further explored, including the analysis of metabolites also. We believe that the application of chip technologies (integrated sample preparation and separation on chip, spray directly to the MS) will gain importance in high-throughput analysis of drugs in the near future. Up to now, we consider a combination of high-throughput off-line sample workup and fast LC-MS separation as the most promising approach for high-speed analysis.

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