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Sensitive determination of a pharmaceutical compound and its metabolites in human plasma by ultra-high performance liquid chromatography–tandem mass spectrometry with on-line solid-phase extraction

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ABSTRACT

This paper describes the determination of a drug candidate and two metabolites in human plasma by column-switching LC–MS/MS after protein precipitation. Starting from a standard method with a quantitation limit of 0.5 ng/mL, a highly sensitive assay was developed, employing UHPLC separation and detection on an API 5000 mass spectrometer. The injected plasma equivalent was increased from 6 to 20 μL; conventional column trapping for compound enrichment and removal of matrix constituents was combined with high-pressure analytical separation using small particle columns to improve resolution and signal-to-noise ratio. Quantitation limits were thus lowered to between 5 and 20 pg/mL, offering the possibility to provide bioanalytical support for microdosing studies in humans. Excellent assay quality and robustness were achieved by both methods.

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1. Introduction

Microdose studies have gained importance as a tool in clinical development of drug candidates during the last years [1,2]. A common microdose design is to measure human clearance and absolute bioavailability by the simultaneous dosing of an intravenous microdose of labeled drug with a clinical dose administered by the intended route (e.g., oral or subcutaneous) [3]. As only very low levels (less than 1/100th of the predicted pharmacological dose but not more than 100 μg [2]) of the drug are used, analytical methods are limited because extreme sensitivity is needed. Accelerator Mass Spectrometry (AMS) is the most common method for ^{14}C microtracer analysis [4], and this powerful technique has supported the extension of microdosing into other areas than PK: drug–drug interaction studies, metabolism investigations, concentration determination in cells and tissues [2]. Disadvantage of AMS is that samples have to undergo extensive sample preparation leading to the loss of any structural information. To circumvent this, fractionation of samples by HPLC and subsequent sample preparation have to be applied with the associated increase in time and costs. With the development of sensitive instruments, liquid chromatography–tandem mass spectrometry (LC–MS/MS) can reach the required limits for microdosing studies, and has the power to distinguish in a single run between drug and metabolites. In this case, stable isotope labeled drug is given on top of and at the same time as the unlabeled drug. Applications have been described already [5,6]. Achieving very low quantitation limits by LC–MS/MS usually requires high sample volumes, efficient clean-up and preconcentration of analytes. Powerful separation is needed to remove matrix components which can cause interfering peaks or ionization suppression. For detection, a high-end mass spectrometer should be preferred using the most sensitive and selective SRM transition. Even in ultra-sensitive analysis there are demands for high-throughput capabilities, short run times, and reduced manual labor and costs. Most sensitive analytical methods employ for sample preparation solid-phase extraction [5,7] or liquid–liquid-extraction [6] using as much as 1 mL of plasma to achieve LLOQs down to 1 pg/mL. Also on-line SPE has been described as efficient approach for sample enrichment and clean-up [8–11]. Separations using small particles for ultra-performance LC, orthogonal mechanisms such as hydrophilic interaction LC, narrow-bore and capillary columns, or nano-technologies (LC on chips) can contribute to high sensitivity as well as selectivity [10–15].

In this manuscript, we describe two methods to determine drug X, a difluoro-ethyl-pyrrolidine analogue and its metabolites M1 (N-dealkylation) and M2 (hydrolysis of the amide bond), see Fig. 1 for abbreviated structures, in human plasma samples. The support of pharmacokinetics assessment in clinical trials, including microdosing studies, was required. A standard multi-analyte assay with a quantitation limit of 0.5 ng/mL and a highly sensitive assay to

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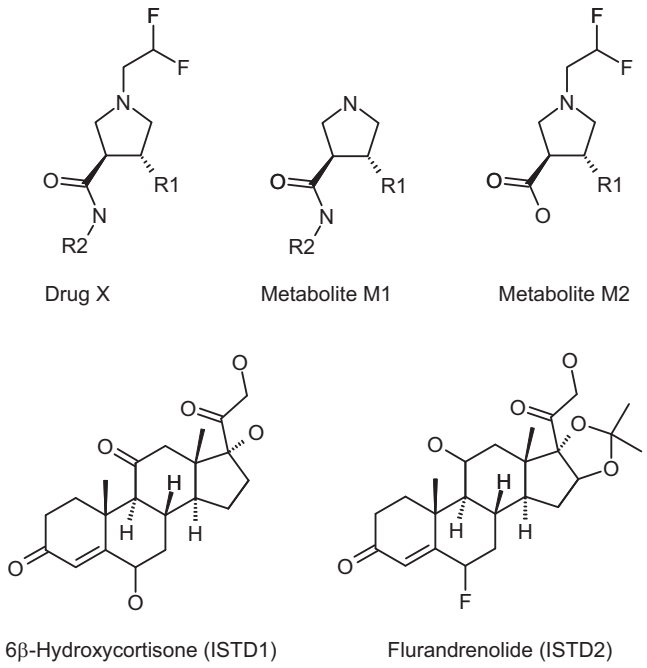

Fig. 1. Structures of drug, metabolites and internal standards. Isotopically labeled analogues are 4-fold deuterated on R1.

detect analyte concentrations as low as 5 pg/mL were developed. The request to simultaneously determine, together with the drug, two polar metabolites with different chromatographic and ionization behavior added an additional challenge. Our attempts at increasing the sensitivity are explained in detail. We combined on-line solid-phase extraction with UHPLC analytical separation, and demonstrate here our method development approach and present validation results. Our method is highly automated, robust, cost-effective and is suggested as generic approach for very sensitive quantitation.

2. Experimental

2.1. Chemicals, solutions and standards

Ethanol and methanol (Lichrosolv for HPLC) were obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Ammonium formate (p.a.), formic acid 98–100% (Suprapur grade), acetic acid (100%, p.a.) and ammonium hydroxide were purchased from Fluka (Buchs, Switzerland). The water used for the preparation of all solutions was obtained from a Milli-Q apparatus (Millipore, Billerica, MA, USA) fed with deionized water. Blank EDTA human plasma was purchased from a blood bank (TRINA Bioreactives, Nänikon, Switzerland). Drug X (MW 520 g/mole, ClogP 3.6), metabolite M1 (MW 456 g/mole, ClogP 2.4), metabolite M2 (MW 409 g/mole, ClogP −0.5) and the deuterated analogues X-d4 (molecular weight MW 524 g/mole), M1-d4 (MW 460 g/mole) and M2-d4 (MW 413 g/mole) were synthesized at F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Fluradrenolide (MW 436.5) was purchased from Sigma–Aldrich Inc. (St. Louis, USA) and 6β-hydroxycortisone (MW 376.7) from Steraloids Inc. (Newport, USA). Stock solutions of analytes and internal standards were prepared in DMSO at 1 mg/mL. These stock solutions were mixed and diluted further with ethanol to provide spiking solutions, which were added to blank EDTA human plasma for the preparation of calibration standards and quality control samples in the ranges 0.5 and 2500 ng/mL (standard assay) or 5 pg/mL to 10 ng/mL (UHPLC assay). Internal standard solutions were prepared in ethanol, containing 25 ng/mL of X-d4, M1-d4 and M2-d4 (standard assay) or 1.5 ng/mL flurandrenolide and 20 ng/mL 6β-hydroxycortisone (UHPLC assay).

2.2. Sample preparation

To 50 μL of plasma standard, QC or study sample, 200 μL of internal standard solution was added (Tecan Genesis RSP 100/4, Tecan Schweiz AG, Männedorf, Switzerland). The samples were vortexed (Heidolph model Reax 2000; Heidolph Instruments, Schwabach, Germany) and centrifuged (Heraeus Multifuge 3 S-R, Thermo Electron LED, Zürich, Switzerland).

2.3. Chromatography

2.3.1. Standard assay

The trapping and analytical columns were Gemini C18, 2 mm i.d., 5 μm with 10 and 50 mm length, respectively (Phenomenex, Torrance, US). The autosampler was a SIL-HTc with integrated system controller SCL-10AD. A trapping pump (LC-10ATvp, Shimadzu, Kyoto, Japan) delivered mobile phase A1 for trapping (5 mM ammonium formate and 0.2% formic acid in water) or alternatively B1 for rinsing after the trapping process (5 mM ammonium formate and 0.2% formic acid in water–acetonitrile 10:90 (v/v)). The dilution pump was a L-6000A (Merck-Hitachi, Tokyo, Japan); it was connected via a T-junction with the trapping pump and delivered 5 mM ammonium formate and 0.2% formic acid in water at a flow rate of 2.5 mL/min. The electrically driven switching valve 7000E (Labsource, Reinach, Switzerland) connected the effluent of the trapping column either to waste or onto the analytical column. A high pressure gradient HPLC system composed of two LC-10ADvp delivered the mobile phases A2 (5 mM ammonium formate and 0.2% formic acid in water–acetonitrile 80:20 (v/v)) and B2 (5 mM ammonium formate and 0.2% formic acid in water–acetonitrile 10:90 (v/v)). All HPLC components were controlled by the Xcalibur 2.0 software. The sample solution (30 μL) was injected onto the trapping column with mobile phase A1 at 0.2 mL/min with simultaneous on-line dilution at 2.5 mL/min for 0.8 min. Polar unwanted sample constituents were rinsed off while analytes and ISTDs were retained. Analytes and internal standards were then transferred to the analytical column in back-flush mode using 100% of solvent A2 at a flow rate of 0.3 mL/min. At 1.6 min, trapping and analytical columns were disconnected, and a rapid gradient separation was performed by increasing solvent B2 to 100% within 1.5 min. At 3.1 min after injection, the initial mobile phase composition was re-established. The trapping column was rinsed with solvent B1 between 1 and 2 min to minimizing possible carry-over effects and then reconditioned with the initial trapping solvent A1 with a flow rate of 2 mL/min. The total run time was 3.6 min.

2.3.2. UHPLC assay

The trapping column was a 10 mm × 2 mm Gemini C18, 5 μm particle size. The analytical column was a 50 mm × 2 mm Luna C18(2)-HST, 2.5 μm particle size placed into the column heater at 60 °C. The autosampler was an HTS PAL (CTC Analytics, Zwingen, Switzerland) equipped with a 200 μL sample loop. Needle and valve rinse was performed using ethanol/water 90:10 (v/v). A 1200-series quaternary pump (Agilent Technologies, Waldbronn, Germany) delivered solvent A1 for trapping (5 mM aqueous ammonium formate) or alternatively solvent B1 for rinsing after the trapping process (5 mM ammonium formate in water–acetonitrile 5:95 (v/v)). The dilution pump was a LC20AT (Shimadzu, Kyoto, Japan) controlled by a CBM-20A module; it was connected with the trapping valve (VICI Valco, Houston, TX, USA) to allow a two-way on-line dilution. The dilution solvent was 5 mM aqueous ammonium formate. A 1200-series binary pump (Agilent) delivered the analytical mobile phases A2 (5 mM aqueous ammonium formate)

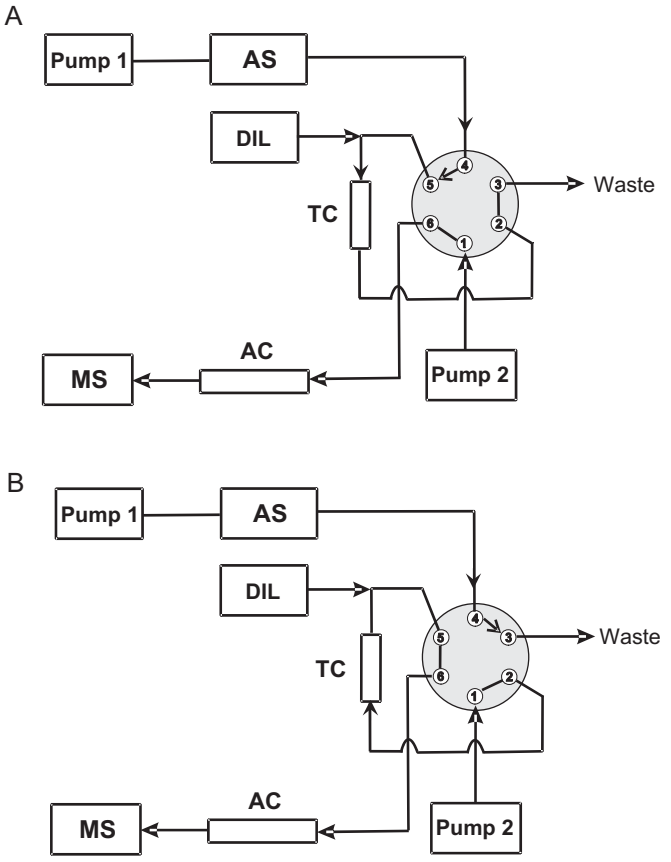

Fig. 2. Scheme of column-switching UHPLC system with on-line dilution: (A) trapping process, (B) elution process. AS: autosampler; TC: trapping column; AC: analytical column; Pump 1: trapping pump; Pump 2: analytical pump; DIL: dilution pump; MS: mass spectrometer.

and B2 (5 mM ammonium formate in water–methanol–acetonitrile 10:30:60 (v/v/v)). The sample solution (100 μL) was injected onto the trapping column with mobile phase A1 at a flow rate of 0.2 mL/min and was diluted with 2.5 mL/min during 1.6 min (Fig. 2A). Analytes and internal standards were then transferred to the analytical column in back-flush mode between 1.8 and 2.2 min using 25% B2 at a flow rate of 0.5 mL/min while on-line dilution occurred at 0.15 mL/min (Fig. 2B). At 2.2 min, the solvent B2 flow rate was increased to 0.6 mL/min and the solvent B2 content to 45%, followed by a rapid gradient within 0.6 min to 100% of solvent B2. At 3.3 min, the initial mobile phase composition (25% B2) was re-established. The trapping column was rinsed with solvent B1 between 1.9 and 2.7 min and then reconditioned with the initial trapping solvent A1 at a flow rate of 2 mL/min. The total run time was 4 min.

2.4. Mass spectrometry

2.4.1. Standard assay

A TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo Fisher Scientific, SanJose, CA, USA) equipped with an electro spray ionization source was used in the positive selected reaction ion monitoring (SRM) mode. Data acquisition was performed on a Dell Optiplex GX270 computer with software Xcalibur 2.0 distributed by Thermo Fisher Scientific. Sheath, auxiliary and sweep gas were nitrogen set at 50, 10 and 5 instrument units, respectively. Argon was used as collision gas at 1.5 mTorr. The spray voltage was set at 4500 V and the capillary temperature at 300 °C. Data acquisition was performed in three time triggered periods using a scan time of 50 ms for each transition.

2.4.2. UHPLC assay

An API 5000 triple quadrupole mass spectrometer (AB/MDS Sciex, Ontario, Canada) equipped with a Turbo V IonSpray source was used in the positive ion selected reaction monitoring (SRM) mode. Data acquisition was performed on a Dell Precision 380 computer with Analyst 1.4.2 software distributed by AB/MDS Sciex. The curtain gas, ion source gas 1, ion source gas 2 and collision gas (all nitrogen) were set at 12, 60, 50 and 5 instrument units, respectively. The spray voltage was 5500 V, the heater temperature was 750 °C, the interface heater was turned on, and the entrance potential was set to 10 V. Data acquisition was performed in three periods using a dwell time of 55 ms for each transition.

The parameters were optimized by infusion and flow injection of the compound solutions at 0.1–1 ng/μL. Unit mass resolution (peak width set to 0.7 Da for Q1 and Q3 at half peak height) was used. Specific settings for each compound are shown in Table 2.

2.5. Data handling and validation procedures

Either Xcalibur 2.0 (TSQ Quantum Ultra) or Analyst 1.4.2 (API5000) were used for integration of chromatograms and calculation of analyte concentrations. The calibration curve ($y = a + bx$) was obtained by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak areas or peak-area ratios analyte/internal standard (y) versus the analyte concentration added to the plasma (x).

The following parameters were addressed according to current method validation guidelines [16,17]: selectivity, inter- and intra-day precision and accuracy, recovery, matrix effect, stability and incurred samples reproducibility. Any interfering peak at the retention time of the analyte should have a response lower than 20% of that of the analyte at the lower limit of quantitation (LLOQ). The precision determined at each concentration level should not exceed 15% CV except for the LLOQ where it should not exceed 20% CV. The mean value of each concentration level should be between 85% and 115% of the nominal value except at LLOQ, where it should be between 80% and 120%. Matrix factors, obtained by comparing the peak areas from spiked plasma sample solutions with those from matrix-free reference solutions, should be in the range of 1.00 ± 0.15 according to internal acceptance criteria. Stability tests in plasma were conducted according to internal guidelines, which are based on a published procedure [18]. Stability was examined by comparing the response (peak areas or area ratios analyte/ISTD) of freshly prepared samples with that of spiked samples maintained at the storage conditions. Five aliquots of each set of samples at two concentrations were analyzed.

3. Results and discussion

3.1. Standard assay development

A generic column switching assay [19] which is standard procedure in our laboratories was modified to optimize separation, peak shape and intensity of the parent drug and its two more polar metabolites. Usually, C18 trapping and analytical columns and mobile phases containing 0.1 or 0.2% formic acid and various contents of acetonitrile or methanol are successfully employed for a variety of drug candidates (unpublished data). However, the most polar metabolite M2 suffered from variable retention time and unsymmetrical peak shape, and the intensity for M1 was not satisfactory. The addition of 5 mM ammonium formate to the mobile phases improved the reproducibility of retention time and the peak shape of M2. The signal-to-noise ratio for M1, the least sensitive substance, increased nearly two-fold. Due to the narrow peaks obtained with fast gradient separation, data acquisition

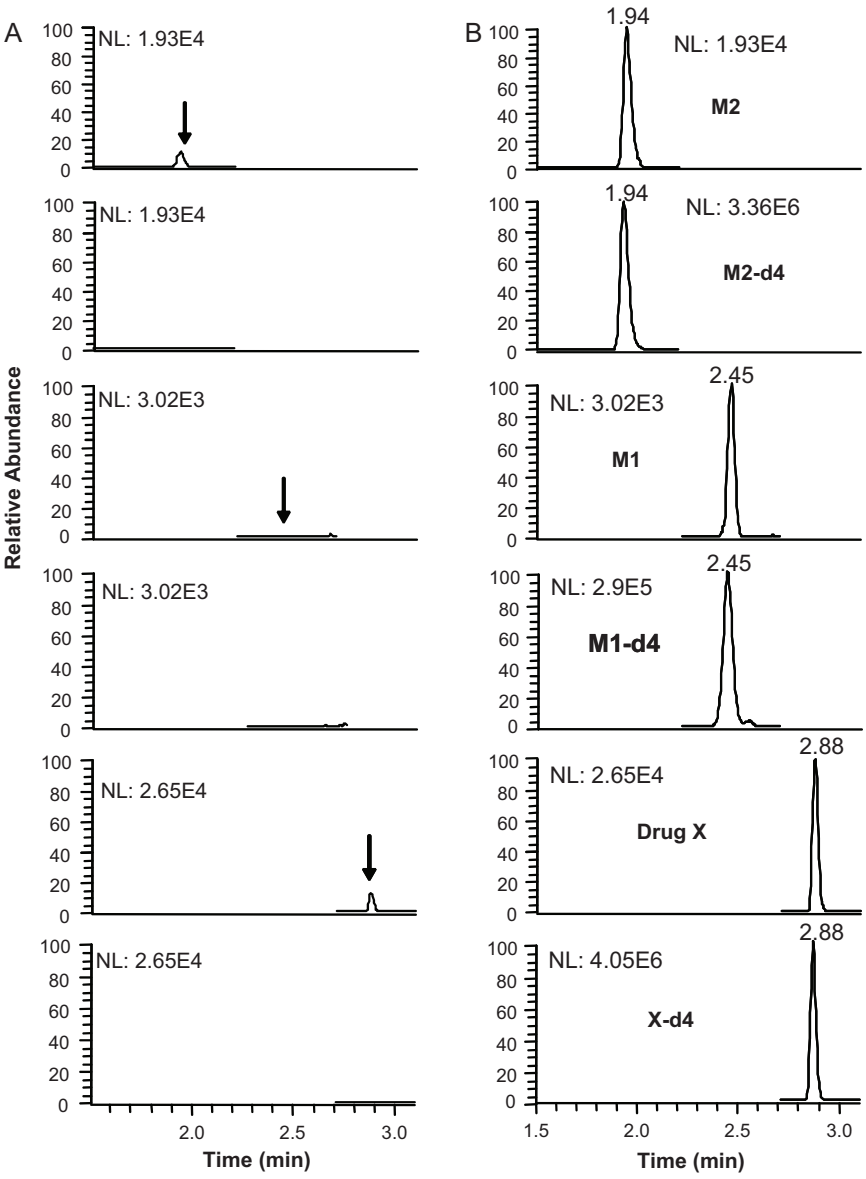

Fig. 3. Extracted ion chromatograms of drug, metabolites and their internal standards in human blank plasma (A) and in human plasma spiked at 0.5 ng/mL (B) obtained with the standard method.

was performed in three time triggered periods to provide a sufficient number of data points across each chromatographic peak. Fig. 3 shows representative extracted ion chromatograms for the drug, metabolites and their deuterated analogues (used here as internal standards) from double blank plasma (without drug and metabolites and internal standards) and from the lowest calibration standard at 0.5 ng/mL.

3.2. Attempts at reducing the quantitation limit

To achieve the required reduction of the LLOQ to support microdosing studies, the assay was transferred to an API5000 mass spectrometer, leading to approximately five-fold increased sensitivity. Further method optimization by using three parts methanol and six parts acetonitrile in solvent B2 for analytical gradient separation yielded in an additional two-fold improvement, but this was still not sufficient to quantify the compounds in the low pg/mL range. Increasing the injection volume to about 500 μL should in theory provide the necessary sensitivity. However, this would result in a 10-fold prolongation of the sample loading time onto the

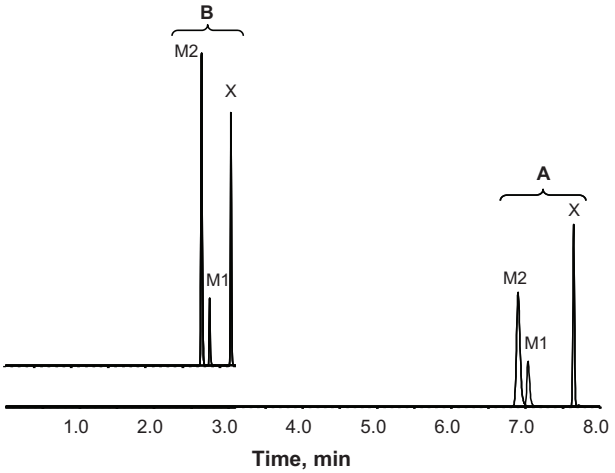

Fig. 4. Comparison of peak shapes and retention times of drug and metabolites using either regular LC (A) or the UHPLC method (B).

trapping column. Evaporation and reconstitution was not an option because of strong adsorption effects of the analytes on container walls. Solid-phase extraction would be suited as off-line enrichment procedure but is associated with increased manual labor and significant costs. By replacing the 2 mm × 10 mm trapping column with a 5 mm × 4.6 mm Onyx C18 column which possessed a low backpressure, the loading and the dilution flow could be increased to a total of 5 mL/min to speed up the trapping and rinsing process. In combination with conventional analytical separation, an entire run time of 9 min was obtained (Fig. 4 portion A). With theses measures, the LLOQ could be lowered to 10–20 pg/mL.

To further increase sensitivity and shorten LC run time we employed small particle analytical columns at high flow rates and developed a UHPLC assay, which is described in the following sections.

3.3. UHPLC assay development

3.3.1. Coupling of normal pressure trapping with high pressure analytical separation

The standard trapping column (2.0 mm × 10 mm, Gemini C18 Mercury, 5 μm particle size) was re-installed because increased

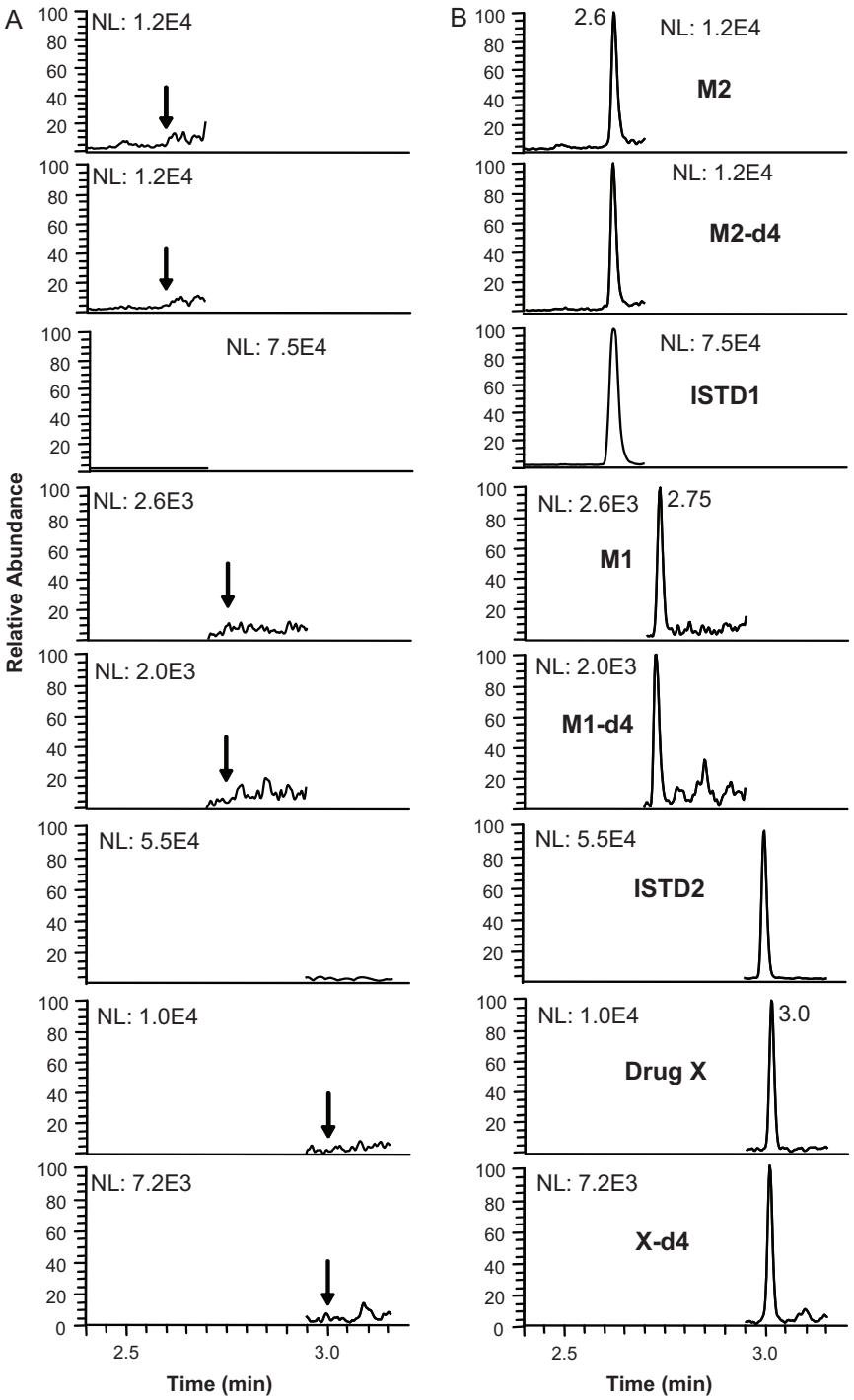

Fig. 5. Extracted ion chromatograms of drug, metabolites, deuterated analogs and their internal standards in human blank plasma (A) and in human plasma spiked at 20 pg/mL (B) obtained with the UHPLC method.

Table 1
Comparison of retention, peak shape and intensity of drug and metabolites on different analytical columns. Trapping column: Gemini C18 10 mm × 2 mm, 5 μm; injection volume 100 μL; gradient: from 25% B to 100% B within 0.8 min at 0.6 mL/min.

Analytical column (dimension 50 mm × 2 mm)	Pressure (psi)	Temperature (°C)	Rt/peak width at 20% height (min)/response(cps × 10^3)		
			M2	M1	Drug X
Zorbax SB-C18 1.8 μm	3900	80	2.39/0.06[a]/7.9	2.79/0.03/3.1	2.93/0.03/11
Zorbax SB-CN 1.8 μm	3800	80	2.25/0.09[a]/7.7	2.77/0.04/3.5	2.84/0.04/6.8
Aquity BEH130 C18 1.7 μm	4600	80	2.52/0.05/9.6	2.69/0.03/2.8	2.92/0.03/12
Hypersil Gold PFP 1.9 μm	4200	60	2.52/0.05/21	3.03/0.035/5	2.95/0.03/11[b]
Hypersil Gold Phenyl 1.9 μm	4400	60	2.59/0.045/6.5	2.79/0.04/2.5	2.98/0.03/18
Luna C18(2) HST 2.5 μm	4600	60	2.64/0.03/11	2.74/0.03/2.7	3.02/0.03/10
Ascentis Express C18 2.7 μm	3200	60	2.52/0.04/13	2.67/0.04/2.6	2.93/0.03/11
Atlantis T3 3 μm	2400	60	2.69/0.04/11	2.81/0.035/2.9	3.07/0.035/11
Gemini C18 5 μm	1900	60	2.67/0.04/12	2.75/0.035/1.2[c]	3.01/0.035/11

[a] Not suited because of peak tailing or shoulder.
[b] Not suited because of endogenous interference peak.
[c] Insufficient response.

sensitivity and hence lower injection volumes were expected with this assay. Optimizing the transfer of the analytes and their internal standards from the trapping to the analytical column was a critical step during method development. Elution with less than 25% of solvent B2 was not practical because it increased the elution time for the drug significantly. On the other hand, a mobile phase containing more than 22% of solvent B2 did not concentrate the first eluting metabolite M2 on the head of the analytical column in a narrow band, resulting in broad, tailing and unsymmetrical peaks. Analytical columns with small particles are sensitive to system dead volumes and prone to peak broadening if the connections and transfer process are not optimized. To improve the peak shape, on-line dilution was performed as depicted in Fig. 2. The first dilution step occurred when the sample was loaded onto the trapping column (Fig. 2A), as already done in the standard assay to properly trap the analytes out of the highly organic injection solution. After valve switching, the effluent from the trapping column (containing 25% of solvent B2) was diluted again with aqueous solution before entering the analytical column (Fig. 2B), resulting in a solvent B2 content of 19%. This supported focusing of all analytes in a narrow zone on top of the analytical column and gradient start at a lower organic solvent content. As a result, the peak shape for M2 improved. The peak shape of the later eluting metabolite M1 and of the drug was satisfactory already without the dilution procedure; however, peak width decreased and signal increased for all analytes.

3.3.2. Selection of analytical columns and separation conditions

Several analytical columns of 2 mm i.d., 50 mm length and small particle sizes between 1.7 and 2.5 μm were tested. The resulting retention times and peak widths for the drug and its two metabolites are shown in Table 1. In addition, data for 3- and 5-μm particle columns and from a fused-core (Ascentis Express) column obtained under UHPLC-like conditions are shown. Response, peak shape and separation on Ascentis Express and Atlantis C18 columns were comparable to those obtained with smaller particle columns, showing that UHPLC like separations are not limited to sub 2 μm columns. We selected the Luna C18(2) HST 2.5 μm column for further validation experiments because it provided good retention and symmetrical peak shape for the first eluting peak (M2), narrow peaks and sufficient response for all analytes, and because it was less sensitive to system dead volumes compared to smaller particle columns. Reducing the gradient time from 1.5 to 0.8 min yielded in higher signals and improved peak shape for M2. To compensate for the higher flow, the sprayer temperature on the API5000 was increased from 450 °C to 750 °C. This had in additional a positive effect on sensitivity because it doubled the signal intensity for all analytes. Compared with the conventional LC–MS/MS method, the UHPLC method used a 5-fold lower injection volume and yielded in peaks which were approximately 1.5 fold higher and half as wide. The run time was reduced to 4.0 min (see Fig. 4 portion B). No signal suppression or enhancement at the retention times of all analytes and internal standards was detected during compound infusion with simultaneous injection of blank matrix [20].

3.3.3. Selection of suitable internal standards

The deuterated drug which was used as ISTD in the standard assay would be co-administered to the "cold" drug in potential microdosing studies and thus forming the deuterated metabolites. Hence, there was a need to explore other compounds to use as ISTDs. First, the available structural analogues were tested: ethoxylated M2, methoxylated M2, 1-isopropyl-pyrrolidine drug analogue, trifluoroethyl drug analogue, and drug analogues in which a pyrrolidine moiety was replaced by cyclopentylidene or fluoro-cyclopentyl. None of these had a retention time close enough to the metabolites M1 or M2. The fluoro-cyclopentyl analogue eluted close to the drug; however, insufficient precision was observed during a test sequence with human plasma and therefore,

Table 2
Mass spectrometric parameters and quantitation data for the UHPLC-MS/MS assay.

Compound	Transition (*m*/*z*)	MS settings		Calibration range (pg/mL)	Calibration curve parameters		
		CE (eV)	DP (V)		Slope	Intercept	R^2
Drug X	520.2 → 392.1	31	120	5–10000	0.00434	0.00746	0.9972
X-d4	524.2 → 396.1	31	120	5–10000	0.00430	−0.0029	0.9977
Metabolite M2	410.1 → 205.1	32	105	20–5000	0.00090	0.000156	0.9982
M2-d4	414.1 → 209.1	32	105	5–5000	0.00065	−0.000159	0.9984
Metabolite M1	456.2 → 328.2	28	100	10–10000	0.00829	0.000183	0.9962
M1-d4	460.2 → 332.2	28	100	10–10000	0.00589	0.000185	0.9977
6β-Hydroxycortisone[a]	377.2 → 255.1	35	140	n/a	n/a	n/a	n/a
Flurandrenolide[b]	437.2 → 361.1	27	145	n/a	n/a	n/a	n/a

[a] ISTD1 for M1, M1-d4, M2 and M2-d4.
[b] ISTD2 for X and X-d4.

Table 3
Precision/accuracy (%) obtained with the UHPLC-MS/MS assay.

Conc. (pg/mL)	Drug X	X-d4	Metabolite M2	M2-d4	Metabolite M1	M1-d4
Intra-day ($n = 5$)						
5	3.0/110.8	12.1/105.3	*>20/>120*	9.6/106.7	*>20/99.2*	*No Peak*
10	4.7/98.5	7.7/94.3	*>20/119.0*	8.3/100.1	10.3/84.2	9.0/113.0
20	3.9/106.0	4.2/103.5	12.2/115.8	5.4/109.6	5.6/96.6	6.2/107.6
50	3.0/100.9	3.6/102.4	2.1/107.6	2.8/104.0	3.7/97.2	5.3/101.3
500	2.4/102.2	2.3/100.4	2.2/101.7	2.2/101.7	4.2/99.1	3.1/96.8
5000	2.8/98.5	2.4/98.1	2.7/98.2	2.7/98.2	4.4/102.7	4.0/102.4
10,000	3.1/85.5	2.3/88.7	*3.4/<85*	*1.5/<85*	1.7/96.0	1.1/95.2
Inter-day ($n = 3$ days $\times$ 5 replicates)						
20	4.7/102.8	5.6/103.7	14.9/111.9	9.2/108.3	6.6/101.1	7.4/103.5
100	6.5/101.9	5.0/101.7	8.5/104.0	8.7/104.1	8.9/101.8	8.4/101.7
1000	3.8/97.5	2.1/97.4	8.5/99.2	9.6/100.7	5.5/99.0	6.6/99.1

it could not be used as internal standard. Other not drug-related compounds, which were available in our laboratory, such as cortisone analogues and CYP450 inhibition substrates (e.g. midazolam and dextromethorphan), were also tested as internal standards. The closest retention time to the drug X and X-d4 was achieved with flurandrenolide, while 6β-hydroxy cortisone (6β-OHE) matched the retention time of M2 and M2-d4. No match was found for M1 and M1-d4; however, 6β-OHE was also used as ISTD for these pair of compounds because its retention time was only 0.12 min longer. Although 6β-OHE and flurandrenolide possess different structures than the analytes, they were well-suited as ISTDs, as demonstrated during method validation. They did not interfere with the ionization of the analytes, were stable during the analytical run, and did not suffer from matrix suppression. Fig. 5 shows representative extracted ion chromatograms for the analytes (unlabeled and labeled) and 6β-OHE and flurandrenolide used here as ISTDs from double blank plasma and from a calibration standard at 20 pg/mL.

3.4. Validation results

Full method validation was performed for the standard assay in the concentration range 0.5–2500 ng/mL. The inter-assay precision and accuracy for human plasma ranged between 0.5–2% (up to 17.6% at LLOQ) and 94–107%, respectively. The recovery ranged from 76% to 89% and the matrix factor from 0.9 to 1.1. The method proved to work reliably and robust throughout analysis of several thousand study samples. The acceptance criteria for routine analysis [16] were met in all analytical runs, and the deviations from the original value in incurred samples re-analyses were always below 20%. The drug and metabolites were found to be stable in human plasma when stored at ambient temperature for 24 h, at −20 °C for 4 months and for three consecutive freeze–thaw cycles from −20 °C to ambient temperature. The stock solutions of all compounds stored at −20 °C were stable for at least 4 weeks. Injection solutions of the compounds were found to be stable for at least 24 h when stored in the autoinjector at room temperature.

Method qualification (abbreviated method validation) was carried out to characterize the performance of the UHPLC assay. Concentration ranges and calibration curve parameters are compiled in Table 2. Inter- and intra-day precision and accuracy data are shown in Table 3. A valid concentration range between 5 pg/mL and 10 ng/mL was obtained for the drug X and deuterated drug X-d4. The deuterated metabolite M2-d4 was valid in range 5 pg/mL to 5 ng/mL. M1 and M1-d4 could be measured between 10 pg/mL and 10 ng/mL, while M2 was only quantifiable between 20 pg/mL (due to variations caused by underlying endogenous compound peaks) and 5 ng/mL. The use of the internal standards 6β-OHE and flurandrenolide, despite not being structural analogues, was of advantage to improve the precision and accuracy in particular at low analyte concentrations. No significant matrix effect (matrix factors ranged between 0.9 and 1.1) was observed for all analytes and ISTDs, which was in agreement to the findings of the infusion experiment.

4. Conclusions

On the example of a drug candidate and two metabolites, the development of a sensitive method for potential use in microdosing studies was described, starting from a standard LC–MS/MS assay. It was demonstrated that on-line SPE by column-switching under normal pressure could be combined with high-pressure separation. It was also shown that, in comparison to conventional LC using short narrow-bore columns with 3–5 μm particles, UHPLC with sub-2 μm columns and also UHPLC-like separations using larger particle columns could provide higher sensitivity (two-fold) and shorter run times (about factor 2.5) while maintaining the selectivity and reducing the sample volume by a factor of 5, resulting in absolut quantitation limits for drug X of 0.1 pg on column with UHPLC *vs.* 1 pg with conventional LC. The simultaneous determination of the drug and the two polar metabolites together with their deuterated analogues required more thorough method optimization than one-analyte assays usually need. The required sensitivity increase could not just be achieved by an increased sample equivalent and a more sensitive detection. Special emphasis was directed to successfully combine column trapping with UHPLC analytical separation, designing the on-line dilution process and optimizing the gradient program to obtain narrow, symmetrical peaks. Usually, SPE and liquid–liquid extraction are needed to clean the samples for injection onto a small particle column, but due to the on-line extraction, no elaborate off-line samples preparation was necessary. The suitability of this particular assay for real-world samples still needs to be proven, but the currently available data raise no concerns for reliable routine application. The approach is being extended to two more drug candidates for their determination at low pg/mL levels in human microdosing studies.

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