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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.

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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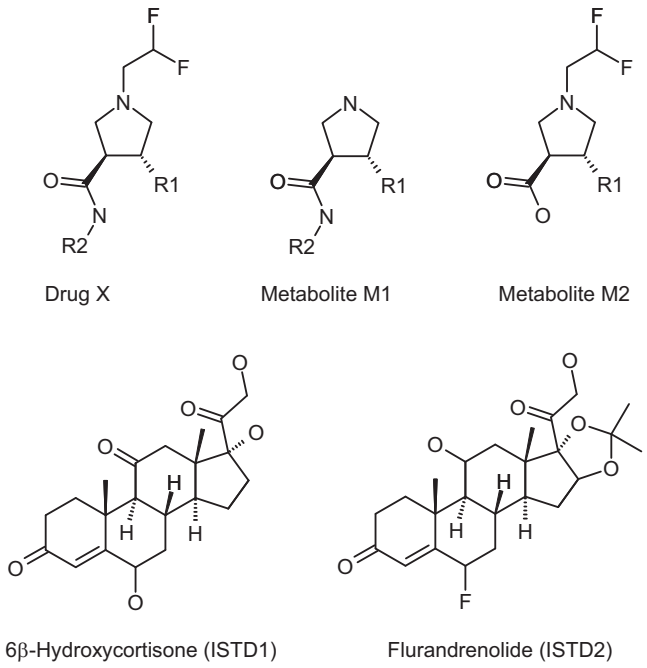

Fig. 1. Structures of drug, metabolites and internal standards. Isotopically labeled analogues are 4-fold deuterated on R1.

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)