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Parallel ultra high pressure liquid chromatography–mass spectrometry for the quantification of HIV protease inhibitors using dried spot sample collection format

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ABSTRACT

An assay was developed and validated for the quantification of eight protease inhibitors (indinavir (IDV), ritonavir (RTV), lopinavir (LPV), saquinavir (SQV), amprenavir (APV), nelfinavir (NFV), atazanavir (AZV) and darunavir (DRV)) in dried plasma spots using parallel ultra-high performance liquid chromatography and mass spectrometry detection in the multiple reaction monitoring mode. For each analyte an isotopically labeled internal standard was used and the assay based on liquid-solid extraction the area response ratio (analyte/IS) was found to be linear; from 0.025 μg/ml to 20 μg/ml for IDV, SQV, DRV, AZV, LPV, from 0.025 μg/ml to 10 μg/ml for NFV, APV and from 0.025 μg/ml to 5 μg/ml for RTV using 15 μl of plasma spotted on filter paper placed in a sample tube. The total analysis time was of 4 min and inter-assay accuracies and precisions were in the range of 87.7–109% and 2.5–11.8%, respectively. On dried plasma spots all analytes were found to be stable for at least 7 days. Practicability of the assay to blood was also demonstrated. The sample drying process could be reduced to 5 min using a commercial microwave system without any analyte degradation. Together with quantification, confirmatory analysis was performed on representative clinical samples.

1. Introduction

In bioanalysis, liquid chromatography coupled to mass spectrometry has established itself as the technique of choice, in the multiple reaction monitoring mode (LC-MRM/MS), for the quantification of pharmaceuticals in biological matrices. With the continuous improvement of instrument sensitivity multi-compounds assays using protein precipitation for sample preparation can be rapidly developed using small sample volumes (<50 μl). Dried blood spot (DBS) is a microsampling technique where the paper is the substrate on which the biological fluid, in general blood, is applied. While DBS has been used in the newborn screening field for the last twenty years, more recently significant efforts have been spent to investigate this sample collection format in different fields and in particular in pharmaceutical bioanalysis [1]. The major benefits are (i) microliter sample volumes, (ii) simplified sample shipment and storage, and (iii) straightforward sample preparation. Another field that would benefit from the DBS format is therapeutic drug monitoring (TDM) since the patient could ultimately perform sample collection themselves. Moreover, sample storage and shipment does not require refrigeration. As TDM aims to optimize a drug treatment by maximizing efficacy and reducing toxicity [2], it calls for reliable, precise and fast analytical methods at controlled cost. One area of interest for TDM is the monitoring of antiretroviral drugs in human immunodeficiency virus (HIV) infection [3]. Current antiretroviral (ARV) therapy combines different drugs to enhance activity and minimize the risk of drug resistance. HIV drugs are classified into several therapeutic categories by U.S. Food and Drug Administration (FDA): nucleoside or nucleotide reverse transcriptase inhibitor (NRTIs or NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, entry inhibitors and HIV integrase strand transfer inhibitors. PIs have been widely used for HIV infection treatment and TDM of PIs is currently considered a useful tool for the optimization of antiretroviral therapy in most international guidelines [3]. Various LC–MS methods have been reported for the analysis of PIs in plasma [4–7] but also in blood based on DBS [8,9].

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High-throughput quantitative analysis was also demonstrated for PIs using matrix-assisted laser desorption/ionization source interfaced to a triple quadrupole mass spectrometer [10,11]. For routine use efficiency, simplicity, robustness as well as cost of an analytical method are to be taken into consideration. Also short analysis times, including sample preparation, sample analysis and data processing, are highly desirable not only for sample throughput but also for fast delivery of the results. In these regards, ultra high-pressure liquid chromatography (UHPLC) allows the reduction of the analysis time and to increase the throughput significantly while maintaining very good separation efficiency. However, the method needs generally to be revalidated, because the assay selectivity may have changed. Another way to decrease or to tune the analysis time without changing the LC conditions is to apply parallel LC [12]. In this case a reduction of analysis time of 50% can be achieved. In the present work an assay was developed to quantify eight protease inhibitors for human plasma and blood using parallel ultra high-pressure liquid chromatography hyphenated to positive electrospray tandem mass spectrometry in multiple reaction monitoring mode.

To retain all the inherent advantages of DBS and to simplify sample collection and sample preparation a tube-based device was applied [13]. This device has several advantages over card format: (i) both sample collection and sample preparation are performed within the same device; and (ii) larger sample volumes can be collected. To reduce the drying time on the filter paper, a microwave-based drying approach was evaluated.

2. Experimental

2.1. Chemicals and reagents

Methanol was obtained from Fisher Scientific (Waltham, MA, USA). Acetonitrile was obtained from Biosolve B.V. (Valkenswaard, Netherlands). Acetic acid was obtained from Sigma–Aldrich (St. Louis, MO, USA). Indinavir (IDV), ritonavir (RTV), lopinavir (LPV) were obtained from Ontario Chemicals Inc. (Ontario, Canada). Saquinavir (SQV) and amprenavir (APV) were obtained from Moravek Biochemicals (Brea, CA, USA). Nelfinavir mesylate hydrate (NFV-mesylate-nH$_2$O) was obtained from Sigma–Aldrich. Atazanavir (AZV) was obtained from American Radiolabeled Chemicals (Saint Louis, MO, USA). Darunavir (DRV), indinavir-d_6 (IDV-d_6), nelfinavir-d_3 (NFV-d_3), amprenavir-d_4 (APV-d_4), darunavir-d_9 (DRV-d_9), atazanavir-d_5 (AZV-d_5), ritonavir-$^{13}C_3$ (RTV-$^{13}C_3$) and lopinavir-d_8 (LPV-d_8) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Saquinavir-d_5 (SQV-d_5) was obtained from F.Hoffmann-La Roche AG (Basel, Switzerland). Structures of analytes are shown in Fig. 1.

2.2. Tube-based sample collection device

Paper disks of 8.5 mm diameter were punched out from Protein Saver™ 903® cards (Whatman, Dassel, Germany) using a paper drill (STAGO GmbH, Neuffen, Germany). The disks were then inserted manually by pressure at the bottom of the inner side of the lid of 1.5 ml or 2.0 ml Safe-Lock (Eppendorf, Schönenbuch, Switzerland) tubes without any airgap. An exact volume of plasma or blood was deposited onto the paper disk.

2.3. Preparation of stock and spiking solutions

Stock solutions of each analyte were prepared by dissolving each analyte in methanol up to 10,000 μg/ml. From these stock solutions, 1.25, 2.5, 10, 25, 50, 100, 250, 500 and 1000 μg/ml spiking solutions contain all analytes (except for NFV 1.21, 2.41, 9.64, 24.1, 48.2, 96.4, 241, 482 and 964 μg/ml) were prepared in 50% MeOH/H$_2$O (v/v) just before fortifying plasma or blood samples. Stock solutions of each labeled internal standards (IS) were prepared by dissolving each IS in methanol up to 1000 μg/ml. All stock solutions were stored at −20 °C until further use and were found to be stable at least for two weeks.

2.4. Preparation of spiked human plasma and blood samples

Human citrate blood was obtained from the Centre de Transfusion Sanguine (Geneva University Hospital, Geneva, Switzerland) from healthy volunteers who gave informed consent and stored at 4 °C for a maximum of two weeks. Human citrate plasma was obtained from this blood by centrifugation of 1200 rpm (121 × g) for 30 min and stored at −20 °C until use. Plasma aliquots were thawed and sonicated for 3 min just before spotting or spiking. Spiked plasma samples were prepared by mixing 20 μl of appropriate spiking solutions with 980 μl of human plasma to obtain 25, 50, 200, 500, 1000, 2000, 5000, 10,000 and 20,000 ng/ml analytes (except for NFV 24.1, 48.2, 193, 482, 965, 1929, 4823, 9647 and 19,293 ng/ml). Spiked human blood samples were prepared by mixing 10 μl of appropriate spiking solutions with 490 μl of human blood to prepare samples with the same concentrations as for plasma. Spiked plasma samples were stored at −20 °C while spiked blood samples were prepared just before use and kept at 4 °C until spotting. Nine levels of spiked human plasma and blood were spotted by depositing an exact volume of 15 μl onto paper disks embedded in the lid of 1.5 ml tube-based devices, and used as calibration standards (Cal) or quality control samples (QC).

2.5. Sample collection and preparation

The procedure is summarized in Fig. 2. After spotting 15 μl of plasma or blood, by using an Eppendorf pipette (Eppendorf), onto the paper disk embedded into the tube-based device, all tube-devices were placed in a microwave oven containing 100 ml of water (InterTronic Solutions Inc., Quebec, Canada) for 5 min at 1200 W to dry and stabilize the samples. 500 μl of internal standard solution (15 ng/ml) in methanol was added into each tube. The lid of tube-based device was closed tightly and all devices were placed into a rack that was sealed in a zip-lock bag. The rack was turned upside down and was placed in TPC-120 ultrasonic cleaning (Telsonic AG, Bronschhofen, Switzerland) in such a way that the tube lids were in contact with the tank bottom. After 15 min of sonication, the filter paper was removed, and then methanol was evaporated in a vacuum centrifuge concentrator (Univapo 150 ECH, Uniequip, Planegg, Germany). 200 μl of a mixture consisting of 0.1% acetic acid in H$_2$O/CH$_3$CN (65/35, v/v) was added as reconstitution solvent and tubes were placed for 10 min at 10 °C in a Thermomixer comfort (Eppendorf) for mixing, followed by a centrifugation step at 14,000 rpm (16,435 × g) for 5 min at 4 °C in a Centrifuge 5804R (Eppendorf). 100 μl was transferred into a glass insert vial (BGB Analytik Vertrieb GmbH, Germany) and then 5 μl was injected.

2.6. Stability investigation for the drying process

The stability of the analytes during drying process was investigated according to the procedure described by Timm et al. [14]. In brief, 15 μl of 1000 ng/ml spiked human plasma was spotted onto ten filter papers embedded into 2.0 ml tube-based devices. Five of them were heated by the microwave oven as described in Section 2.5. The five remaining devices were dried at ambient temperature for 2 h. Furthermore, five additional devices were spotted by 15 μl of 1000 ng/ml spiked plasma just before homogenization. The homogenization step was done by adding 20–30 stainless steel grinding beads of 2 mm diameter (Retsch, Haan, Germany)