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Rapid, simultaneous determination of lopinavir and ritonavir in human plasma by stacking protein precipitations and salting-out assisted liquid/liquid extraction, and ultrafast LC–MS/MS

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ABSTRACT

Lopinavir and ritonavir are co-formulated in Kaletra® approved for the treatment of human immunodeficiency virus infection. A validated analytical method is mandatory for clinical development and therapeutic drug monitoring. Here we are reporting a method for rapid, simultaneous determination of lopinavir and ritonavir in human plasma with stacked protein precipitations and salting-out assisted extraction (SALLE), and ultrafast LC–MS/MS detection. With stacked protein precipitations and SALLE, the sample preparation for a 96-well plate can be completed within 20 min by an automated pipette. Due to the unique cleanliness of SALLE extracts post double protein precipitations, the extracts were injected into an ultrafast liquid chromatography and tandem mass spectrometry system (LC–MS/MS) after simple dilution. An Agilent Zobax Extend-C18 Rapid resolution HT column (1.8 μm, 2.1 mm × 30 mm) was used for the separation. A mixture of acetonitrile:water (55:45, v/v) with 0.1% formic acid was used as the mobile phase. LC ran for approximately 48 s at a flow rate of 0.5 mL min^{-1}, tandem mass spectrometric data collection started at 15 s and lasts for 30 s. The method was validated with reference to Industry Guidance for Bioanalytical Method Validation and then used for clinical samples. The method is ultrafast, and robust. Results of incurred samples demonstrated excellent method of reproducibility. This ultrafast analysis speed did not compromise with the data quality. To our knowledge, this is the fastest analytical method for simultaneous determination of lopinavir and ritonavir.

1. Introduction

Developed by Abbott Laboratories, Kaletra® was approved for the treatment of human immunodeficiency virus infection. Kaletra® is formulated from two protease inhibitors lopinavir and ritonavir. In order to support clinical development and monitor the drug exposure, a reliable and fast analytical method is mandatory to provide trustworthy data. There have been quite a few analytical methods for lopinavir and ritonavir reported in literature [1–9]. However, most of these methods took the conventional sample preparation approaches like protein precipitation, conventional liquid/liquid extraction and solid phase extraction. Most of these sample preparation techniques take at least 1 h for the preparation. The reported methods ran for 4–25 min per injection. Slow sample preparation and long sample cycle time limit the speed of sample analysis and the drug development decision making. In bioanalytical sample preparation, each sample preparation technique has its advantages and disadvantages, for example, protein precipitation is simple but a slow drying down process is normally involved. The protein precipitation extracts are not clean enough and thus generate possible matrix effect. Conventional liquid/liquid extraction is simple and clean, but a drying down is also involved because the extraction solvent is not compatible with reversed phase liquid chromatography. Solid phase extraction is complicated and much more expensive than protein precipitation and conventional liquid/liquid extraction [10]. With the demand of the fast pace development for new therapeutic drugs, high throughput sample preparation and analysis have been widely adopted in pharmaceutical industry. As a high throughput strategy, multi-well plates were introduced to increase the sample preparation efficiency. The 96-well is the most commonly used format of high throughput bioanalytical sample preparation. Recently a fast high throughput salting-out assisted liquid/liquid extraction technique (SALLE) has been developed and applied at Abbott Laboratories [11,12]. SALLE is conducted by adding concentrated inorganic or organic salt solution into a mixture of drug-containing biological sample and a water-miscible solvent like acetonitrile, methanol or acetone. The mixture of biological sample and water-miscible organic solvent can be forced to form phase separation by an increase of ionic strength from solvated salts. Due to specific physical and chemical proper-

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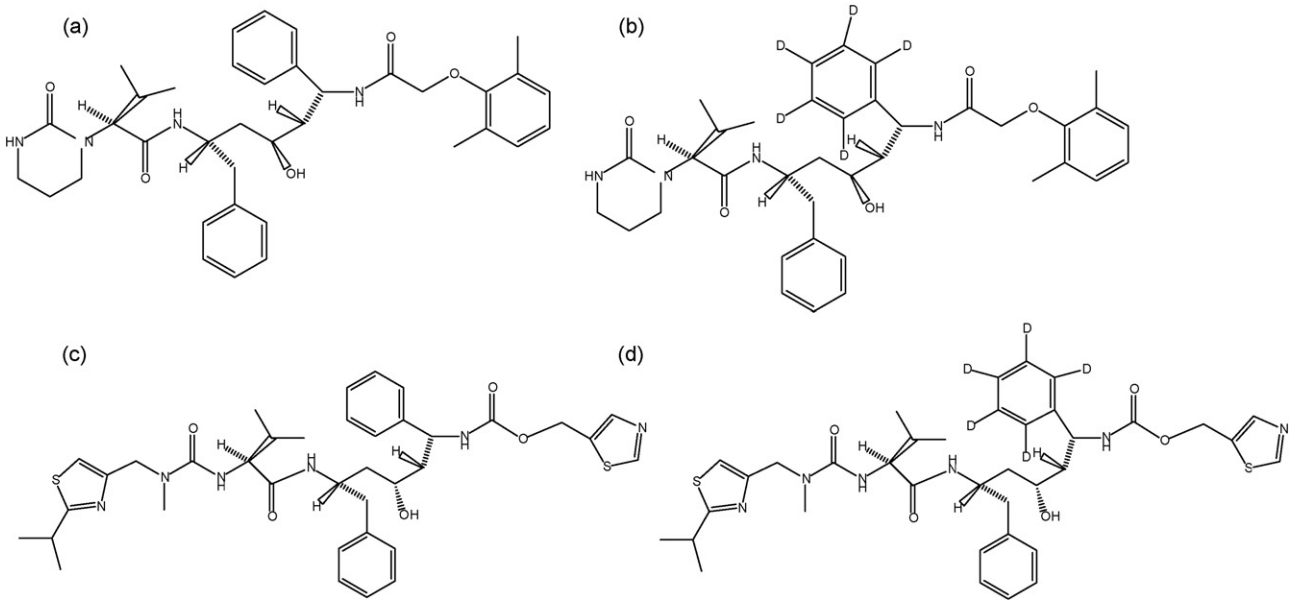

Fig. 1. Structures of (a) lopinavir, (b) lopinavir IS, (c) ritonavir and (d) ritonavir IS.

ties of drugs or drug-like compounds [13–16], these compounds tend to follow water-miscible organic solvent into the new phase. In the extraction of lopinavir and ritonavir from human plasma, the samples went through double protein precipitation by acetonitrile and $ZnSO_4$ sequentially to remove plasma proteins. $ZnSO_4$ was concentrated enough to force the supernatant to form two phases. Lopinavir and ritonavir followed organic phase. The extract was diluted with water and injected into an ultrafast LC–MS/MS system. The sample preparation takes about 20 min for a 96-well plate and the sample injection takes 48 s each sample. With the speed of sample preparation with SALLE and ultrafast LC–MS/MS, the fast delivery of the bioanalytical data has been achieved.

2. Experimental

2.1. Reagents

Lopinavir and ritonavir are reference standards from Abbott Laboratories (North Chicago, IL, USA). Stable isotope labeled lopinavir and ritonavir from Abbott Laboratories were used as internal standards (IS). The structures of lopinavir, lopinavir IS, ritonavir and ritonavir IS are shown in Fig. 1(a), (b), (c) and (d) respectively. Acetonitrile was from EMD Sciences (Gibbstown, NJ, USA), and water was prepared by de-ionizing distilled water through a Milli-Q system from Millipore (Billerica, MA, USA). Zinc sulfate was from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was from J.T. Baker (Phillipsburg, NJ, USA). Human plasma was from Biological Specialty (Colmar, PA, USA).

2.2. Instrumentation

A MicroLab AT 2 Plus automated liquid handler from Hamilton (Reno, NV, USA) was used to handle all liquid transfers. An LC-20AD$_{XR}$ liquid pump from Shimadzu (Kyoto, Japan) was used to deliver the mobile phase isocratically. Autosampler SIL-20AC$_{XR}$ was also from Shimadzu. A rack changer/C from Shimadzu was also used to hold up to a dozen of 96-well plates. Liquid pump, autosampler and rack changer were controlled by a Shimadzu HPLC controller (CBM-20A) though web-based software. An ABI-3000 mass spectrometer from Applied Biosystems Inc. (Foster City, CA, USA) was used to monitor the chromatography effluent. The data collection and processing were performed with Analysts software v1.4.2 from Applied Biosystems Inc. Other instrumentations included system switch valve from Valco (Houston, TX, USA), centrifuge from Jouan (Waltham, MA, USA), balances from Mettler Toledo (Columbus, OH, USA).

2.3. Standard and quality control preparation

Calibration standards and quality control samples (QC) were prepared by spiking the stock solutions into human plasma potassium (K_2) EDTA using class "A" volumetric glassware or gas-tight syringes. Separate stock solutions were prepared and used for preparing calibration standards and QCs. Total 10 levels of calibration standards and three levels of QCs were prepared. Low and high standards were also treated as quality controls in the validation experiments, the low standard was also called as lower limit of quantitation sample (LLOQ) and the high standard was also called upper limit of quantitation sample (ULOQ). The prepared standards and QCs were transferred to polypropylene tubes for frozen storage at approximately −20 °C upon preparation.

2.4. Sample preparation

Thawed samples completely in room temperature water, and mixed thoroughly. Add 50 μL of internal standard working solution (∼500 ng mL^{-1} of lopinavir IS and ritonavir IS in 50:50 (v/v) acetonitrile:water) to each sample except the well designated blank. Add 50 μL of 50:50 (v/v) acetonitrile:water to the well designated blank to compensate the composition. Add 50 μL of samples and mix 5 times by aspirating and dispensing 100 μL. Add 200 μL of acetonitrile to the extraction plate. Add 100 μL of 3 M zinc sulfate to each well of the 96-well plate and mix 5 times by aspirating and dispensing 300 μL in the extraction plate using the Hamilton. Centrifuge the plate at approximately 4000 rpm for approximately 4 min and at approximately 10 °C. Transfer 100 μL of the organic layer from the extraction plate into the injection plate. Add 100 μL of water to the injection plate and mix well using the multi-tube vortex. Inject 10 μL of each sample into LC–MS/MS.