Simultaneous determination of lopinavir and three ester prodrugs by LC-MS/MS in lysates of BeWo cells

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method with positive electrospray ionization (ESI) mode has been validated for the simultaneous determination of lopinavir (LPV) and three prodrugs, succinic acid ester of lopinavir (SLPV), glutaric acid ester of lopinavir (GLPV), and diglycolic acid ester of lopinavir (DLPV) in BeWo cell lysate. A methanolic precipitation was employed after addition of an internal standard ritonavir (RTV), followed by centrifugation, separation of the supernatant liquid, evaporation, and reconstitution. A reversed-phase Phenomenex C18 column was used for separation of lopinavir and three prodrugs with a mobile phase which comprises methanol (solvent A), acetonitrile (solvent B), and 50 mM ammonium acetate buffer adjusted to pH 5.5 with acetic acid (solvent C), with isocratic elution (A:B:C = 34:34:32, v/v). The detection of target compounds was conducted using multiple reaction monitoring (MRM) with the following transitions of m/z 729→447, 745→563, 743→429, 629→447 and 721→296 to measure SLPV, DLPV, GLPV, LPV and RTV (I.S.), respectively. The calibration ranges were determined using quadratic regression over concentration ranges of 7-743 ng/mL (DLPV), 7.5-745 ng/mL (GLPV), 7-729 ng/mL (SLPV) and 6-189 ng/mL (LPV). The reverse calculated residuals for LPV and these prodrugs were all less than 15% different from nominal (range). The method we established was fully validated for accuracy ($\leq 15\%$ different from nominal) and precision ($\leq 15\%$ RSD). The results proved that the method is accurate and specific, and also this method has been successfully applied to test the uptake of lopinavir and prodrugs into BeWo cells.

Keywords:

Lopinavir

Novel ester prodrugs of lopinavir

LC-MS/MS

BeWo trophoblast cell cultures

Placenta

HIV protease inhibitors

Footnotes:

Abbreviations:

BSA	bovine serum albumin
DLPV	diglycolic acid monoester of lopinavir
DPBS	Dulbecco's phosphate buffered saline
GLPV	glutaric acid monoester of lopinavir
LPV	lopinavir
MRP2	multidrug resistance-associated protein 2
Pgp	P-glycoprotein
RTV	ritonavir
SLPV	succinic acid monoester of lopinavir

1. Introduction

Anti-HIV drugs are administered to HIV infected pregnant women for both maternal therapy and prevention of mother-to-child HIV transmission (1). Advances in anti-HIV drug development are remarkable, but a single effective anti-HIV drug product for pregnant women is still not available, in part due to the low penetration into the fetal compartment. The co-formulated, ritonavir-boosted protease inhibitor lopinavir (Kaletra) is currently the first choice of HIV protease inhibitors used for pregnant women with HIV infection (2). However, LPV has poor placental penetration due to low diffusion rate, and can be effluxed by ABC transporters, mainly P-glycoprotein (P-gp), but also Multidrug Resistance Protein (MRP2) (4). In Kaletra, low dose ritonavir is used to improve the pharmacokinetic profile of lopinavir by inhibiting LPV metabolism by CYP3A4 and/or LPV efflux by P-gp as a competitive inhibitor (3).

Several studies demonstrated inadequate or undetectable lopinavir in fetal plasma (5) (6) (7) (8). Furthermore, prolonged use of ritonavir, which inhibits the efflux transporter expressed in the placenta, may disturb normal physiological defending function resulting in failure to protect the fetus from xenobiotics (9). Therefore, it is valuable to discover novel protease inhibitors or modify lopinavir to improve its transfer across the placenta.

A prodrug represents a pharmacologically inert chemical derivative, which can be converted to active drug in vivo by enzyme- or nonenzyme-mediated hydrolysis (10). The carrier mediated prodrug approach is a relatively newly developed prodrug strategy, in which prodrugs are designed to resemble the substrates of targeted carriers (transporters). Lopinavir has potent anti-HIV activity with low viral resistance, and also its pharmacokinetic and toxicity profile have been established, (15, 16) thus making a lopinavir prodrug approach reasonable. Until now, there

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4 has been no prodrug of lopinavir designed and tested to improve uptake in models of the human
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6 placenta. Since fatty acid transporters are highly expressed on the placenta during pregnancy
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8 (17), we chose the carrier-mediate prodrug approach and thus designed dicarboxylate monoester
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10 prodrugs of lopinavir, which we hypothesize will be substrates of fatty acid transporters in order
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12 to improve placental penetration of lopinavir.
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16 Three dicarboxylate acid esters of lopinavir have been synthesized in our laboratory, and
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18 testing their pharmacokinetic properties is in progress. To complete the evaluation, a convenient
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20 and robust method to determine the uptake of the prodrugs into the BeWo model of placental
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22 trophoblast cells is required. The results of the investigation are expected to further our
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24 understanding of the structure–activity relationships between substrates and the transport system
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26 to direct the selection of compounds for further development. Our laboratory has previously used
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28 liquid scintillation analysis to test the uptake of one of these three ester prodrugs in trophoblast
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30 cells, but this method does not distinguish between the prodrug and the parent drug. The method
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32 was limited in that it fails to distinguish between prodrugs and released parent (lopinavir). In
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34 contrast, we believe that LC-MS/MS will enable transport and metabolism to be evaluated
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43 The rate-limiting step in the exchange of substances across the human placenta is the
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45 syncytiotrophoblast, a broad, thin, multinucleated cell layer with tight junctions. As a model of
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47 the human term placental syncytiotrophoblast layer, BeWo human placental choriocarcinoma
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49 trophoblast cells (BeWo cells) are commonly used. They have very similar structural,
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51 enzymatic, endocrine functions and nutrient transporter system to that of human trophoblast.
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53 The BeWo cell monolayer model has a good correlation (95%) of the relative transporter rate
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55 with that of ex vivo human placental perfusion model, which is considered the most clinically
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relevant model. (18). Compared to placental perfusion, BeWo cells monolayer model has some advantages, such as easier handling, high throughput screening, and accessibility (not requiring fresh placenta tissue) (19, 18). Therefore BeWo cells have been widely used to rapidly rank order of uptake of compounds into human placental trophoblast. (19) Taken together, BeWo cell monolayer in vitro model will be used to assess the uptake of prodrugs. In order to perform these studies, a sensitive and reliable isocratic LC-MS/MS method was developed for the simultaneous determination of lopinavir and prodrugs in the cell lysate.

2. Experimental

2.1. Chemicals and reagents

Lopinavir (LPV) and Ritonavir (RTV) were purchased from Beta Pharma, Inc. (Branford, CT, USA). Succinic acid ester of lopinavir (SLPV), glutaric acid ester of lopinavir (GLPV), and diglycolic acid ester of lopinavir (DLPV) were synthesized in our laboratory, with purity >95% and their structures established by proton NMR (Wang et al, unpublished data). The structures are shown in **Figure 1A**. These three prodrugs were prepared by reacting LPV with dicarboxylic acid anhydride as shown in **Figure 1B**.

Acetonitrile and methanol HPLC Grade were purchased from Avantor Performance Materials, Inc. (Center Valley, PA, USA). Ammonium acetate and DMSO were obtained from Fisher Scientific (Valley Pike Middletown, VA, USA). High purity water was obtained in-house using a NANO pure Diamond Life Science ultrapure water System from Millipore Corporation (Billerica, MA, USA). 12 wells plate was obtained from Greiner Bio-one (Monroe, NC, USA).

2.2. Cell culture

BeWo Trophoblast Cells (Schwarz/b30 clone) were obtained as a kind gift from Dr. Kenneth Audus at the University of Kansas (Lawrence, KS), and grown in complete growth medium consisting of 88% DMEM/high glucose, 10% fetal bovine serum (FBS, Atlantic biologicals), 1% non-essential amino acid and 1% penicillin. Cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO₂. A density of 100,000 Cells (passage 38) were seeded into a T-75 flask (for propagation or preparation of cell matrix) or a Corning 3997 12-well plate (for uptake studies). To prepare blank cell matrix, after the cell layer was 90% confluent, 9.5 mL methanol was added into T-75 flask, incubated for 15 minutes, then the supernatant liquid was harvested.

2.3. Instrument and analytical conditions

The HPLC system used for separation was a Shimadzu (Shimadzu, Kyoto, Japan) chromatographic system, which consisted of a Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A. The auto-sampler HTS PAL was from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater was from Eppendorf (Westbury, NY, USA). A Phenomenex (Torrance, CA, USA) C18 guard column (4.0 mm × 2.0 mm; 3 µm) was used with Phenomenex Gemini C18 analytical column (100 mm × 2.0 mm I.D., 3.0 µm). The mobile phase comprised 34% of 50 mM ammonium acetate buffer (pH 5.5 adjusted with acetic acid), 34% methanol and 32% acetonitrile with a flow rate of 0.4 mL/min; the column temperature was maintained at 35°C. The mass spectrometer was a Waters API Quattro Micro triple quadrupole mass spectrometer from Waters Corporation (Milford, MA, USA).

2.4. Mass spectrometric conditions

The mass spectrometer used positive ion electrospray (ESI+) with multiple reaction monitoring (MRM). Tuning and optimizing of mass spectrometer parameters were performed before injection in order to achieve maximum sensitivity. The final conditions for ESI+ were: capillary voltage 2.4 kV, source temperature 130°C, desolvation temperature 350°C; cone voltage and collision energy were also optimized (see Table 1). High purity argon was used as collision gas. All data were evaluated using MassLynx 4.1 software. The mass spectrometer parameters were summarized in **Table 1**.

2.5. Stock solution preparation and stock dilution

The stock solutions (7 mM) were prepared in DMSO and stored at -80 °C. A series of standard working solutions was then prepared by appropriately diluting the standard stock solutions of prodrug and lopinavir with cell extracts. Internal standard (ritonavir) was diluted into internal standard working solution to a concentration of 3 µg/mL in methanol extracts.

2.6. Preparation of calibration standards and quality control samples in cell methanol extraction

The methanol extract of BeWo cells was used to provide the matrix for the study. Working solutions of prodrugs and lopinavir were prepared by spiking appropriate volumes into the methanol extraction of BeWo cells to obtain calibration curve standards and QC. Calibration standards were freshly prepared for each analytical run at concentration levels for LPV (6, 16, 31 157, 314, 629 ng/mL), SLPV (7, 18, 36, 182, 364, 729 ng/mL), DLPV (7, 19, 37, 186, 372, 745 ng/mL), GLPV (7, 19, 37, 185, 371, 743 ng/mL) and quality control samples, including the lower

limit of quantification (LLOQ), low (LQC), medium (MQC) and high (HQC) concentration quality controls (Table 5), which were prepared in the same procedure.

2.7 Sample preparation

Compounds were dissolved in 500 μ L cell extract with internal standard ritonavir 336 ng/mL, and then centrifuged at 14,000 g for 15 min at room temperature. After collecting 450 μ L of supernatant liquid, samples were dried under a gentle, steady nitrogen stream at 55 $^{\circ}$ C. Finally, the samples were reconstituted with mobile phase (as described in section 2.3). The samples were centrifuged again at 14,000 g for five minutes and 25 μ L of the supernatant liquid was injected into the LC-MS/MS system.

2.8. Validation of method

The method was validated for linearity, accuracy and precision according to the FDA bioanalytical guidance for these parameters [21].

2.9.1. Linearity and lower limit of quantification (LLOQ)

Calibration curves for standard samples in cell extracts were constructed by plotting ratios of the peak area of each drug to that of internal standard versus standard concentrations. The final concentrations of calibration standards obtained for plotting the calibration curve were listed in the **Table 2**. A correlation coefficient (r) of 0.995 or better was used as criterion along with reverse calculated residuals criterion of $\pm 20\%$ or $\pm 15\%$ for LLOQ and all other points, respectively.

2.9.2. Selectivity

Six individual blank cell extracts were processed according to the sample preparation procedure. The selectivity of this assay was evaluated by comparing of LLOQ level samples with blank samples to measure putative interference in the cell extract samples.

2.9.3. Accuracy and precision

Accuracy and precision were determined from QC samples (LLOQ, LQC, MQC, HQC) in three independent runs. The LLOQ of the assay was assessed by running six replicates independent of the QC samples as the lowest concentration on the calibration curve and quantitatively determining the concentration from the calibration curve. Acceptable precision and accuracy were within $\pm 20\%$ for the LLOQ and $\pm 15\%$ for the remaining QC's. Intra-assay precision and accuracy were determined from 6 replicates of each QC sample in a single assay. Inter-assay precision and accuracy were determined by analyzing three different validation runs with a total of 12 replicates (22).

2.9.4. Recovery and Matrix Effects

Extraction recovery was determined by comparing the peak area of each drug to that of internal standard in the pre-extracted cell extraction samples to that of post-extracted dry residue spiked samples. A post-column infusion experiment was also performed to assess potential matrix effects by the following experiment: a 1000 ng/mL solution (all prodrugs, LPV and RTV) in the mobile phase was prepared and continuously infused at 20 $\mu\text{L}/\text{min}$ post-HPLC column directly into the mass spectrometer using a "tee" connection. After the baseline was stabilized, a blank sample was injected, the profiles of which were compared with the retention time of

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4 analytes to check for suppression or enhancement. A post-extract addition study was also
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6 performed to determine the percent matrix effects associated with the methanol extraction of
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8 cellular constituents over the ionization of lopinavir, prodrug and ritonavir. The peak area
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10 responses of the post-extracted cell methanol extraction standard samples ($n = 6$) were compared
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12 with the responses of analytes from stock solutions directly diluted in the mobile phase at
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14 equivalent final concentrations. The matrix effect for lopinavir and prodrugs was determined at
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16 QC of low, medium and high concentrations (50, 100, 500 ng/mL) based on six replicates.
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23 *2.9.5. Stability studies*

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25 Post-preparative stability was assessed using cell methanol extraction spiked with
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27 compounds at two levels MQC and HQC. These quality control samples were processed with a
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29 calibration curve. These samples were maintained at 4 °C in the autosampler. After 24 hours, the
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31 samples were injected again and processed with the previous calibration curve. A criterion of $\pm$
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33 20% of the nominal concentration was used to assess the 24 h post-preparative stability.
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40 *2.10. Application of the validated method*

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42 The validated method was used for determination of the uptake of lopinavir and the
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44 prodrugs in BeWo human placental trophoblast cells in 12 well plates. After the cells were 90%
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46 confluent, they were considered ready for the experiments. The cell medium was aspirated from
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48 the wells and the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS;
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50 1ml). The cells were incubated with 10 μ M drug in 0.05% bovine serum albumin (BSA) in
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52 DPBS (500 μ L) at 37 °C for 15 min, respectively. The buffer containing drug was removed from
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54 the wells. The wells were washed twice with 1 mL ice cold 0.5% BSA in DPBS and 1 mL ice
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56 cold phosphate buffered saline. (20) Cells were lysed and analytes extracted from the cells by
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exposing the cells to methanol (0.5ml) for 15 minutes on ice. The post treatment was same as the QC samples mentioned above. The concentration of protein was measured by BCA protein assay (Thermo Scientific™ Pierce™) (21). The uptake results were calculated by the concentration of drugs multiplied by 0.5 mL normalized by the molar amount (μg) of protein related to transporter expressed in each well after 15 min incubation, using the BCA protein assay (0 $\mu\text{g/mL}$ -2000 $\mu\text{g/mL}$, $r^2 = 0.9998$).

2.11. Software

ChemDraw Office 2006 was used to draw structures and the route of prodrug synthesis. GraphPad Prism (v.5) was used for statistical analyses.

3. Results and Discussion

3.1. Methodological results

3.1.1. Calibration curve and lower limit of quantization

In this method, the linear ranges, regression types, correlation coefficients and weighting factors for measuring both LPV and its prodrugs in cell extracts were summarized and listed in the **Table 2**. To improve the goodness of fit, quadratic (rather than linear) equations were fit to the data. The correlation coefficients (r^2) from three independent runs for all drugs we tested were at least 0.9975.

3.1.2. Selectivity

Six blank cell extraction samples were used to investigate interference. Acceptable selectivity was considered to be demonstrated when the responses to blank samples were all less than 20% of lowest limit of quantitation (LLOQ) of test samples in the corresponding MRM

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4 channels. The retention times of DLPV, SLPV, GLPV, LPV and RTV were approximately 2.06,
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6 2.48, 2.70, 4.56 and 3.68 min, respectively. An example chromatogram is shown in **Figures 2A**
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8 **and 2B**, and the results are summarized in the **Table 2**. The results showed that the
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10 chromatograms were free of interference after the treatment.
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16 *3.1.3. Extraction recovery rates and matrix effects*

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18 A post-column infusion profile is presented in **Figure 2C**, which indicates negligible
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20 matrix effects at the retention time of lopinavir and its prodrugs. The extraction recovery rates of
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22 LPV and three prodrugs in cell extracts are listed in the **Table 3**. The matrix effect of LPV and
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24 three prodrugs were also listed in the **Table 4**. The results suggested that the relative extraction
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26 recovery rates of LPV and prodrugs are all above 70% and matrix effect was not observed in this
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28 method. Therefore, this method is qualified to be used to determine biological sample
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38 *3.1.4. Precision and accuracy*

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40 According to the FDA guidelines for bioanalytical method validation(22), the RSD%
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42 values for intra-assay and inter-assay precision should be $\leq 15\%$. The precision and accuracy
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44 data are listed in the **Table 5**, which met the requirements for determination of biological sample
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53 *3.2.1. Application of the analytical method*

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55 After incubation with LPV and its prodrugs, the uptake of compounds into the cells was
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57 measured by the established LC-MS/MS method. The result are shown in the **Figure 3**. The
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uptake results showed that all the prodrugs had higher uptake compared to lopinavir (which was below the LLOQ). GLPV showed the highest uptake among these three prodrugs, which was about 16- fold higher compared to the LLOQ of LPV. SLPV showed 9-fold higher uptake compared to LLOQ of LPV, while DLPV showed two fold higher uptake compared to LLOQ of LPV.

3.3. Conclusion

In this study, we synthesized three ester prodrugs of lopinavir and developed a bioanalytical assay to measure them. In order to test their uptake into BeWo cells, an LC-MS/MS method with high specificity and sensitivity, as well as simultaneous quantitative analyses of lopinavir and its prodrugs in the BeWo cells was established and validated. We also successfully applied this method to the in vitro uptake study of these drugs in BeWo cells. The results showed that all the prodrugs had higher uptake into BeWo cells compared to lopinavir, which suggested that the prodrugs may have better penetration into the fetal compartment compared to lopinavir.

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References

- (1). Schmit JC, Cogniaux J, Hermans P, Van Vaeck C, Sprecher S, Van Remoortel B, et al. Multiple drug resistance to nucleoside analogues and nonnucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. *J Infect Dis.* 1996;174(5):962-8.
- (2). <http://aidsinfo.nih.gov/contentfiles/lvguidelines/perinatalgl.pdf>. [Accessed by 07/16/2013]
- (3). Chandwani A, Shuter J. Lopinavir/ritonavir in the treatment of HIV-1 infection: a review. *Ther Clin Risk Manag.* 2008;4(5):1023-33. PMCID: 2621403.
- (4). Patterson KB, Dumond JB, Prince HA, Jenkins AJ, Scarsi KK, Wang R, et al. Protein binding of lopinavir and ritonavir during 4 phases of pregnancy: implications for treatment guidelines. *J Acquir Immune Defic Syndr.* 63(1):51-8. PMCID: 3625477.
- (5). Marzolini C, Rudin C, Decosterd LA, Telenti A, Schreyer A, Biollaz J, et al. Transplacental passage of protease inhibitors at delivery. *Aids.* 2002;16(6):889-93.
- (6). Optimising HIV Treatment--Building on Experience. Proceedings of a symposium. 15 March 2002, Seville, Spain. *J Acquir Immune Defic Syndr.* 2003;34 Suppl 1:S1-100.
- (7). Ivanovic J, Nicastrì E, Anceschi MM, Ascenzi P, Signore F, Pisani G, et al. Transplacental transfer of antiretroviral drugs and newborn birth weight in HIV-infected pregnant women. *Curr HIV Res.* 2009;7(6):620-5.
- (8). Stek AM, Mirochnick M, Capparelli E, Best BM, Hu C, Burchett SK, et al. Reduced lopinavir exposure during pregnancy. *Aids.* 2006;20(15):1931-9.
- (9). Sarkadi B, Homolya L, Szakacs G, Varadi A. Human multidrug resistance ABCB and ABCG transporters: participation in a chemoinnity defense system. *Physiol Rev.* 2006;86(4):1179-236.

- 1
2
3
4 (10). Bundgaard H, Johansen M. Prodrugs as Drug Delivery Systems .4. N-Mannich Bases as Potential
5
6 Novel Prodrugs for Amides, Ureides, Amines, and Other Nh-Acidic Compounds. Journal of
7
8 Pharmaceutical Sciences. 1980;69(1):44-6.
9
- 10
11 (11). Jana S, Mandlekar S, Marathe P. Prodrug design to improve pharmacokinetic and drug delivery
12
13 properties: challenges to the discovery scientists. Curr Med Chem.17(32):3874-908.
14
- 15
16 (12). Mahato R, Tai WY, Cheng K. Prodrugs for improving tumor targetability and efficiency.
17
18 Advanced Drug Delivery Reviews. 2011;63(8):659-70.
19
- 20
21 (13). Han HK, Amidon GL. Targeted prodrug design to optimize drug delivery. AAPS PharmSci.
22
23 2000;2(1):E6. PMCID: 2751001.
24
- 25
26 (14). Lipka E, Crison J, Amidon GL. Transmembrane transport of peptide type compounds: prospects
27
28 for oral delivery. J Control Release. 1996;39(2-3):121-9.
29
- 30
31 (15). Sham HL, Kempf DJ, Molla A, Marsh KC, Kumar GN, Chen CM, et al. ABT-378, a highly
32
33 potent inhibitor of the human immunodeficiency virus protease. Antimicrob Agents Chemother.
34
35 1998;42(12):3218-24. PMCID: 106025.
36
- 37
38 (16). Kumar GN, Jayanti V, Lee RD, Whittern DN, Uchic J, Thomas S, et al. In vitro metabolism of
39
40 the HIV-1 protease inhibitor ABT-378: species comparison and metabolite identification. Drug Metab
41
42 Dispos. 1999;27(1):86-91.
43
- 44
45 (17). Johnsen GM, Weedon-Fekjaer MS, Tobin KA, Staff AC, Duttaroy AK. Long-chain
46
47 polyunsaturated fatty acids stimulate cellular fatty acid uptake in human placental choriocarcinoma
48
49 (BeWo) cells. Placenta. 2009;30(12):1037-44.
50
- 51
52 (18). Li H, van Ravenzwaay B, Rietjens IM, Louisse J. Assessment of an in vitro transport model using
53
54 BeWo b30 cells to predict placental transfer of compounds. Arch Toxicol.
55
56 (19). Poulsen MS, Rytting E, Mose T, Knudsen LE. Modeling placental transport: correlation
57
58 of *in vitro* BeWo cell permeability and *ex vivo* human placental perfusion. Toxicol In Vitro.
59
60 2009;23(7):1380-6.
61
62
63
64
65

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3
4 (20) Johnsen GM, Weedon-Fekjaer MS, Tobin KA, Staff AC, Duttaroy AK. Long-chain
5
6 polyunsaturated fatty acids stimulate cellular fatty acid uptake in human placental choriocarcinoma
7
8 (BeWo) cells. Placenta. 2009;30(12):1037-44.
9
10
11 (21) Pierce. [cited 2013 07/11]; Available from: [http://www.piercenet.com/instructions/](http://www.piercenet.com/instructions/2161296.pdf)
12
13 [2161296.pdf](http://www.piercenet.com/instructions/2161296.pdf).
14
15
16 (22). FDA Industry Guideline. 2001.
17
18
19
20
21
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24
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31
32
33
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Legends for Figures

Figure 1. A: Novel prodrugs SLPV, GLPV, DLPV and parent drug LPV. **B:** Synthesis of prodrugs.

Figure 2. A: Example chromatogram of extracted LLOQ, A) DLPV (7 ng/mL), B) ALPV (7 ng/mL), C) SLPV (7 ng/mL), D) LPV (7 ng/mL). **B:** Blank Samples with different extraction channel: A) DLPV, B) GLPV, C) SLPV, D) RTV, E) LPV. **C:** Post-column infusion profile of lopinavir and its prodrugs: A) DLPV; B) GLPV; C) SLPV; D) RTV; E) LPV.

Figure 3. The results of the uptake experiment for each prodrug normalized to total cell protein amount. Note: LPV was detected but below the LLOQ. The line is the LLOQ of lopinavir multiplied by 0.5 mL and then normalized by the average amount of protein in wells.

A.

Succinic acid ester of Lopinavir (SLPV)

Glutaric acid ester of Lopinavir (GLPV)

Diglycolic acid ester of Lopinavir (DLPV)

Lopinavir (LPV)

B.

Figure

Figure

Table 1. Mass spectrometer parameters and approximate retention times (Tr).

Analyte	~ Tr(min)	Multiple reaction monitoring transitions (parent ion→product ion)	Cone Voltage (V)	Entrance Potential (V)	Exit Potential (V)	CE (V)
SLPV	2.48	729.4→447.4	30	12	12	16
DLPV	2.06	745.4→563.1	30	12	12	30
GLPV	2.70	743.2→429.3	25	12	12	14
RTV	3.68	721.4→296.0	30	12	12	18
LPV	4.56	629.4→447.4	20	12	12	10

Table 2. Results of regression analysis on calibration curves of lopinavir, its prodrugs in the cell methanol extraction.

Analyte	Calibration range (ng/mL)	Regression type	Correlation coefficient (<i>r</i>)	weight
LPV	6.3-188.7	Quadratic	>0.9975	1/x
DLPV	7.4-743.2	Quadratic	>0.9996	1/x ²
GLPV	7.5-745.5	Quadratic	>0.9977	1/x ²
SLPV	7.3-729.5	Quadratic	>0.9990	1/x ²

Table 3.The recovery rate of lopinavir and its prodrugs.

Compound Name	Extraction Recovery (%)		
	500 ng/mL (%)	250ng/mL(%)	10 ng/mL(%)
DLPV	90.7±8.1	91.4±5.8	84.5±11.1
SLPV	96.4±3.2	90.4±6.2	93.3±1.7
GLPV	84.0±4.3	86.6±3.9	88.9±5.9
	150 ng/mL(%)	90 ng/mL	10 ng/mL(%)
LPV	89.1±1.1	89.4±2.9	102.2±3.3

Table 4.The Matrix Effect of lopinavir and its prodrugs.

Compound Name	Matrix Effect		
	500 ng/mL (%)	200 ng/mL(%)	10 ng/mL(%)
DLPV	6.2	-4.9	6.9
SLPV	-4.8	-10.1	5.2
GLPV	3.1	-9.0	-2.7
	150ng/mL (%)	90 ng/mL(%)	10 ng/mL(%)
LPV	-1.3	2.2	10.4

Table 5. Intra-assay precision and accuracy of prodrugs and LPV.

Compound		QC Concentrations (ng/mL)			
		522 ng/mL	75 ng/mL	19 ng/mL	7.5 ng/mL
DLPV	Accuracy (%)	13.2	4.7	11.2	17.7
	RSD (%)	14.9	2.4	7.0	8.16
		510 ng/mL	73 ng/mL	18 ng/mL	7.3 ng/mL
GLPV	Accuracy(%)	15.0	4.5	-7.2	6.4
	RSD(%)	4.5	8.9	11.3	13.7
		510 ng/mL	73 ng/mL	18 ng/mL	7.3 ng/mL
SLPV	Accuracy(%)	-5.2	9.9	-5.5	-6.9
	RSD(%)	11.6	6.8	13.9	10.3
		63 ng/mL	38 ng/mL	13 ng/mL	6.3 ng/mL
LPV	Accuracy(%)	-0.8	-2.8	9.9	13.8
	RSD(%)	7.9	5.6	13.7	13.4

Table 6. Inter-assay precision and accuracy of prodrugs and LPV.

Compound		QC Concentrations (ng/mL)			
		522 ng/mL	75 ng/mL	19 ng/mL	7.5 ng/mL
DLPV	Accuracy (%)	-4.0	-4.6	9.7	10
	RSD (%)	14.3	9.7	5	9.1
		510 ng/mL	73 ng/mL	18 ng/mL	7.3 ng/mL
GLPV	Accuracy(%)	14.9	8.1	-4.8	6.7
	RSD(%)	5.2	7.1	12.1	9.4
		510 ng/mL	73 ng/mL	18 ng/mL	7.3 ng/mL
SLPV	Accuracy(%)	-8.4	-3.0	-7.8	-6.3
	RSD(%)	9.0	12.9	9.7	7.8
		63 ng/mL	38 ng/mL	13 ng/mL	6.3 ng/mL
LPV	Accuracy(%)	-4.6	-5.07	2.4	6.1
	RSD(%)	11.7	10.6	4.8	19.7

Table 7. Post-preparative stability results.

	73 ng/mL	18 ng/mL		73 ng/mL	18 ng/mL
SLPV (%)	-9.7	-10.7	GLPV (%)	-8.2	-5.3
	75 ng/mL	19 ng/mL		38 ng/mL	13 ng/mL
DLPV (%)	-16.8	-14.0	LPV (%)	-17.0	-16.0