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Original Article

A RAPID ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC-UV) METHOD FOR THE QUANTIFICATION OF RITONAVIR IN HUMAN PLASMA

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ABSTRACT

Objective: An accurate, simple, and rapid HPLC-UV based method for the quantitative determination of ritonavir (RTV) in human plasma is developed.

Methods: The method involved deproteinization of the sample with 0.125N NaOH and methyl tertiary butyl ether and evaporated to dryness. The residue was reconstituted with mobile phase (20 mM Sodium Acetate and Acetonitrile–55:45 v/v). The analysis was done using C8 column (250 x 4.6 mm ID) and detection at a wavelength of 212 nm

Results: The method range was linear over the range 0.5–10.0 µg/ml as derived using calibration curve method. Mean intra-and inter-day variations over the ranges of the standard curves were less than 10 % and mean extraction recoveries from human plasma ranged from 96 to 110 %.

Conclusion: A rapid and accurate method for quantitation of RTV in plasma was validated. The assay spans the concentration range of clinical interest. The easy sample preparation and small sample size makes this assay highly suitable for pharmacokinetic studies of RTV in HIV-infected patients with TB.

Keywords: Ritonavir, Pharmacokinetics, HPLC, Plasma extraction, HIV

INTRODUCTION

The clinical treatment of patients with human immunodeficiency virus (HIV-1) infection has been advanced by the development of highly active antiretroviral therapy (HAART). However, some patients develop drug resistance and metabolic complications. This undesirable outcome may result from a failure to achieve effective antiretroviral drug plasma concentrations.

Therefore monitoring plasma drug concentrations is essential to ensure optimal drug efficacy, to prevent viral resistance, to manage drug interactions, to avoid adverse effects, and to assess non adherence [1-2]. Ritonavir (RTV), is an antiretroviral medication along with other medications to treat HIV/AIDS. Ritonavir, originally developed as an inhibitor of HIV protease, is widely used as a booster for other protease inhibitors. Additionally, because RTV is metabolized by cytochrome P450 enzymes, monitoring may be useful for evaluation of drug-drug interactions, such as those with a potent CYP 3A4 inducer or inhibitor that has been shown to increase or decrease the RTV plasma concentration.

In recent years, several HPLC methods for simultaneous determination of anti-retroviral drugs in plasma have been published [3-23]. Most of the methods using HPLC, though they are reliable and sensitive, have some critical disadvantages involving solid phase extraction, special columns, gradient elution programs, cumbersome operation procedures, larger sample volumes, multiple wavelengths, special detectors and/or longer run time. Other methods for quantification of antiretroviral drugs by tandem mass spectrometry (LCMS/MS) [24-29] and matrix-assisted laser desorption/ionization-triple quadrapole tandem mass spectrometry (MALDI-QqQ-MS/MS) [30, 31] have also been published and these sophisticated equipments may not be available in conventional hospital laboratories and also requires high analytical cost. Therefore we aimed to develop a simple, economical and reliable chromatographic method for the determination of ritonavir (RTV) in plasma using lopinavir (LPV) as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

RTV (Code No: RD322) and LPV (Code No: R8FB6) were kindly provided by Cipla Ltd. Acetonitrile used was of HPLC grade (Merck, India). Sodium Acetate, methyl tert butyl Ether and Sodium hydroxide from Qualigens (India) were used. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). Pooled human plasma from healthy volunteers was obtained from Lions Blood Bank, Chennai, India.

Chromatographic system

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), diode array detector (SPD-M10Avp) and autosampler with built-in system controller (SIL-HTA). ClassVP-LC workstation was used for data collection and acquisition. The analytical column was a C8, 250 x 4.6 mm ID, 5 µm particle size (Lichrospher 100 RP-8e, Merck, Germany) protected by a compatible guard column.

Chromatographic conditions

The mobile phase consisted of 20 mM Sodium Acetate in water, pH 4.8 (adjusted with 1N HCl) and acetonitrile (55:45 v/v). Prior to preparation of the mobile phase, the aqueous Sodium acetate and acetonitrile were degassed separately using a Millipore vacuum pump and filtered through 0.4 µm nylon membrane filter. The UV detector was set at a wavelength of 212 nm. The chromatogram was run for 13 min at a flow rate of 1.5 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height ratios (analyte/internal standard) vs. Concentration curve. The linearity was verified using estimates of coefficient of determinants (r) and correlation coefficient (R2) values.

Preparation of standard solution

A stock standard (1 mg/ml) was prepared by dissolving RTV in methanol. The working standards of RTV in concentrations ranging from 0.5 to 10.0µg/ml were prepared in human plasma.

Sample preparation

To 200 µl each of calibration standards and test samples, 20 µl of LPV (internal standard) was added at a concentration of 100µg/ml. This was mixed with 400 µl of 0.125N NaOH and 2.0 ml of methyl tertiary butyl ether, the contents were vortexed vigorously, and centrifuged at 5,000 rpm for 5 min. 1.5 ml of the clear supernatant was taken into the test tube, evaporated to dryness in a nitrogen evaporator (Turbo vap) for 20 min. The dried residue was reconstituted in 100 µl of mobile phase; 75 µl was injected into the HPLC column.

Accuracy and linearity

The accuracy and linearity of RTV standards were evaluated by analyzing a set of standards ranging from 0.5 to 10.0µg/ml. The within day and between day variations were determined by processing each standard concentration in duplicate for six consecutive days.

Precision

In order to evaluate the precision of the method, patient samples at three different concentrations of RTV (0.73, 1.15 and 3.34 µg/ml) were analysed in duplicate on three consecutive days.

Recovery

Varying concentrations of RTV (1.5, 3.5, and 7.5 µg/ml) were prepared in drug-free human plasma and extracted as described above along with the internal standard. The percentage of the drug recovered from the plasma samples was determined by comparing the peak height ratio after extraction with those of unextracted methanolic solutions containing same concentrations of RTV as in plasma. Recovery experiments were carried out on three different occasions.

Specificity

Co elution from endogenous compound was investigated by analysing blank plasma samples obtained from six each of male and female subjects. Interference from certain antiretroviral drugs, namely, nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir at a concentration of 10µg/ml and anti-tuberculosis drugs such as, isoniazid, pyrazinamide, rifampicin, ethambutol, streptomycin was also evaluated.

Limit of detection (LOD) and lower limit of quantitation (LLOQ)

The (LOD) and (LLOQ) were calculated using slope analysis of 0.5–20 ng/ml for RTV using the formula LOD= 3 x S. D/Slope and LOQ = 10 x S. D/slope.

Pharmacokinetic application of methods to human plasma samples

Serial blood samples at pre-dosing (0 hour) and at 1, 2, 4, 6, 8 and 12 h post dosing were drawn in heparinized containers from six HIV-infected adult patients with tuberculosis who were receiving ritonavir (100 mg daily) as part of the treatment regimen.

All the blood samples were centrifuged and plasma was separated and stored at-20oC until assay. Estimation of plasma RTV was undertaken within 24 to 48 h of blood collection. Informed written consent was obtained from the parent before blood draws were made.

RESULTS AND DISCUSSION

Method development

The analytical method developed was based on the protocol set out in the International Conference on Harmonization (ICH) guidelines [32]. Under the chromatographic conditions described above, peaks of RTV was presented in separate retention time with other elutants as seen in the chromatograms representing high and low concentrations (fig. 1a, b). The retention times of RTV and the internal standard (LPV) were 10.0 and 11.4 min respectively. Blank plasma samples did not give any peak at the retention times of RTV (fig. 1c).

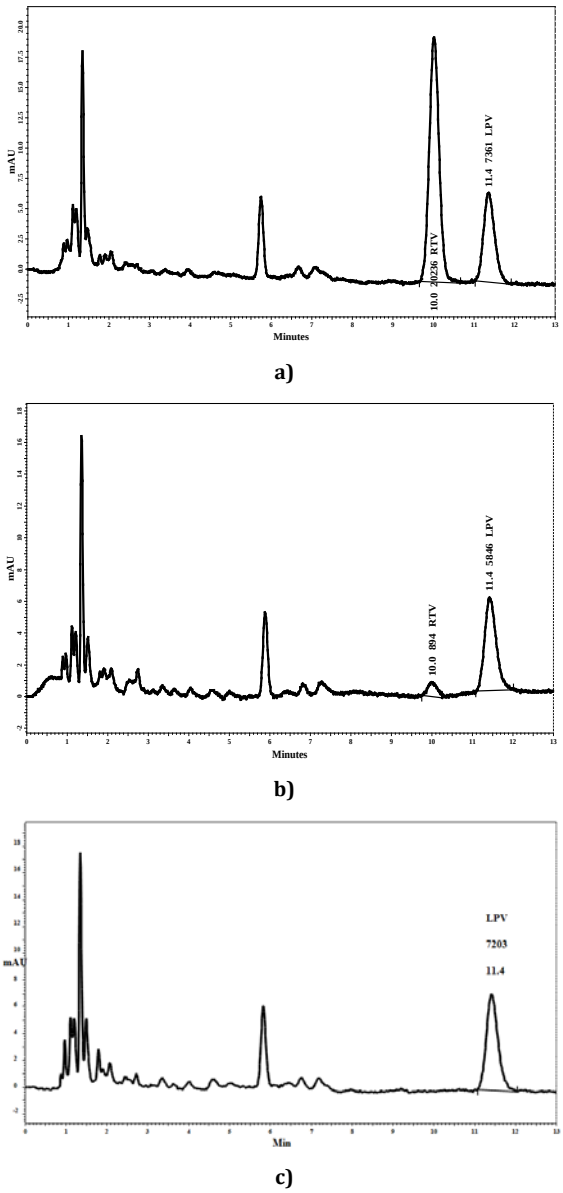

Fig. 1: (a) Chromatogram of extracted RTV plasma standard 10.0 µg/ml (contains 20 µl of LPV added at the concentration of 100 µg/ml); (b) Chromatogram of extracted RTV plasma standard 0.5 µg/ml (contains 20 µl of LPV added at the concentration of 100 µg/ml); (c) Chromatogram of extracted blank plasma (contains 20 µl of LPV added at the concentration of 100 µg/ml)

No endogenous substances or antiretroviral drugs such as nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir or first-line anti-tuberculosis drugs such as isoniazid, pyrazinamide, ethambutol and streptomycin was coeluted as cross checked using pure standards with the RTV chromatogram.

Ritonavir concentrations ranging from 0.5-10.0 µg/ml were checked for linearity. The calibration curve parameters of RTV from six individual experiments for standard concentrations ranging from 0.5 to 10.0µg/ml showed a linear relationship between peak height ratio and concentrations. The mean (+SD) correlation coefficient, slope and intercept values were 0.9997, 0.266 and-0.0232 respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma RTV are shown in fig. 2. The within-day and between-day percent variation (CV) for

standards containing 0.5 to 10.0µg/ml ranged from 3.4 to 7.9% and 2.0 to 10.3% respectively (table 1).

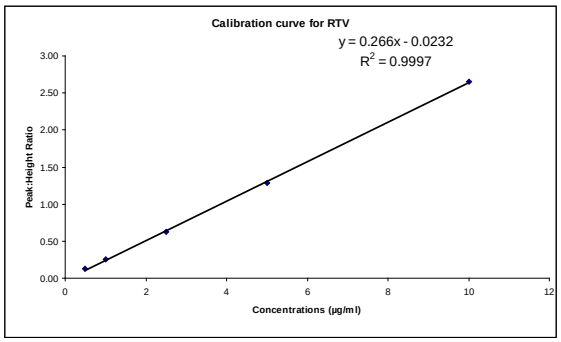

Fig. 2: Linear response of spiked plasma RTV concentrations ranging from 0.5–10.0 µg/ml

Table 1: Linearity and reproducibility of plasma RTV standards

S. No.	Mean peak height ratio±SD (RSD %)		
	Concentrations (µg/ml)	Intraday (n=6)	Inter day (n=6)
1	10.0	2.747±0.055(2.0)	2.650±0.090(3.4)
2	5.0	1.267±0.032 (2.5)	1.287±0.095 (7.4)
3	2.5	0.620±0.026 (4.2)	0.623±0.027 (4.3)
4	1.0	0.223±0.023 (10.3)	0.252±0.017 (6.8)
5	0.5	0.117±0.012 (10.3)	0.127±0.010(7.9)

Data given is the mean of 6 samples±Standard deviation and Relative Standard deviation in the parantheses,

Table 2: Precision of plasma RTV assay

S. No.	mean±SD
Sample 1	0.72±0.04
Sample 2	1.17±0.04
Sample 3	3.21±0.10

The values given are the mean of 3 concentrations with standard deviation

The reproducibility of the method was further evaluated by analyzing three plasma samples containing different concentrations of RTV. The RSD for these samples ranged from 2.6 to 5.0% (table 2). The % variations from the actual concentrations ranged from 92 to 104%. (LOD) and lower (LLOQ) were 140 ng/ml and 0.4 ug/ml respectively**.** The percent recovery of RTV from plasma ranged from 98 to 108% (table 3). The mean concentrations of RTV in serial blood samples collected at predosing (0 hour) and at 1, 2, 4, 6, 8, 12 and 24 h post dosing were 0.17, 0.63, 1.38, 1.60, 1.22, 0.91, 0.40 and 0.09 µg/ml respectively (fig. 3).

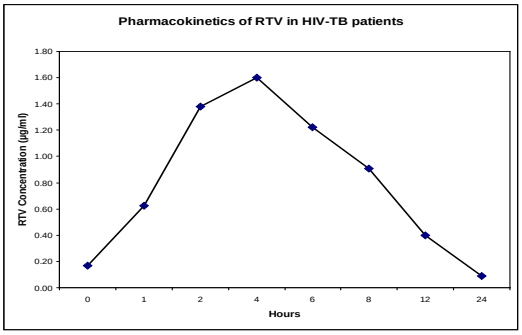

Fig. 3: RTV concentrations in HIV-TB patients. The above values are mean plasma concentrations of RTV obtained from 6 patients who were administered oral dose of 100 mg of RTV

Table 3: Recovery

S. No.	Base	Added (µg/ml)	Actual (µg/ml)	Obtained (µg/ml)	Recovery (%)
1	1.0	0.5	1.5	1.59	106
2	1.0	2.5	3.5	3.85	110
3	5.0	2.5	7.5	7.24	96

DISCUSSION

LPV/RTV is a major antiretroviral in salvage regimens, has been proven effective in Chinese HIV-positive adults with first-line treatment failure [33-35]. Thus, the establishment of a quantitative method for determination of RTV in clinical pharmacokinetic studies and therapeutic drug monitoring is necessary.

There have been recent reports of HPLC methods for the simultaneous determination of antiretroviral drugs which includes RTV. However, these methods have several disadvantages in terms of cost performance [24-29], time consumption [4–20], necessary equipments [30, 31], the use of expensive disposable solid phase extraction cartridges [8] and gradient elution control.

We describe the development, validation and application of a simple HPLC method for quantitative determination of RTV in human plasma using LPV as internal standard. The principal advantages of our method are a rapid liquid-liquid drug extraction from plasma in a simple one-step sample preparation and using a small sample volume (200 microlitres) without any loss of analyte, an isocratic elution on a reversed phase C8 column and a shorter run time when compared to the existing methods.

The developed method was simple, specific and did not require any expensive equipment or extra processing steps. A gradient elution program is commonly used for determination of LPV and RTV [36-38]. However, this requires binary pump system, a difficult elution procedure, long run time and results in consumption of large volume of reagent. Under isocratic conditions, as described here, the run time was short (13 min) and there was no need for the analytical column to be washed or re-equilibrated between runs. This condition provided good resolutions for the analyte peaks and separated them from endogenous interferences in plasma and companion antiretroviral drugs.

The method was quite robust as evidenced by a discrete peak denoting RTV. The percent variations (within-day and between-day) for the standards were below 10%. The method reliably yielded an acceptable range of recovery. Further, data from the accuracy and precision experiments yielded satisfactory results.

In view of its potent anti-retroviral activity, RTV is used in the treatment regimen along with other anti-retroviral and anti-tuberculosis drugs in HIV positive patients with and without tuberculosis, who fail to first-line anti-retroviral drugs. It therefore becomes necessary to rule out interference of both these classes of drugs in the assay of RTV and establish the specificity of the method. The method was highly specific for RTV; drugs such as nevirapine, efavirenz, zidovudine, tenofovir, didanosine, stavudine, lamivudine, lopinavir, indinavir and saquinavir or anti-tuberculosis drugs such as rifampicin, isoniazid, pyrazinamide, ethambutol, streptomycin or any endogenous substance interfered in the assay.

When this method was applied to estimate RTV in plasma samples collected serially at different time points from adult HIV-TB patients who received 100 mg of RTV along with LPV and other anti-tuberculosis and second line anti-retroviral drugs, we obtained a mean peak concentration of 1.59 µg/ml, which was within the therapeutic range of RTV (9).

Thus the method spans the range of clinical interest and could be applied to pharmacokinetic studies in both adults and children. Most of the methods published were simultaneous determination of anti-retroviral drugs which requires longer run time and permits quantitation of limited samples. The method has the potential to implement at low budget hospital environment, as it does not require high operational cost instruments such as NMR or tandem mass spectrometers.

CONCLUSION

A sensitive, specific and validated method for quantitative determination of RTV in plasma is described. This simple, rapid, accurate and reproducible method utilizes a single step direct extraction without involvement of expensive solid phase cartridges. The chromatogram yields a well-resolved peak for RTV with good intra-and inter-day precision. This simple HPLC method can be conveniently used as a routine clinical application and enables study of the drug pharmacokinetics in conventional hospital and research laboratories.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Hammer SM, Eron JJ Jr, Reiss P, Schooley RT, Thompson MA, Walmsley S, *et al.* Antiretroviral treatment of adult HIV infection recommendations of the international AIDS society–USA panel. Jama 2008;300:555-70.
2. Thompson MA, Aberg JA, Cahn P. Antirétroviral treatment of adult HIV infection: 2010 recommendations of the International AIDS Society–USA panel. Jama 2010;304:321–33.
3. Janoly A, Bleyzac N, Favetta P, Gagneu MC, Bourhis Y, Coudray S, *et al.* Simple and rapid high-performance liquid chromatographic method for nelfinavir, M8 nelfinavir metabolite, ritonavir and saquinavir assay in plasma. J Chromatogr B 2002;780:155-60.
4. Walson PD, Cox S, Utkin, Gerber N, Crim L, Brady M, *et al.* Clinical use of a simultaneous HPLC assay for indinavir, saquinavir, ritonavir and nelfinavir in china and adults. Ther Drug Monit 2003;5:588-92.
5. Turner ML, Reed-Walker K, King JR, Acosta EP. Simultaneous determination of nine antiretroviral compounds in human plasma using liquid chromatography. J Chromatogr B 2003;784:331-41.
6. Kou, HuiJuan, Min Ye, Qiang Fu, Yang Han, XiaoLi Du, *et al.* Simultaneous quantification of lopinavir and ritonavir in human plasma by high performance liquid chromatography coupled with UV detection. Sci Chin Life Sci 2012;55:321-7.
7. Hirano, Atsushi, Masaaki Takahashi, Eri Kinoshita, Masaaki Shibata. High performance liquid chromatography using UV detection for the simultaneous quantification of the new non-nucleoside reverse transcriptase inhibitor etravirine (TMC-125), and 4 protease inhibitors in human plasma. Biol Pharm Bull 2010;33:1426-9.
8. Laure Elens, Sophie Veriter, Vincent Di Fazio, Roger Vanbist, Daniel, Boesmans, *et al.* Quantification of 8 HIV-protease inhibitors and 2 non-nucleoside reverse transcription liquid chromatography with diode array detection. Clin Chem 2009;55:170-4.
9. Masskai Takahashi, Masao Yoshida, Tsuyoshi Oki, Naoya Okumura, Tatsuo Suzuki and Tsuguhiro Kaneda. Conventional HPLC Method used for Simultaneous Determination of the seven HIV protease inhibitors and nonnucleoside reverse transcription inhibitors efavirenz in human plasma. Biol Pharm Bull 2005;28:1286-90.
10. Hirbayashi Y, Tsuchiya K, Kimura S, Oka s. Simultaneous determination of six HIV protease inhibitors (amprenavir, indinavir, lopinavir, ritonavir and squinavir), the active metabolism of nelfinavir (M8) and non-nucleoside reverse transcriptase inhibitor (efavirenz) in human plasma by high-performance liquid chromatography. Biomed Chromatogr 2006;20:28-36.
11. Tribut O, Verider Mc, Arvieux C, Allian H, Michelet C, Bentue–Ferrer D. Simultaneous quantitative assay of atazanavir and 6 other HIV protease inhibitors by isocratic reversed–phase liquid chromatography in human plasma. Ther Drug Monit 2005;27:265-9.
12. Verbesselt R, Van Wijngaerden E, De Hoon J. Simultaneous determination of 8 HIV protease inhibitors in human plasma by isocratic high-performance liquid chromatography wtih combined use of UV and fluorescence detection: amprenavir, indinavir, atazanavir, retonavir, saquinavir, nelfinavir and M8-nelfinavir metobolite. J Chromatogr B Analyt Technol Biomed Life Sci 2007;845:51-60.
13. Poirier JM, Robidou P, Jaillon P. Simple and simultaneous determination of the HIV–protease inhibitors amprenavir, atazanavir, indinavir, lopinavir, nelfnavir, ritonavir and saquinavir plus M8 nelfinavir metabolite and the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine in human plasma by reversed–phase liquid chromatography. Ther Drug Monit 2005;2:186-92.
14. Choi So, Rezk NL, Kashuba AD. High-performance liquid chromatography assay fro the determination of the HIV–protease inhibitor tipranavir in human plasma in combination with nine other antiretroviral medications. J Pharm Biomed Anal 2007;43:1562-7.
15. Albert V, Modamio P, Lastra CF, Marino EL. Determination of saquinavir and ritonavir in human plasma by reversed–phase high–performance liquid chromatography and the analytical error function. J Pharm Biomed Anal 2004;36:835-40.
16. D'Avolio A, Baietto L, Siccardi M, Sciandra M, Simiele M. An HPLC-PDA method for the simultaneous quantification of the HIV integrase inhibitors raltegravir, the new non–nucleoside reverse transcriptase inhibitors etravirine, and 11 other antiretroviral agents in the plasma of HIV–infected patients. Ther Drug Monit 2008;30:662-9.
17. Rouzes A, Berthoin K, Xuereb F, Djabarouti S, Pellegrin I, Coupet AC, *et al.* Simultaneous determination of the antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by high–performance liquid chromatography–mass spectrometry. J Chromatogr B: Analyt Technol Biomed Life Sci 2004;813:209-16.
18. Notari S, Bocedi A, Ippolito G, Narciso P, Pucillo LP, Tossini G, *et al.* Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography. J Chromatogr B: Analyt Technol Biomed Life Sci 2006;831:258-66.
19. Dailly E, Raffi F, Jolliet P. Determination of atazanavir and other antiretroviral drugs (indinavir, amprenavir, nelfianvir and its active metabolite M8, saquinavir, ritonavir, lopinavir, nevirapine and efavirenz) plasma levels by high performance liquid chromatography with UV diction. J Chromatogr B: Analyt Technol Biomed Life Sci 2004;813:353-8.
20. Rezk NL, Tidwell RP, Kashuba AD. High–performance liquid chromatography assay for the quantification of HIV protease inhibitors and non–nucleoside reverse transcriptase inhibitors in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2004;805:241-7.
21. Rebiere H, MKazel B, Civade C, Bonnet PA. Determination of 19 antiretroviral agents in pharmaceuticals or suspected products with two methods using high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 2007;850:376-83.
22. Ofotokun, Ighovwerha, Edward P Acosta, Jeffrey L Lennox. Pharmacokinetics of an indinavir-ritonavir-fosamprenavir regimen in patients with human immunodeficiency virus. Pharmacother: J Human Pharmacol Drug Ther 2008;1:74-81.
23. la Porte CJL, Colbers EPH, Bertz R, Voncken DS, Wikstrom K. Pharmacokinetics of adjusted-dose lopinavir-ritonavir

combined with rifampin in healthy volunteers. Antimicrob Agents Chemother 2004;48:1553–60.

24. Kityo C, Walker AS, Dickinson L, Lutwama F, Kayiwa J, Ssali F, *et al.* Pharmacokinetics of lopinavir-ritonavir with and without nonnucleoside reverse transcriptase inhibitors in ugandan HIV-Infected adults. Antimicrob Agents Chemother 2010;54:2965–73.
25. Marta Boffito, Akil Jackson, Alieu Amara, David Back, Saye Khoo, Chris Higgs, *et al.* Pharmacokinetics of once-daily darunavir-ritonavir and atazanavir-ritonavir over 72 H following drug cessation. Antimicrob Agents Chemother 2011;5:218–23.
26. Naik H, Murry DJ, Kirsch LE, Fleckenstein L. Development and validation of a high-performance liquid chromatography mass spectroscopy assay for determination of artesunate and dihydroartemisinin in human plasma. J Chromatogr B: Analyt Technol Biomed Life Sci 2005;816:233–42.
27. Chi J, Jayewardene AL, Stone JA, Motoya T, Aweeka FT. Simultaneous determination of five HIV protease inhibitors nelfinavir, indinavir, ritonavir, saquinavir and amprenavir in human plasma by LC/MS/MS. J Pharm Biomed Anal 2002;30:675–84.
28. Jiangeng Huanga, Nagsen Gautama, Sai Praneeth R Bathenaa, Upal Royb, JoEllyn McMillanb, Howard E Gendelmanb, *et al.* UPLC-MS/MS quantification of nanoformulated ritonavir, indinavir, atazanavir, and efavirenz in mouse serum and tissues. J Chromatogr B: Analyt Technol Biomed Life Sci 2011;879:2332–8.
29. Else L, Watson V, Tjia J, Hughes A, Siccardi M, Khoo S, *et al.* Validation of a rapid and sensitive high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds. J Chromatogr B Anal Technol Biomed Life Sci 2010;878:1455–65.
30. Jeroen JA, van Kampen, Mariska L Reedijk, Peter C Burgers. Ultra-fast analysis of plasma and intracellular levels of HIV protease inhibitors in children: a clinical application of MALDI mass spectrometry. PLoS One 2010;5:11409.
31. Roland JW Meesters, Jeroen JA van Kampen, Mariska L Reedijk. Ultrafast and high-throughput mass spectrometric assay for therapeutic drug monitoring of antiretroviral drugs in pediatric HIV-1 infection applying dried blood spots. Anal Bioanal Chem 2010;398:319–28.
32. ICH Harmonised Tripartite Guideline. Validation of analytical procedures: Text and Methodology Q2. Courtesy: Somatek Inc, USA; 2005.
33. Manosuthi W, Kiertiburanakul S, Amornnimit W, Prasithsirikul W, Thongyen S, Nilkamhang S, *et al.* Treatment outcomes and plasma level of ritonavir-boosted lopinavir monother-apy among HIV-infected patients who had NRTI and NNRTI failure. AIDS Res Ther 2009;6:30.
34. Josephson F, Andersson MC, Flamholc L, Gisslén M, Hagberg L, Ormaasen V, *et al.* The relation be-tween treatment outcome and efavir enz, atazanavir or lopinavir ex-posure in the NORTHIV trial of treatment-naive HIV-1 infected pa-tients. Eur J Clin Pharmacol 2010;66:349–57.
35. Hsu A, Isaacson J, Brun S, Bernstein B, Lam W, Bertz R, *et al.* Pharmacokinetic-pharmacodynamic analysis of lopinavir-ritonavir in combination with efavirenz and two nucleoside reverse transcriptase inhibitors in extensively pretreated human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 2003;47:350–9.
36. Avolio A, Siccardi M, Sciandra M, Lorena B, Bonora S, Trentini L, *et al.* HPLC-MS method for the simultaneous quantification of the new HIV protease inhibitor darunavir, and 11 other antiretroviral agents in plasma of HIV-infected patients. J Chromatogr B: Analyt Technol Biomed Life Sci 2007;859:234–40.
37. Colombo S, Guignard N, Marzolini C, Telenti A, Biollaz J, Decosterd LA, *et al.* Determination of the new HIV-protease inhibitor atazanavir by liquid chromatography after solid-phase extraction. J Chromatogr B: Anal Technol Biomed Life Sci 2004;810:25–34.
38. Marzolini C, Beguin A, Telenti A, Schreyer A, Buclin T, Biollaz J, *et al.* Determination of lopinavir and nevirapine by high-performance liquid chromatography after solid-phase extraction: application for the assessment of their trans-placental passage at delivery. J Chromatogr B: Analyt Technol Biomed Life Sci 2002;774:127–40.