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Simultaneous determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma by high-performance liquid chromatography

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method with ultraviolet detection at 205 nm has been validated for the determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma. The ritonavir analogue A-86093.0 was used as internal standard. Good chromatographic separation was achieved using a stainless steel column packed with 5 μm Phenomenex phenyl hexyl material operated at 40 °C, and a mobile phase consisting of acetonitrile–10 m*M* potassium phosphate buffer (50:50, v/v). The calibration curve for indinavir was linear over the range of 50 to1000 μg/l while the ritonavir and lopinavir calibration curves were linear over the range of 100 to 15000 μg/l. The lower limit of quantitations for indinavir, ritonavir and lopinavir were 50, 100 and 100 μg/l, respectively, using 500 μl of human plasma. The validation data showed that the assay is sensitive, specific and reproducible for determination of indinavir, ritonavir and lopinavir. This method is being used in a therapeutic drug monitoring service to quantitate these therapeutic agents in patients infected with human immunodeficiency virus. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Indinavir; Ritonavir; Lopinavir

1. Introduction

Indinavir (IDV), ritonavir (RTV) and lopinavir (LOP: ABT 378) are protease inhibitors used in the treatment of human immunodeficiency virus (HIV) infection [1,2]. The novel protease inhibitor KALETRA is a co-formulation of LOP and RTV. RTV, with a low dose in the formulation, inhibits the cytochrome3A-mediated metabolism of LOP leading to an increase in plasma concentration, allowing twice daily dosing.

The emergence of drug resistant mutations is multifactorial, but it is likely to be caused by the repeated exposure to subtherapeutic drug concentrations [3]. These variations in drug exposure may be due to variable pharmacokinetics between patients (drug absorption, distribution and elimination), non-adherence to drug therapy or drug–drug interactions. The consequences of having subtherapeutic PI drug concentrations or adverse events are significant in

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this patient population with limited antiretroviral options [3]. A role for therapeutic drug monitoring (TDM) of these agents has been identified, however, before TDM can be of most value there is a need to develop standardised assays to be performed routinely in clinical laboratories [3,4].

While there are a number of published methods that describe the analysis of IDV and/or RTV in plasma [5] there is only one report that quantitates all three agents, using a complex gradient elution technique [6]. In this paper we describe the development and validation of a simple, isocratic and sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of IDV, RTV and LOP following liquid–liquid extraction of 0.5 ml of plasma. The method is used for therapeutic drug monitoring of these agents.

2. Experimental

2.1. Chemicals

Merck Sharp & Dohme (Rahway, NJ, USA) kindly supplied IDV. RTV, LOP and the internal standard (A-86093.0, an analogue of RTV) were supplied by Abbott Pharmaceutical Products (Abbott Park, IL, USA). The structures of these compounds have been previously published [5]. Acetonitrile and hexane were HPLC grade and were purchased from Mallinckrodt Baker (KY, USA). 1-Chlorobutane, HiperSolv HPLC grade was purchased from BDH Labs. (Poole, UK). Orthophosphoric acid, 85% was purchased from Ajax Chemicals (Sydney, Australia) and disodium hydrogenphosphate 3-hydrate (pro-analysis grade) was purchased from Merck (Darmstadt, Germany). Distilled water was used after recirculation through a Milli-Q water purification system (Millipore, Milford, MA, USA). Blank human plasma was supplied from the Australian Red Cross Blood Bank (Sydney, Australia).

2.2. Equipment

The HPLC system consisted of the following components: a Shimadzu (Rydalmere, Sydney, Australia) Model LC-10AT pump, a DGU-12A degasser, an SIL-10AXL autosampler, a CTO-10A column oven, an SPD-M10A diode array UV detector. Shimadzu Class LC-10 chromatographic software was used for peak detection and integration. The analytical column was a Phenomenex Luna phenyl hexyl column (250×4.6 mm I.D., particle size 5 μm; Phenomenex, Torrance, CA, USA).

2.3. Chromatography

The chromatographic analysis was performed at 40° C on a phenyl hexyl column with a mobile phase composed of acetonitrile and water containing 10 m*M* potassium phosphate buffer adjusted to pH 7.0–85% orthophosphoric acid (50:50, v/v). Absorbance was measured at 205 nm while the flow-rate was maintained at 1.0 ml/min. Aliquots of 50 μl were injected onto the HPLC column.

2.4. Preparation of standards

Stock solutions of IDV, RTV and LOP were prepared by dissolving the appropriate amount of drug pure substance in methanol to yield a final drug concentration of 0.1 g/l IDV and 1 g/l RTV and LOP. Working solutions were prepared from a 1/10 (INV) or 1/100 (RTV, LOP) dilution of the stock. The working solution of the internal standard (A-86093) was prepared from a 1/10 dilution of a stock solution (0.1 g/l in methanol) in water. The standard solutions were stored at −20 °C.

2.5. Preparation of calibration standards and quality control samples

Calibration standards were prepared in plasma covering the concentration range between 50 and 1000 μg/l INV and 100 to 15000 μg/l RTV and LOP by adding appropriate volumes of the working solutions to drug-free plasma. The volume of methanolic working solution never exceeded 50 μl/ml of plasma. Five calibration concentrations were used to define the standard curve (50, 100, 300, 500, 1000 for INV and 100, 500, 2000, 5000 and 15000 for RTV and LOP). These calibration samples were divided into 5-ml glass extraction tubes as 500 μl aliquots, and frozen at −80 °C until assay. Quality control samples were prepared by dilution of an independently prepared stock solution of INV (0.1

g/l), RTV and LOP (1 g/l). The quality control samples were prepared in drug-free human plasma to final concentrations of 150 and 600 for INV; 3000 and 10000 for RTV and LOP.

2.6. Sample pretreatment

Internal standard (100 μl) was added to 500 μl of standard, quality control or patient plasma. The drugs were extracted from plasma using 5 ml of 1-chlorobutane and mixing on a rotating shaker for 10 min. After the samples were centrifuged at 2000 *g* for 10 min the organic layer was transferred to a tapered tube and evaporated under air at 50 °C in a water bath. The extract was reconstituted in 500 μl mobile phase, briefly vortexed, and washed with 3 ml of hexane. The tubes were centrifuged at 2000 *g* for 2 min and the top, organic layer was aspirated and discarded. A second 3 ml hexane wash was performed and after centrifugation the organic layer was discarded. The remaining aqueous layer was transferred to an autosampler vial for analysis.

2.7. Assay validation

A 4-day validation of the analysis of INV, RTV and LOP was performed. The statistical analyses were done using SigmaStat for Windows (Version 2.03, SPSS, Chicago, IL, USA). The acceptance criteria used to validate the assay have been published or are available as guidelines [7,8].

2.7.1. Linearity

Duplicate calibration curves were generated each day for three consecutive days. One reagent blank, plasma blank and control zero (blank plasma with internal standard added) was analysed in each run. The linear regression of the ratio of peak height of INV, RTV, LOP and the internal standard versus the concentration were weighted by $1/x$ (reciprocal of the concentration).

2.7.2. Accuracy and precision

The accuracy and intra-day and inter-day precision of the method were estimated by assaying three replicate quality control samples at two different concentrations for each drug, in three analytical runs. The accuracy was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations expressed as percent deviation (% DEV). The overall mean precision was defined by the relative standard deviation (RSD) of two quality control samples at two different concentrations analysed over 3 days.

2.7.3. Specificity and selectivity

Interference from endogenous compounds was investigated by the analysis of six different blank plasma matrices. Potentials coadministered drugs used in combination therapy with INV, RTV and LOP, including HIV-reverse transcriptase inhibitors (zidovudine, abacavir, lamivudine, stavudine, didanosine, zalcitabine, efavirenz, nevirapine and delavirdine) and protease inhibitors (amprenavir, saquinavir and nelfinavir) were also analysed for interference under the HPLC conditions.

2.7.4. Lower limit of quantitation

The lower limit of quantitation (LLQ) was investigated in plasma using six replicates of the following calibration standards: INV 50 μg/l, RTV and LOP 100 μg/l. The concentration that was chosen as the LLQ was accepted if the percent deviation from the nominal value (measure of accuracy) and the RSD (measure of precision) was less than 20% [8].

2.7.5. Recovery

The recovery of INV, RTV, and LOP was determined on duplicate standards at five concentrations by comparing the peak area of extracted standards with the peak area of recovery standards prepared in mobile phase and injected directly onto the column to produce the identical ‘‘on column’’ concentrations. The recovery of the internal standard was calculated from the average peak area of extracted replicates with the peak area of replicates directly injected onto the column.

2.7.6. Stability

Duplicate quality control samples at two concentrations were used to assess the stability of INV, RTV, LOP and internal standard in mobile phase, and at room temperature for 24 h after extraction. The stability of the drugs and internal standard during sample handling was also verified by subjecting the samples to two and three freeze–thaw cycles.

3. Results and discussion

3.1. Specificity and selectivity

Blank plasma samples from six different individuals showed no interfering endogenous substances in the analysis of INV, RTV and LOP (Fig. 1). However, we have found from experience that the two hexane washes were necessary to prevent endogenous interference and long run times due to late eluting peaks (Fig. 1). Potentially coadministered drugs that were tested had retention times that were different from INV (7.0 min), RTV (17.5 min), LOP (19.4 min) and internal standard (24.4 min). However, efavirenz did coelute with the RTV peak and amprenavir chromatographs near the indinavir peak. We were able to separate RTV and efvavirenz by simply changing the column to a Zorbax SB-CN column (150×4.6 mm, 5 μm; Agilent Technologies, Palo Alto, CA, USA) and performing the analysis at room temperature. However, when a patient receives efavirenz and KALETRA (LOP plus RTV) as co-medication we do not report the RTV concentration as it is not present in therapeutic concentrations but simply acts as a pharmacokinetic enhancer. The use of amprenavir and indinavir in a combined therapeutic regimen is unlikely, however, we recommend diode-array UV detection to prevent misinterpreting an indinavir peak in a patient receiving amprenavir and to identify the presence of a suspected amprenavir metabolite that may interfere with the ritonavir when amprenavir is present in high concentrations.

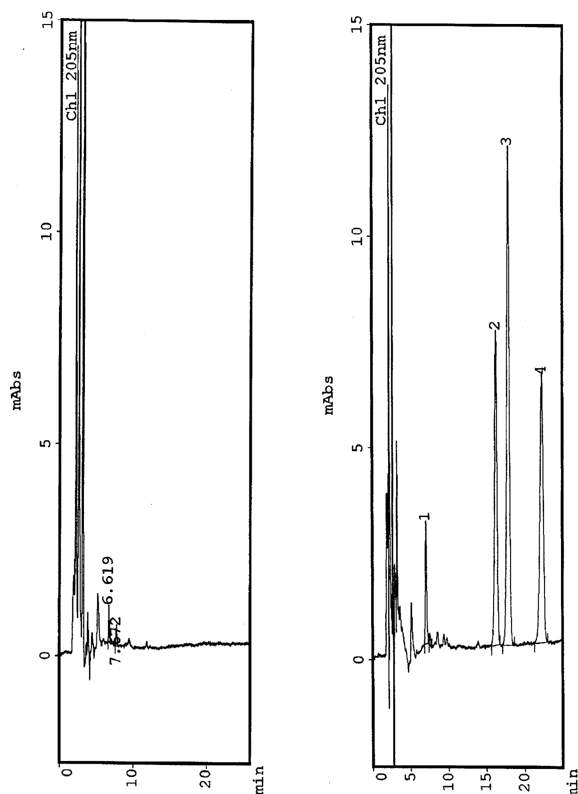

Fig. 1. Chromatograms of blank human plasma (left panel) and a standard extracted from human plasma (right panel) 1=indinavir, 2=ritonavir, 3=lopinavir and 4=internal standard.

3.2. Validation

Calibration curves in human plasma were linear over the concentration range 50 to 1000 μg/l for INV and 100 to 15000 μg/l for RTV and LOP. These ranges were considered adequate for TDM [4]. The slope, intercept and correlation coefficients of the calibration curves were INV: $0.17 \cdot 10^{-2} \pm 0.60 \cdot 10^{-4}$, $0.68 \cdot 10^{-2}$ and 0.999 (mean±SD; $n=6$); RTV: $0.08 \cdot 10^{-3} \pm 0.18 \cdot 10^{-4}$, $-0.32 \cdot 10^{-3}$ and 0.999; LOP: $0.10 \cdot 10^{-2} \pm 0.18 \cdot 10^{-4}$, $0.67 \cdot 10^{-2}$ and 0.999, respectively. The LLQ of 50 μg/l for INV and 100 μg/l for RTV and LOP was chosen and is acceptable for TDM [4]. The accuracy and precision of the LLQ was INV: −6.7% DEV and 7.6% RSD; RTV: 8.7% DEV and 3.8% RSD and LOP: 10.8% DEV and 3.0% RSD.

Table 1 shows the accuracy and precision of the quality control samples prepared in human plasma. The results indicate that the method is accurate and precise. Mean accuracy data over 3 days was better than 10.8% DEV for INV, 12.4% DEV for RTV and 8.2% DEV for LOP, while the between-day precision was better than 7.9% RSD for INV, 5.0% RSD for RTV and 4.9% RSD for LOP. The mean recovery of INV was 93%, RTV 103%, LOP 98% and 94% for the internal standard.

INV, RTV, LOP and internal were stable under all conditions tested, with all results falling within the acceptance criteria of ±15% DEV from the nominal concentration.

Table 1
Accuracy and precision of indinavir, ritonavir and lopinavir in human plasma

Concentration (μg/l)	Accuracy* (% DEV)	Precision (RSD, %)		N
		Within-day (N_w=6)	Between-day (N_b=6)	
INV 150	−8.3	7.9	5.3	18
INV 600	−8.5	3.4	3.3	18
RTV 3000	−0.6	5.0	6.6	18
RTV 10000	2.7	3.3	11.1	18
LOP 3000	0.9	4.9	2.7	18
LOP 10000	−1.0	2.9	2.8	18

Abbreviations: DEV=deviation from the nominal value, RSD=relative standard deviation, N_w=number of replicates per run, N_b=number of different runs, N=total number of replicates.
* Mean over 3 days.

3.3. Applicability

Current therapeutic regimens with highly active antiretroviral therapy (HAART) have proven successful in treating patients with HIV. This success is tempered by rates of virological failure, estimated to be as high as 67% in therapy-naive patients. Additionally, drug resistance and significant drug-related toxicity remain serious concerns. The response to HAART is variable and is attributed to virologic (development of drug resistance), behavioural (non-adherence), immunologic and pharmacologic differences between patients. Pharmacologic differences are due, in part, to variability in drug absorption, distribution, metabolism and excretion. Drug dose is a poor predictor of drug effect because it does not account for the pharmacologic variability of the drug, however, TDM, using a plasma drug concentration to compare with targets of effect and/or toxicity allows tailoring of drug dose to overcome this variability. TDM is also useful in managing drug–drug interactions, identifying non-adherent patients and managing drug overdose. We are using this method in our TDM service and have found significant variability in plasma concentrations of these drugs in patients receiving the same standard dose (Fig. 2). Additionally, a significant number of plasma drug concentrations were below the minimum effective concentration (MEC) necessary to prevent viral replication. TDM is an essential tool to optimise the dose of these drugs in patients infected with HIV.

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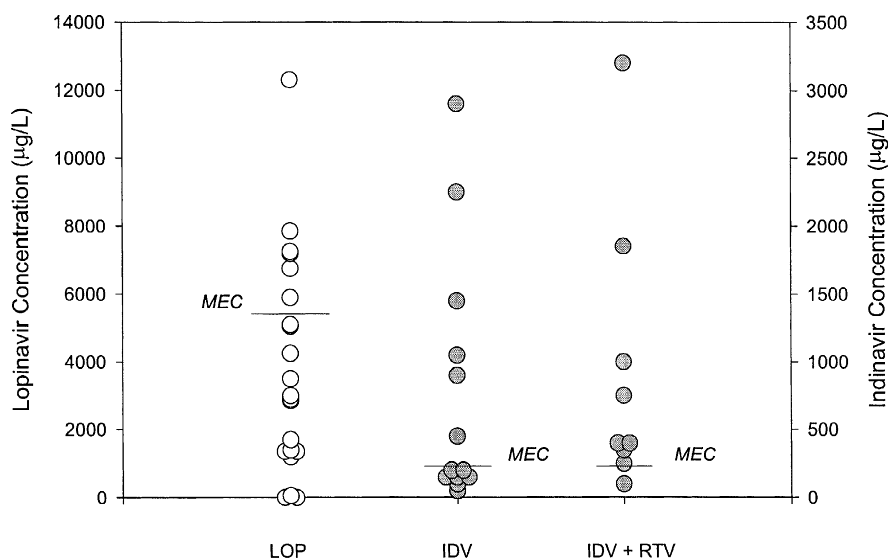

Fig. 2. Trough plasma concentrations of lopinavir (open circles) and indinavir (grey circles) in patients who received identical doses of each drug. MEC is the minimum effective concentration needed to suppress viral replication.

ritonavir, lopinavir, internal standard and indinavir pure substance. We also thank these companies for their generous financial support.

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