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Reversed-phase liquid chromatography with ultraviolet detection for simultaneous quantitation of indinavir and propranolol from ex-vivo rat intestinal permeability studies

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Abstract

A simple, rapid, sensitive and specific reversed-phase high performance liquid chromatographic (RP-HPLC) method involving ultraviolet detection ($\lambda = 210$ nm) was developed for analysis of indinavir along with propranolol in samples obtained from ex vivo intestinal permeability studies. Chromatography was carried out on C-18 column with mobile phase comprising of phosphate buffer-acetonitrile (68:32, v/v) pumped at flow rate of 1 ml/min. The proposed method has a short run time of 12 min and involves a simple sample preparation for the purpose of reducing permeability model artifacts and to concentrate the samples. Fluorescein was used as internal standard. The proposed method has been validated with regard to specificity, detection limit, recovery, accuracy and precision. For both the drugs, method was found to be selective, linear ($R^2 \approx 0.999$), accurate (recovery = 100–105%) and precise (<3% R.S.D.) in the range of 2–20 μg/ml. The limit-of-detection and limit-of-quantification of the method were 40 ng/ml and 100 ng/ml for indinavir, and 30 and 80 ng/ml for propranolol, respectively. Indinavir, a widely prescribed HIV protease inhibitor, suffer from bioavailability problems where involvement of P-glycoprotein mediated drug efflux may play a significant role. The proposed method was successfully applied for intestinal permeability of indinavir to estimate the contribution of P-glycoprotein in limiting its oral bioavailability. The advantage of the developed method lies in the simultaneous determination of propranolol, a passive integrity marker, routinely employed in permeability studies and its selectivity in presence of various P-gp modulators and permeability markers.

Keywords: Indinavir; Propranolol

1. Introduction

Human acquired immuno-deficiency virus (HIV) protease inhibitors form the mainstay in clinical management of HIV-1 infection because of their ability in reducing viral load of plasma and other tissues. However, the complete therapeutic potential of this class of drugs is yet to be exploited due to number of limitations related to their poor and variable transport across important biological membranes, especially gastrointestinal tract (GIT). Adequate and consistent absorption is particularly needed in anti-HIV therapy because the virus surviving after exposure to drug may mutate, and develop resistance. Further, their clinical use is also limited because of the requirement of very high doses and potential drug–drug interactions, which are even of more concern in complicated dosage regimens [1].

Indinavir (CRIXIVAN™) is a FDA approved peptidomimetic HIV protease inhibitor [2]. Indinavir, most frequently prescribed due to strong anti-retroviral activity (than saquinavir) and better tolerability (than ritonavir), however exhibit absorption problems characteristic to this class [3]. Other than factors like high first pass metabolism, indinavir is shown to be a substrate to P-glycoprotein (P-gp) resulting in greater permeability towards the secretory (luminal) direction than in the absorptive direction. P-gp is an ATP-dependent efflux membrane transporter apically expressed throughout the GIT and acting as major biochemical barrier in limiting oral bioavailability [4]. Biopharmaceutical properties of indinavir place it into biopharmaceutic classification system (BCS) Class IV drugs with poor solubility

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and poor permeability (unpublished data). As a result, absorption enhancement by application of conventional drug delivery systems is difficult to achieve and strategy would be to focus on improvement of its passage across the membrane using active extrusion inhibitors known as P-gp inhibitors or chemosensitizers.

Rat everted sac is a well-established in vitro model used to characterize P-gp substrates and inhibitors. A suitable analytical method for determining drugs in the transport buffer in presence of various P-gp modulators is necessary for studying the role of P-gp on overall permeability of indinavir and for explaining the use of P-gp modulators for improving absorption kinetics. The several methods available so far for quantification of indinavir in blood samples involve complicated extraction procedures [5]. P-gp modulation studies are mostly done with radio-chemical analysis but this technique is always not possible because of problems related to the storage, handling and waste disposal of radioactive materials.

We propose a simple and reliable RP-HPLC method for routine analysis of indinavir in intestinal permeability studies, in the presence of propranolol, a model integrity marker.

The ranges of the concentration measured by this method are in line with those measured by other authors [6,7], however, we present a simple and specific method for quantification of indinavir, having short run time of 12 min in comparison to 30 min as proposed by Jayewardene et al. [8]. Above all, we have utilized isocratic-mode with UV detection, which is more feasible and less time-consuming than any other method of detection [9]. Other advantage of this method is that it enables simultaneous determination of propranolol, which is FDA suggested high permeability passive integrity marker routinely used in permeability studies [10]. Hence, the proposed method was further validated for routine usage for its application to intestinal permeability studies.

2. Experimental

2.1. Chemicals

Indinavir and propranolol were kindly provided by Matrix Labs and Baroda Pharma Limited, India respectively. All the solvents used were of HPLC grade (J.T. Baker) and reagents were of analytical grade. Following drugs were used for evaluation of selectivity, quinidine (Sigma–Aldrich Co., USA), digoxin (Burroughs Wellcome Ltd., India), furosemide (Matrix Labs., India), phenol red (HiMedia Lab., India), cyclosporin (Dabur Research Foundation, India), rifampicin (Macleods Pharmaceuticals, India), imipramine (Sigma–Aldrich Co., USA), amitryptyline (Sigma–Aldrich Co., USA), sulindac (Sigma-Aldrich Co., USA), lovastatin (Matrix Labs., India), fluorescein (Sigma-Aldrich Co., USA) and grapefruit juice (Berry Ltd., Australia). Purified water obtained by reverse osmosis (USF ELGA) filtered through 0.45 μm membrane filter was used throughout the study.

2.2. Equipment

A liquid chromatographic system (Shimadzu, Japan) comprising of LC-10AT VP solvent pump, DGU-14AM on line-degasser, autoinjector SIL-10AD VP with temperature control, CTO-10AS VP column oven and SPD-10AVP UV-Vis spectrophotometric detector was used. Shimadzu CLASS-VP software was used for data acquisition, reporting and analysis. The column used for chromatographic separations was Hypersil BDS C-18 (250 mm × 4.6 mm, 5 μm) (Thermo Hypersil, UK) and guard column of Symmetry C18 WATO 54225 (Waters, USA). Mobile phase was pumped in isocratic mode at a flow rate of 1 ml/min at ambient temperature. The analytical wavelength was set at 210 nm and samples of 50 μl were made automatically injected using SIL-10AD VP autosampler (Shimadzu, Japan). In addition, electronic balance (AG 245, Mettler Toledo, Switzerland), sonicator (3210, Branson, USA), Millipore filtration assembly and filters (USA) were used.

2.3. Method development

2.3.1. Mobile phase

The chromatographic separations were accomplished using mobile phase, consisting of 50 mM disodium hydrogen phosphate buffer (pH adjusted to 5.0 with 20% *o*-phosphoric acid) and acetonitrile (62:38), filtered through 0.45 μm filter (Millipore) and deaerated in ultrasonic bath (Branson 3510).

2.3.2. Preparation of standard solutions

Primary stock solution of indinavir and propranolol were prepared in ultra pure water to obtain a concentration of 2 mg/ml. Primary standard solution was diluted to 100 μg/ml that served as the secondary stock, which was further diluted in blank ex vivo samples (obtained from blank run of everted sac experiments, see Section 2.5) to obtain working standards in the range of 2–20 μg/ml.

2.3.3. Extraction procedure for analysis of ex-vivo samples

Quantification of indinavir and propranolol in samples obtained from ex vivo intestinal everted sac model required extraction to eliminate intestinal tissue artifacts that interfere during analysis. This also resulted in increased sensitivity of the method as concentrations upto 100 ng/ml could be quantified. Fluorescein, used as an internal standard was added (50 μl of 100 μg/ml) along with indinavir and propranolol to the blank ex vivo sample (500 μl), in 2.0 ml centrifuge tube. 1.0 ml of ACN was added and the samples were centrifuged at 16000 × *g* for 30 min. A fixed volume (1.4 ml) of supernatant was taken into another micro centrifuge tube and evaporated to dryness in centrivac (Maxi dry lyo, Denmark). The residue thus obtained was reconstituted with 200 μl of mobile phase; vortex mixed for 30 s, centrifuged

at 16000 × *g* for 5 min and injected (50 μl). Same extraction procedure, after addition of internal standard, was followed for analyzing actual samples.

2.4. Method validation

HPLC method was validated to determine various parameters viz. linearity, range, specificity, sensitivity, precision and system suitability. Six-point calibration curves were constructed for both drugs over the concentration range of 2–20 μg/ml. For both indinavir and propranolol, limit of detection (LOD) and limit of quantitation (LOQ) were determined with and without extraction procedure. Percentage recovery (accuracy) and precision were determined with 3 replicates of quality control (QC) samples. QC samples were prepared in blank ex vivo samples at three different concentrations (2, 7, and 12 μg/ml) following the same procedure as for calibration standards, using different primary stock. To assess intra-day variation, calibration curve was prepared three times on the same day. The procedure was further repeated on three consequent days for determination of inter-day variability. The results were expressed as %R.S.D. of slopes. The assay for selectivity consisted of the addition of certain permeability markers and P-gp substrates, such as imipramine, furosemide, cyclosporine A, quinidine, etc. To determine the extraction efficiency of the extraction methodology, drugs with internal standard was injected with and without extraction. The direct injection samples contained equivalent amount of drugs as spiked in the extracted samples. Extraction efficiency was given as

$$\text{Extraction efficiency (\%)} = \frac{(\text{Peak area of extracted sample})}{(\text{Peak area of un-extracted sample})} \times 100$$

2.5. Ex-vivo permeation studies

All animal studies were done according to the guidelines of the Institutional Animal Ethical Committee (IAEC) of National Institute of Pharmaceutical Education and Research (NIPER), Punjab. Male Sprague–Dawley rats (230–250 g) were housed under standard laboratory conditions and fasted for 16 h before the experiment with water ad libitum. After a mild anesthesia, they were killed by cervical dislocation. The specimens were cut open, intestinal tissue from the region of interest was removed, everted and ligated to make a sac. The sacs were placed into 10 ml of pre-oxygenated buffer and 3 ml of Kreb's Ringer Buffer (KRB; containing NaCl (7.0 g), KCl (0.35 g), $CaCl_2$ (0.28 g), $MgSO_4$ (0.28 g), $NaHCO_3$ (2.10 g), KH_2PO_4 (0.16 g) and D-glucose (5.05 g), was placed in the serosal compartment. For investigation of basal-to-apical (B → A) transport, drug solution (indinavir, 50 μM and propranolol, 200 μM) in KRB was placed in serosal side. During inhibition studies, the cyclosporin A in the specified concentration of 25 μM was placed in the apical chamber [11]. An aliquot of 1.0 ml was taken as sample from the receptor phase at 60, 120 and 180 min and replaced with same volume of buffer. The collected samples were extracted according to the extraction procedure and injected to estimate the drug content. Data acquisition and processing was done on Shimadzu Class-VP software.

2.6. Statistical analysis

Data of three-four independent experiments was reported as mean ± standard error of mean (S.E.M.) unless other wise noted. One-way ANOVA were performed on permeability data. A *P*-value of 0.05 and 0.01 were used as the significance levels for all tests. All statistical tests were performed using Jandel Sigma stat version 2.0.

3. Results and discussion

3.1. Chromatography and Specificity

Representative chromatograms of blank and extracted samples are shown in Fig. 1. No interfering peaks were observed near the retention time of indinavir or propranolol or of the internal standard, fluorescein. The retention times of propranolol, fluorescein and indinavir were 5.1, 6.5 and 9.5 min, respectively. The chromatographic run time of 12 min was sufficient for routine sample analysis permitting large number of samples to be analyzed in a short period of time. The retention times of range of P-gp inhibitors (cyclosporin A and grapefruit juice) likely to be used during the study were also measured by injecting solutions of these compounds onto the HPLC column. None of the peaks interfered with any of the components of KRB or P-gp modulators (cyclosporin A and grapefruit juice).

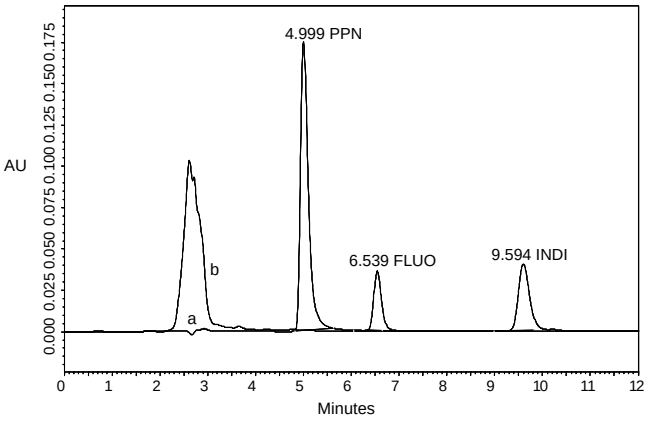

Fig. 1. Representative overlayed chromatograms of (a) blank intestinal extract and (b) intestinal sample extract from ex-vivo rat everted sac studies containing propranolol (PPN), indinavir (INDI) and the internal standard, fluorescein (FLUO). The peaks are annotated with their respective names and retention times. For chromatographic conditions refer Section 2.3. Blank and intestinal samples were extracted as described in Section 2.3.

3.2. Validation

3.2.1. Range and linearity

Six-point calibration curves were constructed for both drugs over the concentration range of 2–20 μg/ml. This concentration range was selected on the basis of anticipated drug concentrations in absorption studies. Ratio of peak areas of drug to that of internal standard versus concentration were plotted and found to be linear within the concentration range. Standard curve was constructed on two consecutive days and regression parameters, slope, intercept and correlation coefficient, were calculated and listed in Table 1.

3.2.2. Accuracy and precision

Accuracy and precision of the method was determined by analyzing quality control (QC) samples at three different concentrations within the calibration range in triplicates ($n = 3$). QC samples prepared in blank ex vivo samples are dilutions from weightings independent of those used for preparing calibration curves. The percent recovery of method was found to be 100 ± 5% and R.S.D. of <2% indicating that method is accurate and precise (Table 1).

Intra- and inter-day repetitions determined the intermediate precision of the present method. It is expressed as percent relative standard deviation (R.S.D.) for a statistically significant number of samples. The %R.S.D. values in the three regression lines prepared on the same day or different days were <2% for both indinavir and propranolol (Table 1).

3.2.3. Extraction efficiency

Recoveries of both indinavir and propranolol from ex vivo intestinal samples were determined by comparing the peak areas of the extracted standards and those of un-extracted standards (Table 2). The extraction efficiency of indinavir was found to be in the range of 92–99% and that of propranolol was found to be in the range of 87–95%.

Table 2
Percentage extraction efficiency of indinavir and propranolol from ex vivo ntestinal sac samples done at different concentration levels[a]

Concentration (μg/ml)	Indinavir		Propranolol	
	Extraction efficiency (%)	S.D.	Extraction efficiency (%)	S.D.
5	98.4	4.4	94.4	1.8
7	94.1	5.3	87.9	2.1
10	92.0	2.4	93.6	2.3
15	99.0	1.3	89.2	1.5

[a] Values are the mean of three determinations.

3.2.4. Sensitivity and selectivity

LOD and LOQ decide about the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method whereas; LOQ is the minimum quantifiable concentration. The signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ, respectively, which was calculated using Shimadzu Class VP software, and further confirmed by taking dilutions from the secondary stock solution till the peak area obtained has 3 (for LOD) and 10 (for LOQ) fold then the standard deviation of six determinations. The developed method was sensitive enough to quantitate 500 ng/ml of indinavir and 400 ng/ml of propranolol without involvement of extraction procedure. However, application of the extraction procedure allowed determinations to be done at much lower levels,

Table 1
HPLC method validation parameters for determination of Indinavir and propranolol

Parameter		Indinavir			Propranolol	
Regression equation						
Range		2–20 μg/ml			2–20 μg/ml	
Slope (±S.D.)		0.0695 (±0.0012)			0.2463 (±0.0025)	
Intercept (±S.D.)		−0.0152 (±0.0044)			−0.014 (±0.0367)	
LOD (3:1 signal-to-noise ratio)[a]		40 (24) ng/ml			30 (19.5) ng/ml	
LOQ (10:1 signal-to-noise ratio)[a]		100 (80) ng/ml			80 (65) ng/ml	
Accuracy and precision						
Concentration (μg/ml)	2	7	12	2	7	12
%Recovery*	104.51	100.53	104.55	104.69	100.71	104.14
%R.S.D.[b]	2.16	0.43	1.45	2.83	2.05	1.61
Intermediate precision						
Intra-day variation#						
Slope (%R.S.D.)		0.0695 (1.83)			0.2463 (1.03)	
Inter-day variation#						
Slope (%R.S.D.)		0.0693 (2.24)			0.2381 (3.80)	

Each standard curve was generated in triplicates across the range.
[a] Values indicated in the parenthesis are obtained based on signal-to-noise ratio, while values outside the parenthesis were obtained by dilution technique.
[b] Precision expressed as %R.S.D. of three determinations.
* Accuracy was determined by % recovery at three concentration levels in triplicates.
# Intra-day and inter-day variation is expressed as slope (%R.S.D.) of three calibration curves prepared on same day and on different days, respectively. Standard curve was plotted between the ratio of areas of drug and internal standard against concentration.

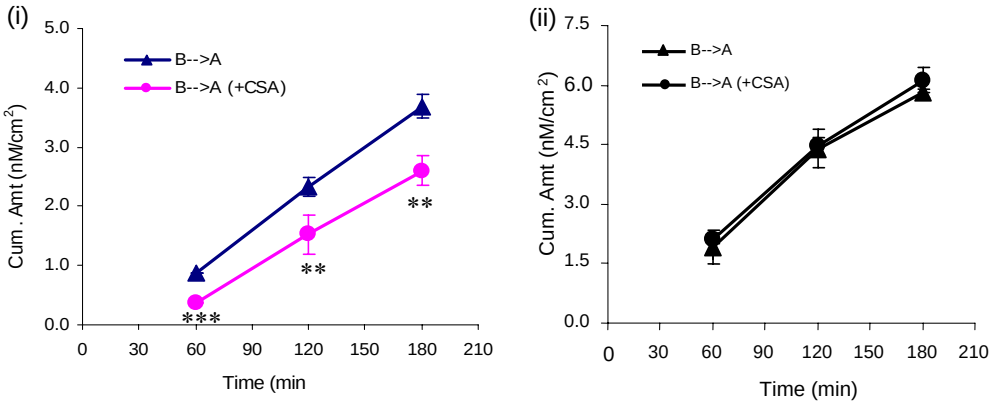

Fig. 2. Time course of basolateral-to-apical (B → A) (i) indinavir (50 μM) and (ii) propranolol (200 μM) in terms of cumulative amount permeated per unit surface area across rat ileal intestinal segment in presence and absence if P-gp inhibitor, cyclosporin A (CSA, 25 μM). $^{**}P < 0.05$, one-way ANOVA. $^{***}P < 0.01$, one-way ANOVA. Drug was added to the basolateral compartment and at the indicated time point, amount of drug appearing in the opposite compartment was determined. Values are mean of three measurements ± S.E.M. Inhibitor solutions were applied to the apical side and the amount permeated into the receptor compartment was measured at indicated time points.

i.e. 100 ng/ml and 80 ng/ml for indinavir and propranolol, respectively (Table 1). LOD and LOQ of extracted samples based on signal-to-noise ratio were found to be 24 and 80 ng/ml for indinavir and 19.5 and 65 ng/ml for propranolol, respectively.

The selectivity of the method was determined by testing various drugs, which were either substrates or modulators to P-gp, or general permeability markers that are used for validating permeability models. The drugs examined were cyclosporin, quinidine, digoxin, phenol red, imipramine, furosemide, amitryptyline, sulindac, rifampicin, lovastatin and prednisolone. The retention times of these drugs are listed in Table 3. Amitriptyline, cyclosporin and digoxin did not elute till 35 min.

Table 3
Retention times of drugs tested for non-interference (λ = 210 nm)[a]

Drug	Retention time (min)	Remarks
Imipramine	16.71	High permeability
Quinidine	4.01	P-gp substrate and inhibitor
Furosemide	6.93	Low permeability
Phenol red	6.12[b]	Control for membrane integrity
Rifampicin	9.49[c]	P-gp and CYP 3A substrate
Sulindac	7.41	P-gp substrate
Lovastatin	7.96	P-gp substrate
Prednisolone	5.54[d]	P-gp substrate
Amitriptyline	>35[e]	High permeability
Cyclosporine A	>35[e]	P-gp substrate and inhibitor
Digoxin	>35[e]	P-gp substrate and inhibitor

[a] Drug substances in appropriate concentrations were injected without involving extraction procedure.
[b] Interferes with Internal standard (FLUO) elution.
[c] Interferes with INDI elution.
[d] Interferes with PPN elution.
[e] Compound did not elute in the due time of chromatographic run.

3.3. Application of method to determine P-gp mediated efflux of indinavir and propranolol in presence of P-gp inhibitor, cyclosporin

Validation data of the present method demonstrated its application in the analysis of indinavir and propranolol in samples obtained from rat intestinal everted sac. P-gp mediated efflux activity was determined through the cumulative amount transported in secretory (B → A) direction. Fig. 2 shows that cyclosporin A (25 μM), a known P-gp modulator, has no significant effect on the secretary transport of propranolol. This was as expected since propranolol is not a substrate to P-gp and its transport across the intestinal epithelium is controlled by passive transcellular diffusion. Therefore, addition of P-gp modulator produces no effect on the permeability of propranolol [12]. In the present study propranolol was used as transcellular permeability marker. However, other membrane integrity markers like phenol red, furosemide and highly permeable drugs like imipramine can also be quantified using the same method (Table 3). Indinavir secretary transport was found to be significantly influenced by P-gp modulation ($P < 0.05$). Cumulative amount drug transport of indinavir after addition of cyclosporin A decreased (Fig. 2). A change of 29% in the cumulative amount transported in basal to apical direction was observed. Indinavir is thus seen to be actively extruded by P-gp and its absorptive transport can be increased by application of suitable P-gp inhibitor [13].

4. Conclusions

Permeability studies demands its quantitation in samples obtained from experimental models like ex vivo rat intestinal

everted sac model. A rapid and reliable isocratic RP-HPLC assay for determination of both indinavir and propranolol simultaneously from ex vivo rat intestinal permeability studies has been developed and validated. All the parameters met the criteria of ICH guidelines for bio-analytical methods. The assay is highly specific, precise and involves simple sample preparation with a short run time making it suitable for routine permeability studies and enabling large number of samples to be analyzed per day. The proposed method has an advantage of simultaneous determination of propranolol, a passive integrity marker, routinely employed in permeability studies and its selectivity in presence of various P-gp modulators and permeability markers. This method has been applied for elucidating P-gp role in intestinal absorption of indinavir.

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