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Simultaneous determination of plasma lopinavir and ritonavir by chemometrics-assisted spectrophotometry and comparison with HPLC method

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Abstract: Chemometrics-assisted spectrophotometry for the determination of two protease inhibitors, lopinavir and ritonavir, in plasma was evaluated. A set of calibration mixtures (calibration set) was designed according to central composite design. The UV spectra obtained from the calibration set were subjected to partial least square regression (PLS-1) to construct the prediction models for lopinavir and ritonavir in unknown samples, which were then validated in a randomly selected set of synthetic mixtures of the drugs. An optimum model was obtained in the wavelength ranges of 215–249 nm and 240–279 nm with principal components 8 for both lopinavir and ritonavir respectively. The prediction models were used to analyse the two drugs in plasma and the results were compared with those obtained by high-performance liquid chromatography (HPLC). The PLS-1 model and the HPLC method were found to be comparable.

Keywords: protease inhibitor, lopinavir, ritonavir, chemometrics, PLS-1

INTRODUCTION

Lopinavir (LPV) and ritonavir (RTV) are important HIV-1 protease inhibitors, alone or in combination, for highly active antiretroviral therapy (HAART) [1]. However, there is a relationship between protease inhibitor concentration and pharmacological activity as well as toxicity or side effects. In addition, wide variability in pharmacokinetics of protease inhibitors was also found [2]. To avoid drug toxicity and improve efficacy, therapeutic drug monitoring is required for drugs with a narrow therapeutic range including protease inhibitors [1, 3-5]. Several analytical methods have been

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described for the determination of protease inhibitors and other antiretroviral agents in biological samples [1, 3-17]. In the past decade, a combination of antiretroviral drugs including LPV and RTV in human plasma was usually determined by high-performance liquid chromatography – ultraviolet spectroscopy (HPLC-UV) or HPLC-UV/fluorescence [3-9]. Nowadays, liquid chromatography – mass spectrometry (LC-MS) and LC-MS/MS are the most widely adopted methods for quantitating protease inhibitors including LPV and RTV in several types of samples, e.g. human plasma [10-12], dried blood spot [13], dried plasma spot [14] and peripheral blood mononuclear cell [1, 15-17]. Although LC-MS/MS seems to be the most effective instrument due to its sensitivity and specificity when compared with conventional HPLC-UV, the price of the instrument and professional operation has been the limitation on routine therapeutic drug monitoring. On the other hand, a spectrophotometric measurement without prior chromatographic separation is interesting but the overlap of the absorbance spectra remains problematic. However, it is possible to solve this problem with chemometric approaches. Among these, multivariate calibration methods such as multiple linear regression (MLR), principle component regression (PCR) and partial least square (PLS) utilising spectrophotometric data are important for the determination of drugs in combination [18-19]. In our previous study [20], multivariate calibration methods, i.e. PCR and PLS-1, were developed for determination of LPV and RTV in syrup. Although those results were comparable with the developed HPLC method, the previous PCR and PLS-1 models cannot be applied to plasma samples owing to the difference between the syrup matrix and the human plasma matrix. A chemometrics-assisted spectrophoto- metric method, PLS-1, is therefore developed for the determination of LPV and RTV in plasma samples in this study. The same sample set was also analysed by HPLC. Results of the two methods are then compared to determine the reliability of the chemometric approach. To our knowledge, the determination of LPV and RTV in human plasma by PLS-1 has not been reported.

MATERIALS AND METHODS

Reagents, Apparatus and Software

All standard drugs of working standard grade were kindly donated by the Government Pharmaceutical Organisation (GPO), Thailand. Reagent-grade hexane and ethyl acetate were obtained from J. T. Baker. Acetonitrile and methanol (HPLC-grade) were purchased from Labscan. Sodium dihydrogen phosphate was obtained from Merck. The drug-free plasma samples were expired plasma kindly donated by Ramathibodi Hospital, Bangkok. These plasma samples were not in a category which needs ethics committee approval.

UV absorbance was recorded in a 10-mm quartz cell using a PerkinElmer (Lambda 35) UV-Visible spectrophotometer. Manipulation of the spectral data was carried out with UV Winlab software. The chromatographic system consisted of a Shimadzu LC-10AD, a CBM-10A (system controller) and a SPD-10V UV-Vis detector. Hypersil Gold C18 (250 × 4.6 mm i.d., particle size 5 μm) was supplied by Thermo Scientific (USA). A 1.73-GHz Intel personal computer with Windows XP operating system, together with the Unscrambler® (version 9.8) programme and other statistical analyses, was used to calculate the PLS model.

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Preparation of Standard Solutions and Plasma Samples

Stock solutions of LPV (1 mg L^{-1}) and RTV (0.5 mg L^{-1}) were prepared by dissolving the required amount of each drug in methanol. Proper dilution of each stock solution was prepared with 50% methanol to obtain standard solutions for further use in the experiments. The linear concentration range of each drug was determined by one-component calibration. The absorbance spectra were recorded over a range of 200–400 nm using 50% methanol as blank.

Each plasma sample was prepared by spiking 400 µL of drug-free blank plasma with 100 µL of the desired standard mixture solution. Prior to the analysis, interference from the plasma samples was removed by liquid-liquid extraction. The plasma samples and organic solvent were mixed and then centrifuged at 3000 rpm for 15 min. The organic layer was transferred to a clean tube and evaporated under nitrogen at 50°C in a water bath. The extract was reconstituted by adding 2 mL of 50% methanol and centrifuged at 3000 rpm for 5 min., and 1.5 mL of the supernatant was then removed for analysis. For the HPLC method, the samples were cleaned and then filtered through a nylon membrane filter.

Chemometric Method

Two sets of standard LPV- and RTV-spiked plasma samples were prepared. The concentration of each drug in the two sets was within its respective linearity range. One set served as the calibration set, which consisted of 16 drug mixtures in plasma and four blank plasma samples. The second set was the validation set, which contained ten drug mixtures in plasma. The composition of the calibration set was selected based on central composite design (CCD) [21] while that of the validation set was randomly selected. Absorbance data of the two sample sets were collected at wavelengths between 200-400 nm. These data were applied to construct a model using Unscrambler® programme. The resulting model was then used to determine the LPV and RTV concentrations in the validation set (test set).

HPLC Method

HPLC conditions, comprising acetonitrile and 25 mM phosphate buffer pH 6 (50:50 v/v) and a C18 column (Hypersil Gold C18, 250 × 4.6 mm i.d., particle size 5 µm), were developed and validated. The flow rate of the system was 1 mL min^{-1}. The presence of the desired drugs was monitored by UV detection at 254 nm. Drug-spiked plasma samples were used for method development. To prove the reliability of the developed HPLC method, important validation parameters, i.e. linearity, accuracy, precision, specificity and system suitability, were investigated. Linearity was established by using the drug-mixture-spiked plasma. Six concentrations of LPV (2–20 µg mL^{-1}) and RTV (0.5–8 µg mL^{-1}) were assessed; indinavir (IDV) at a concentration of 2 µg mL^{-1} was used as internal standard. Linearity was demonstrated by plotting the peak area ratio of the drugs to the internal standard versus concentration of the drugs. Regression parameters were then computed. For accuracy and precision, drug-free plasma was spiked with three concentrations of LPV (3–18 µg mL^{-1}) and RTV (0.75–7 µg mL^{-1}) with five determinations for each concentration. The accuracy of the developed method was expressed as per cent recovery of the amount of added drug vs. amount detected, while precision was expressed as the relative standard deviation (% RSD) of per cent recovery at each concentration. The specificity of the method was confirmed by comparing chromatograms of the drug-spiked plasma samples with those of the drug-free plasma samples.

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Three parameters, namely repeatability, resolution and tailing factor, provided the basis for the system suitability testing. Repeatability was based on five replicates of injected-drug mixtures (10 μg LPV mL^{-1} and 3 μg RTV mL^{-1}). Injection repeatability was expressed as % RSD of the peak area ratio of the drug to internal standard obtained from five-replicate injections. The resolution (R_s) of each drug and its adjacent peak was calculated and the tailing factor of the peak of the desired drug was also investigated.

RESULTS AND DISCUSSION

Since the spectra of LPV and RTV strongly overlap in the wavelength region of 200–270 nm [20], determination of one drug in the presence of the other by univariate calibration is not possible. Instead, a multivariate calibration technique, i.e. partial least square regression (PLS-1), is chosen for this study. Due to the complexity of plasma matrix and the absence of collinearity data, the PCR is not chosen.

PLS-1 Modelling

To construct the PLS-1 model, the one-component calibration (univariate calibration) of each drug was initially executed. A linearity range was determined in the concentration ranges of 2–20 μg mL^{-1} and 1–6 μg mL^{-1} for LPV and RTV respectively. The concentrations of LPV and RTV in the calibration set assigned for the CCD and the randomly selected concentrations of these drugs in the validation set were within the linearity range (2–12 μg mL^{-1} for LPV and 1–4 μg mL^{-1} for RTV). The composition of the calibration set corresponding to CCD is illustrated in Table 1. Individual analytes were independently modelled by PLS-1 with the optimum wavelength region and number of principal components (PCs) or factors. The optimum wavelength region was selected by visual observation; a region which was less interfered by another drug was chosen for testing.

Owing to the above-mentioned strongly overlapping drug spectra, several wavelength regions and PCs were studied to obtain the fitted models. Leave-one-out cross-validation (LOO-CV) was used to validate the PLS-1 model in the model development. The optimum number of factors (PCs) with minimum prediction error of sum squares for an optimum model was determined from the model development. The optimum prediction models were achieved in the wavelength regions of 215–249 nm with 8 PCs for LPV and 240–279 nm with 8 PCs for RTV. The resulting PLS-1 model for plasma samples contained more PCs than those in the PLS-1 model for syrup [20]. In plasma, the PCs of 8 were found to be the optimum components for both LPV and RTV models while the optimum PCs for LPV and RTV in syrup were 2 and 4 respectively. These may be due to a more complicate matrix in plasma compared to that in syrup.

In the calibration step (model development), statistical parameters such as the root mean square error of calibration, the correlation coefficient (r^2) and the relative error of prediction were also computed. In addition, the independent set of plasma samples containing different compositions of LPV and RTV (test set sample) that did not contribute to the calibration step was used to evaluate the proposed calibration models. The statistical parameter expressing the predictive applicability of a regression model was the standard error of prediction. All relevant statistical parameters of the optimal PLS-1 model for LPV and RTV determination were acceptable for all proposed calibration models as shown in Table 2.

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Table 1. Composition of calibration set corresponding to CCD

Sample	Concentration (μg mL^{-1})		Coded level [22]	
	LPV	RTV	LPV	RTV
1	0	2.0	- α	0
2	16.0	2.0	+ α	0
3	8.0	0	0	- α
4	8.0	4.0	0	+ α
5	2.3	0.6	- 1	- 1
6	2.3	3.4	- 1	+ 1
7	13.7	3.4	+ 1	+ 1
8	13.7	0.6	+ 1	- 1
9	8.0	2.0	0	0
10	8.0	2.0	0	0
11	8.0	2.0	0	0
12	8.0	2.0	0	0
13	8.0	2.0	0	0
14	8.0	2.0	0	0
15	8.0	2.0	0	0
16	8.0	2.0	0	0

Table 2. Statistical parameters of the optimum PLS-1 model for LPV and RTV determination

Parameter	LPV	RTV
Spectral range (nm)	215 – 249	240 – 279
PCs	8	8
PRESS	0.6371	0.0213
RMSEC	0.1675	0.0365
r^2	0.9976	0.9986
REP (%)	2.50	1.82
SEP	0.3706	0.2144
SEN	1.02×10^{-3}	7.96×10^{-4}
SEL	9.60×10^{-3}	6.16×10^{-3}

Note: PRESS = prediction error of sum squares; PCs = number of principle component; RMSEC = root mean square error of calibration; r^2 = correlation coefficient; REP = relative error of the prediction; SEP = standard error of prediction; SEN = sensitivity; SEL = selectivity.

The proposed models were also validated in terms of linearity, accuracy, precision and limit of quantitation in order to show that the chemometric method is appropriate to the investigation of LPV and RTV in plasma samples by spiking plasma with different concentrations of the two drugs. Table 3 shows that all parameters meet the requirements of the bioanalytical method validation guidelines [23].

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Table 3. Results of validation of PLS-1 model for LPV and RTV

Parameter[a]	LPV	RTV
Spectral range (nm)	215 – 249	240 – 279
PCs	8	8
Linearity	0.9988	0.9975
Accuracy	104	96
Repeatability	5.07	5.85
Inter-day precision	5.00	4.73
LOQ	1.77	0.51

[a] Linearity is expressed as the correlation coefficient (r^2) and accuracy as the average % recovery (n=15). Precision is expressed as relative standard deviation (% RSD) and consists of repeatability (n=15) and inter-day precision (n = 5). Limit of quantitation (LOQ) was calculated by the formula: LOQ = 10(SD/S), where SD is the standard deviation of the response and S is the slope of calibration curve.

HPLC Method Development and Method Validation

LPV and RTV can be well separated from each other and from other interferences present in plasma. The retention times of LPV, RTV and the internal standard IDV are 12.23 min., 10.55 min. and 5.16 min. respectively. Both LPV and RTV exhibit a linear relationship in a graph of the peak area ratio vs. concentration over the entire concentration range (2–20 μg mL^{-1} for LPV and 0.5–8 μg mL^{-1} for RTV) with acceptable linear regression parameters (Table 4). The stability of the proposed method was evaluated by studying the variation in the peak area ratio of LPV and RTV in drugs-spiked plasma.

Table 4. Summary of validation results and system suitability parameters for HPLC method

Parameter (Unit)	LPV	RTV
Linearity range (μg mL^{-1})	2-20	0.5-8
r^2	0.9917	0.9983
Slope	0.0913	0.7261
Intercept	0.0183	0.1118
Repeatability (% RSD, n=5)	1.43-2.62	0.73-3.37
Intermediate precision (% RSD, n=5)	2.10-3.89	2.03-7.86
Recovery (%)	100-104	86-101
Repeatability of peak area ratio (% RSD, n=5)	1.92	0.57
Limit of quantitation (μg mL^{-1})	2.96	0.96
Resolution	4.91	5.18
Tailing factor	1.08	1.09

As summarised in Table 4, the repeatability (intra-day precision) of the determination is indicated by the % RSD value for plasma analyses, which range between 1.43–2.62% for LPV and 0.73–3.37% for RTV. The intermediate precision (inter-day precision) RSD values obtained for the drugs-spiked plasma samples are below 3.89% for LPV and 7.86% for RTV over the entire concentration range of 3–18 μg mL^{-1} for LPV and 0.75–7 μg mL^{-1} for RTV. The results show that

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the precision of the proposed HPLC method is satisfactory for the analysis of LPV and RTV in plasma samples. The accuracy of the method is expressed as per cent recovery of the added drug. The mean recoveries are 100–105% for LPV over the concentration range of 3–18 μg mL^{-1} and 86–101% for RTV over the concentration range of 0.75–7 μg mL^{-1}. All figures of merit for method validation meet the requirements of the bioanalytical guidelines [23].

The specificity of the method is illustrated in Figure 1. A comparison of the chromatogram of the drug-spiked plasma and that of the drug-free plasma shows that the endogenous substances in plasma do not elute at the retention times of LPV and RTV, indicating that LPV and RTV can be separated from the sources of interference present in plasma. Additionally, the precision of retention time, resolution and tailing factor (asymmetric peak), the three criteria used to assess the system suitability of the HPLC conditions, are all acceptable (Table 4).

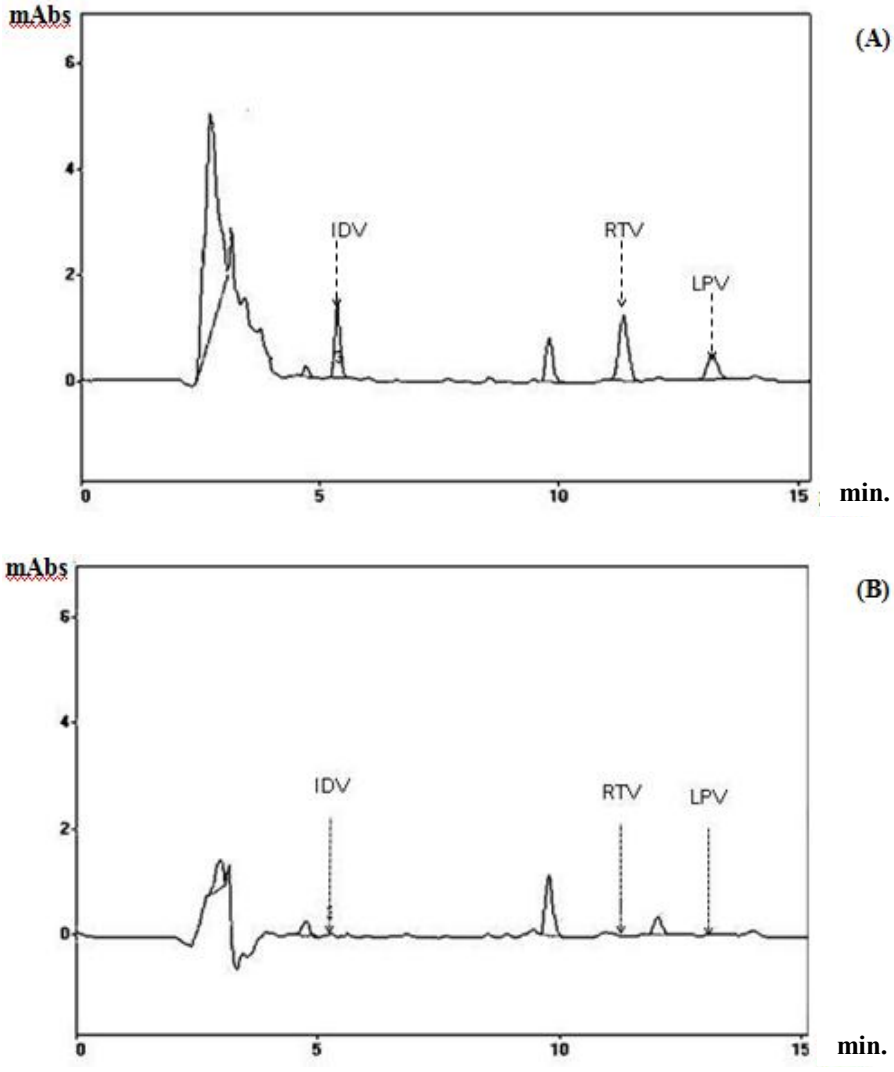

Figure 1. (A) Chromatogram of LPV (10 μg mL^{-1}), RTV (3 μg mL^{-1}) and IDV (2 μg mL^{-1}) spiked in plasma; (B) Chromatogram of drug-free plasma sample with expected retention times of IDV, RTV and LTV

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Comparison of Chemometric and HPLC Methods

A set of 10 mixture samples, apart from those used in calibration step, were prepared and analysed using the developed HPLC method and PLS-1 model. The mean recoveries of LPV and RTV from human plasma obtained from the two methods were then compared by using t-test statistic. As shown in Table 5, the mean recovery results for the LPV- and RTV-spiked in plasma are not significantly different at the 95% confidence limit. These results indicate that the simple and rapid chemometric method can be used as an alternative to the HPLC method for the determination of LPV and RTV in plasma.

Table 5. Mean recoveries of LPV and RTV obtained using PLS-1 optimum model compared with HPLC method

LPV			RTV		
Parameter	**HPLC**	**PLS-1**	**Parameter**	**HPLC**	**PLS-1**
Mean % recovery (n=10)	106	103	Mean % recovery (n=10)	96	98
% RSD	3.37	4.29	% RSD	3.15	6.63

CONCLUSIONS

The developed PLS-1 model, employing UV absorbance data successfully determined LPV and RTV concentrations in plasma. Validation of the reliability of the PLS-1 prediction model gave acceptable validation results and was not significantly different from the reference HPLC method in the determination of LPV and RTV, which shows that this chemometrics-assisted spectrophotometric method can be used as an alternative to HPLC in the determination of the drug mixtures.

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