Development and Validation of a Liquid Chromatographic Method for the Simultaneous Analysis of Six Protease Inhibitors Using a Polymer Column

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A liquid chromatographic method for the simultaneous determination of six human immunodeficiency virus (HIV) protease inhibitors, indinavir, saquinavir, ritonavir, amprenavir, nelfinavir and lopinavir, was developed and validated. Optimal separation was achieved on a PLRP-S 100 Å, 250 x 4.6 mm I.D. column maintained at 60 °C, a mobile phase consisting of tetrahydrofuran-potassium phosphate buffer (0.1M, pH 5.0)-tetrabutylammonium hydrogen sulphate (0.1M, pH 5.0)-water (35:30:10:25 %v/v) at a flow rate of 1.0 ml/min, with ultraviolet detection at 254 nm. The method was found to be linear over the ranges investigated with r^2 values of 0.9997-0.9915 for the six drugs. The limit of quantitation for the six drugs was 0.16 to 5.12 µg, while the limit of detection was 0.08 to 2.12 µg. The intra-day and inter-day precision was within the ranges of 0.39 to 1.14% and 0.55 to 1.46%, respectively.

Key words: Protease inhibitors, liquid chromatography, poly(styrene-divinylbenzene), method development, method validation.

INTRODUCTION

The use of antiretroviral (ARV) drugs has resulted in a decline in Human Immunodeficiency Virus/Acquired Immuno-deficiency Syndrome (HIV/AIDS) related mortality and morbidity [1-2]. Though the number of patients receiving antiretroviral therapy (ART) in developing countries has increased, ARVs are still not affordable and accessible to many others. Lack of well-established quality specifications is one of the factors that has limited recent efforts by the WHO to accelerate access to ARVs by promoting the local manufacture of quality generics through compulsory licensing under the World Trade Organization agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS) [4]. Surveys of the general quality of locally manufactured and imported generic drugs in the Kenyan market indicate a failure rate of about 10-25 % [5-8]. Hence, there is need for validated methods of analysis for evaluating generic ARVs to ensure that acceptable standards of quality, safety and efficacy are achieved.

The protease inhibitors (PIs) have transformed ART as they have been shown to increase life expectancy in HIV/AIDS patients [1-2]. The United States Food and Drug Administration has approved amprenavir, indinavir, nelfinavir, ritonavir, saquinavir, atazanavir, fos-amprenavir, tipranavir and a fixed dose combination of lopinavir/ritonavir for the treatment of HIV/AIDS patients [9-11].

Most HIV-protease inhibitors currently in clinical use have no compendial methods of analysis (12). Different liquid chromatographic (LC) techniques for simultaneous quantification of specific combinations of PIs in biological samples have been reported [13-26]. However, these methods have various limitations such as use of a gradient elution [14-15, 23-24], long run time [15, 20, 22, 24] and use of multiple chromatographic systems per analysis [15].

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Other methods employ the rather expensive LC-MS-MS system [21] or variable wavelength detection [13-16, 18], which may not be available in quality control laboratories in developing countries. This paper describes the development and validation of a simple, isocratic, precise and selective liquid chromatographic method for simultaneous analysis of six protease inhibitors namely amprenavir, indinavir, nelfinavir, lopinavir, ritonavir and saquinavir, using a poly(styrene-divinylbenzene) column.

EXPERIMENTAL

Chemicals and Reagents

Indinavir sulphate working standard (98.5% w/w) was from Ranbaxy Laboratories Limited (Pune, India), while saquinavir (Roche Diagnostics GMBH, Mannhelm, Germany) was a kind donation from Roche Products Limited (Nairobi, Kenya). Nelfinavir mesylate (99.9% w/w) was from Hetero Labs Limited (Hyderabad, India) and amprenavir (99.4% w/w) was obtained from GlaxoSmithKline (Nairobi, Kenya). Ritonavir and lopinavir/ritonavir capsules were from Abbott Laboratories Limited (Queenborough, U.K.).

Potassium dihydrogen phosphate (Aldrich Chemical Co. Ltd, Gillingham-Dorset, U.K.), dipotassium hydrogen phosphate (Acros Organics, Geel, Belgium), tetrabutyl ammonium hydrogen sulphate (Acros Organics Geel, Belgium) were all of analytical grade. Methanol (Fischer Scientific U.K. Ltd, Loughborough, U.K.) and tetrahydrofuran (PROLABO, Fontenay S/Bois, France) were of analytical grade and were freshly glass distilled before use. Water was freshly distilled in the laboratory.

Equipment and Materials

The liquid chromatographic system consisted of a Merck Hitachi intelligent pump model L-6200A (Hitachi Ltd, Tokyo, Japan), coupled to a Valco model sample injection valve (Valco Company, Texas, U.S.A.) equipped with a 20 µl loop. A 112-Gilson fixed wavelengths UV detector (Gilson, Middleton, U.S.A.) and a HP 3396 series II (Hewlett Packard, Avondale, PA, U.S.A.) integrating recorder were used.

A laboratory packed poly(styrene-divinylbenzene) (PSDVB) analytical column, PLRP-S 100 Å, 8 µm (Polymer Laboratories, Church Stretton, Shropshire, U.K.), 250 mm x 4.6 mm I.D. was used for all experiments.

Standard solutions

Stock solutions of indinavir, nelfinavir, saquinavir and amprenavir were prepared by dissolving appropriate amounts of accurately weighed standard substances in methanol. Stock solutions of ritonavir and lopinavir were prepared from commercial products. The stock solutions were kept at 4 °C until used. The working solution used for method development and validation was prepared by diluting stock solutions with mobile phase to a concentration of 1.0 mg/ml for indinavir and ritonavir and 0.5, 0.3, 0.2 and 1.3 mg/ml for nelfinavir, saquinavir, amprenavir and lopinavir, respectively.

RESULTS AND DISCUSSION

Method Development

Wavelength: Although the compounds under study showed highest ultraviolet absorbance at approximately 210 nm for all the compounds and also at 263 nm and 239 nm for amprenavir and saquinavir, respectively, a wavelength of 254 nm was chosen due to its versatility. There was sufficient absorbance of all compounds at 254 nm to allow analysis.

Column selection: Preliminary separation of the drugs was compared on five co-polymer columns: RoGel, PRP-1, PLRP-S 100 Å, PLRP-S 300 Å and PLRP-S 1000 Å of dimensions 250 x 4.6 mm I.D. Retention times decreased progressively from RoGel, PRP-1, PLRP-S 100 Å, PLRP-S 300 Å, and finally PLRP-S 1000 Å. The RoGel column had the lowest efficiency and high retention with only three compounds eluting within 35 min. Only three compounds were resolved on both PLRP-S 300 Å and 1000 Å columns. On the PRP-1 column, four compounds were resolved, with IDV and APV

as well as RTV and LPV co-eluting. The PLRP-S 100 Å column yielded five peaks with APV and IDV co-eluting, whereas RTV and LPV were partially separated. It also gave the best column efficiency, short retention times and asymmetry factors and so it was chosen for further work.

Organic modifiers: Preliminary work was done with mobile phases consisting of organic solvent-potassium phosphate buffer (0.1 M, pH 6.0)-water (X:10:90-X, v/v). The amounts of acetonitrile and methanol required to elute the compounds were too high (50-70%). Tertiary butanol (30%) gave long retention times and could not be increased due to its high viscosity. Thirty percent tetrahydrofuran gave long retention times while 35 % gave acceptable retention times and peak separation of all the six compounds. Hence 35 % tetrahydrofuran was chosen for further work.

Effect of pH: Variation of the pH of the potassium phosphate buffer had little effect on the retention of RTV, LPV and APV, but it had a significant effect on the retention of SQV and NFV as shown in Figure 1. A pH of 5.0 was found to be optimal with respect to retention time and separation of the analyes.

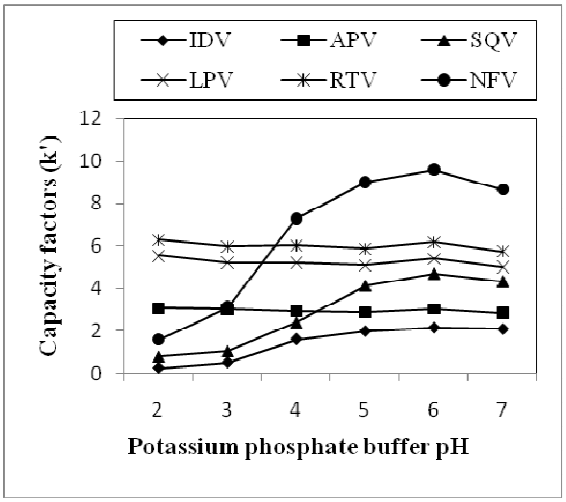

Figure 1: Effect of potassium phosphate buffer pH on the capacity factors of the working solution. Column: PLRPS-100 Å, 250 x 4.6 mm. Column temperature: 60 °C. Mobile phase: Tetrahydrofuran-potassium phosphate buffer (0.1 M, pH 5.0)-water (35:30:35, v/v). Flow rate: 1.0 ml/min. Detection: 254 nm.

Potassium phosphate buffer concentration: Potassium phosphate buffer (0.1 M, pH 5.0) was varied from 10 to 40 %. The optimal buffer concentration at 30 % was chosen for further work (Figure 2).

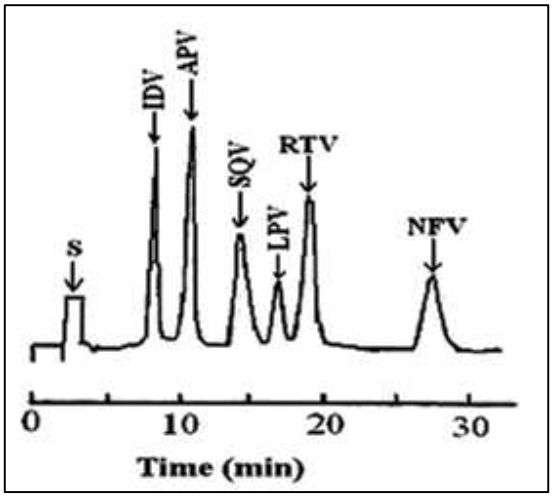

Figure 2. Separation of the working mixture using buffer concentration 30 %. Column: PLRPS-100 Å, 250 x 4.6 mm. Column temperature: 60° C. Mobile phase: Tetrahydrofuran-potassium phosphate buffer (0.1 M, pH 5.0)-water (35:30:35, v/v). Flow rate: 1.0 ml/min. Detection: 254 nm. S: Solvent front.

Effect of tetrabutylammonium hydrogen sulphate: To further improve on the selectivity, varying concentrations of tetrabutylammonium hydrogen sulphate (0.1 M, pH 5.0) were added to the mobile phase consisting of tetrahydrofuran-potassium phosphate buffer (0.1 M, pH 5.0) -water (35:30:35, v/v). An asymmetry factor of 1.4 was achieved at a tetrabutylammonium hydrogen sulphate concentration of 20 %. In addition, resolution between SQV and LPV also improved. However 10 % tetrabutylammonium hydrogen sulphate (0.1 M, pH 5.0) was chosen since resolution and symmetry were improved at this concentration.

Effect of column temperature: The effect of column temperature was investigated at 50, 60 and 70 °C. High back pressure and poor peak resolution were observed between RTV and

LPV at 50 °C. Even though 70 °C gave better peak symmetry and improved resolution between RTV and LPV, 60 °C was preferred to avoid the unstable baseline observed at 70 °C.

Optimized conditions: The optimal chromatographic conditions characterized by a relatively short run time as well as good resolution and asymmetry factors for all the peaks were established as: PLRP-S 100 Å 8 µm column maintained at 60^{o} C; a mobile phase consisting of tetrahydrofuran-potassium phosphate buffer (0.1M, pH 5.0)-tetrabutylammonium hydrogen sulphate (0.1 M, pH 5.0)-water (35:30:10:25, v/v) pumped at a flow rate of 1.0 ml/min and UV detection at 254 nm. Figure 3 shows a typical chromatogram of the working solution obtained under the optimum separation conditions.

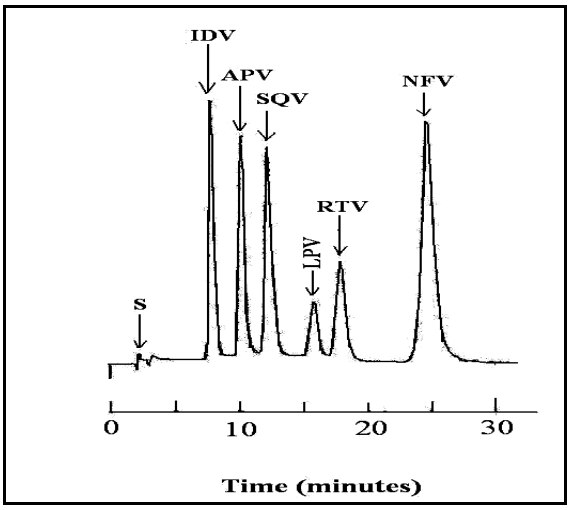

Figure 3. A typical chromatogram of the working solution. Column: PLRP-S 100 Å, 250 x 4.6 mm maintained at 60 ^{o}C. Mobile phase: Tetrahydro-furan-potassium phosphate buffer (0.1M, pH 5.0)-tetrabutylammonium hydrogen sulphate (0.1 M, pH 5.0)-water (35:30:10:25 %v/v). Flow rate: 1.0 ml/min. Detection: 254 nm. S: Solvent front.

Method Validation

Table 1 shows the validation parameters for the method. For linearity, the concentrations of the components in the working solution were taken as 100 %. The detector response was linear in the range of 20-200 % for IDV, APV, SQV and NFV, and 20-130 % for RTV and LPV with r^2 values of 0.9997-0.9915. The detection limits were between 0.08 µg and 2.12 µg at a signal to noise (S/N) ratio of 3. The limits of quantitation with S/N 10 ranged from 0.16 µg for APV to 5.12 µg LPV and all the coefficients of variation were less than 20%. The coefficient of variation calculated for all the compounds at the optimal conditions ranged from 0.39 to 1.14 % for intra-day and 0.55 to 1.68 % for inter-day precision. This shows that the method is reliable over a wide range of drug concentrations.

CONCLUSION

Previously reported methods for assay of various protease inhibitors recorded run times of 30-60 min and with use of gradient elution [13-26]. The method developed in the present study offers the advantage of isocratic elution, relatively short run time and the use of a fixed wavelength detector (254 nm). Furthermore, it uses a polymer column, which is known to be more robust than silica-based columns. Therefore, the method is suitable for routine analysis of raw materials or dosage forms of the protease inhibitors singly or in combination using the same chromatographic conditions.

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Table 1. Validation parameters for the protease inhibitors indinavir, amprenavir, saquinavir, lopinavir, ritonavir and nelfinavir

Parameter	IDV	APV	SQV	LPV	RTV	NFV
Linearity (n=3, k=6)						
Range (mg/ml)	0.20 - 2.06	0.04 - 0.40	0.06 - 0.60	0.53 - 2.26	0.13 - 0.83	0.10 - 1.00
Slope	3180	8065	7314	3512	12543	82473
Intercept	612	3609	3581	79	604	2406
r^2	0.9992	0.9997	0.9983	0.9915	0.9975	0.9994
LOD (µg)	0.580	0.080	0.092	2.120	0.325	0.250
LOQ (µg)	1.140	0.160	0.168	5.120	1.080	0.500
CV	7.1	12.5	15.2	7.3	7.4	11.3
Repeatability, RSD (n=6)	0.52	1.07	0.81	1.14	0.73	0.39
Inter-day Precision (n=6, 3 days)	1.58	1.68	1.49	1.32	1.32	0.55

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