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Two-dimensional LC–MS/MS determination of antiretroviral drugs in rat serum and urine

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ABSTRACT

A simple, rapid, reliable and highly sensitive on-line two-dimensional reversed-phase liquid chromatography–tandem mass spectrometric (2D-LC/MS/MS) method to determine antiretroviral drugs viz., abacavir (ABC), nevirapine (NVP) and indinavir (IDV) in rat serum and urine was developed and validated. The analytes were extracted on-line from rat serum and urine by a restricted access material (RAM) column and back-flushed into the reversed-phase C_{18} column for separation by LC. Detection was carried out by ESI-MS/MS. The developed method showed good selectivity, accuracy and precision for quantification of the antiretroviral drugs in rat serum and urine. Quantification limits for abacavir and nevirapine were 4.0 ng ml^{-1}, whereas for indinavir 4.7 ng ml^{-1}. The calibration graphs were linear in the range of 4–50 ng ml^{-1} for abacavir, nevirapine and indinavir. The method was successfully applied to study the pharmacokinetics of antiretroviral in rats.

1. Introduction

Determination of drugs, metabolites and endogenous compounds in biological fluids viz., blood serum, plasma and urine is not only important in clinical research but also a challenge to analytical chemists [1]. The clean-up procedures to remove matrix components are the most important steps in such analyses. A great amount of work was done and different techniques viz., liquid–liquid and solid-phase extraction were developed. These techniques are no doubt quite efficient but still need improvement in recovering low concentration of drugs from biofluids. Often, the sample pretreatment is the most time-consuming step and usually depends on the complexity of the matrix to be treated. The traditional clean-up procedures for removal of proteins are based on (i) precipitation of proteinaceous material using organic solvents, buffers followed by extraction of the drug by liquid–liquid and solid-phase extraction; (ii) enzymatic deproteinization (employing proteolytic enzymes); (iii) membrane filtration; (iv) precipitation by metal-complex formation; (v) salting-out; and (vi) thermal denaturation. These procedures separate the analytes of interest from proteinaceous material preventing the adsorption of protein and other interferences onto the analytical column. The pretreatment step covering both clean-up and preconcentration is the bottle-neck of the analytical procedures and an important issue in bioanalytical studies [2].

Direct injection of the blood serum into the LC system is highly complicated due to the presence of high concentration of proteins and the endogenous compounds [3]. The proteins in the biological fluids could be precipitated, denatured and adsorbed onto the packing material leading to high backpressures resulting in column blockage. It also decreases the sensitivity, retention time, column efficiency and capacity of the LC column. Restricted access materials (RAM) are porous chromatographic supports specifically designed for the removal of endogenous macromolecules from biofluids. These materials work on the principles of size-exclusion chromatography and used for analysis of low molecular weight drugs in biofluids. There are five basic types [4] of RAM columns according to the nature of the barrier and the structure of the sorbent: (a) mixed-functional dual-zone materials; (b) internal surface reversed-phase packings; (c) shielded hydrophobic phases (SHP); (d) semi-permeable surfaces; and (e) polymeric materials. In SHP packing, the hydrophilic network consists of bonded polyethylene oxide with embedded phenyl groups [5]. These columns enable direct injection of the biological samples into HPLC system with out pretreatment.

Antiretroviral drugs are used for treatment of infections by retroviruses, primarily human immunodeficiency viruses (HIV) that can lead to acquire immunodeficiency syndrome (AIDS). Presently four classes of antiretroviral drugs are available viz., (i) nucleoside/tide reverse transcriptase (NRTI) inhibitors, (ii) non-nucleoside (NNRTI) inhibitors, (iii) protease (PI) inhibitors and (iv) fusion inhibitors.

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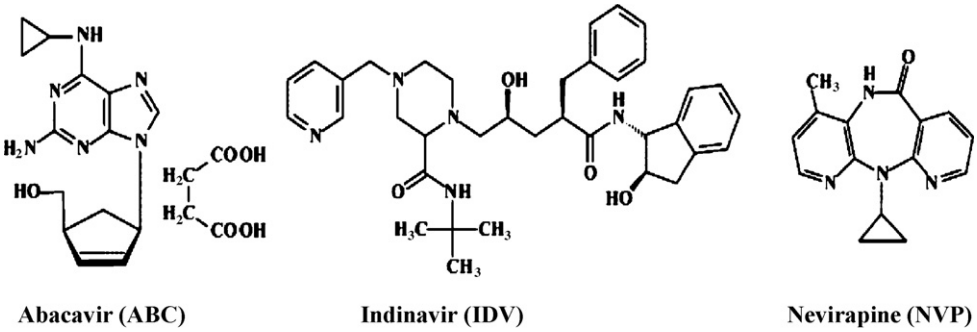

Fig. 1. Chemical structures of ABC, IDV and NVP.

Abacavir (ABC), [(1R)-4-[2-amino-6-(cyclopropylamino)purin-9-yl]-1-cyclopent-2-enyl]methanol is a novel purine carbocyclic nucleoside analogue that exhibits selective activity against HIV-1 and HIV-2 [6–8]. Nevirapine (NVP), 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2′,3′-e], is a non-nucleoside reverse transcriptase inhibitor (NNRTI) widely used as a part of highly active antiretroviral therapy (HAART) for treatment of HIV/AIDS, and prevention of mother-to-child transmission of HIV (PMTCT) [9]. Indinavir (IDV) is another potent HIV protease inhibitor with virological, immunological, and clinical characteristics [10]. It is most frequently prescribed due to strong anti-retroviral activity and better tolerability [11].

Analysis of ABC, NVP and IDV in biological fluids was well studied [12–16]. Notari et al., [17] reported simultaneous determination of anti-HIV drugs in human plasma using a Symmetry^{TM} C_{18} (250 mm × 4.6 mm I.D.) column with (0.01 M aqueous KH_2PO_4 and acetonitrile) as mobile phase at 1.0 ml/min in a gradient elution mode and automated solid-phase extraction by Oasis HLB cartridge. Aymard et al., [18] have determined 12 anti-retroviral agents in human plasma using solid–liquid extraction by C_{18} cartridges. Ravitch et al., [19] measured abacavir and its two major metabolites, in human urine and cerebrospinal fluids by reversed-phase HPLC. D'Avolio et al., [20] have quantified 12 antiretroviral agents along with IDV and NVP. Choi et al., [21] have reported simultaneous determination of IDV with nine other antiretroviral drugs in plasma. However none of the reports deal with the use of RAM columns for analysis of antiretroviral drugs in biological fluids. Recently two-dimensional liquid chromatography (2D-LC) coupled with mass spectrometry (2D-LC/MS, 2D-LC/MS/MS) has become popular as it generates excellent resolution, enabling on-line sample clean-up and easy recovery of analytes from biological matrices. These approaches minimize the sample losses and enhance the sensitivity of detection. The present manuscript describes the development of a validated 2D-LC–ESI-MS/MS method for determination of ABC, NVP and IDV in rat serum and urine treated on-line with a RAM column in first dimension and back-flushed to a Inertsil C_{18} column in second dimension. The method was applied to study the pharmacokinetics of ABC, NVP and IDV in rats.

2. Experimental

2.1. Chemicals

All the reagents were of analytical-grade unless stated otherwise. Glass-distilled and de-ionized water (Nanopure, Barnsted, USA), HPLC-grade acetonitrile (Qualigens Fine-chem. Mumbai, India) and ammonium acetate (S.D.Fine-chem. Ltd., Mumbai, India) were used. Abacavir (ABC), nevirapine (NVP), and Indinavir (IDV) (Fig. 1) were gifted by local manufacturers in Hyderabad, India.

2.2. Liquid chromatography–ESI-tandem spectrometry (LC–ESI-MS/MS)

The on-line 2D-LC system consisted of a Finnigan Surveyor LC Pump Plus, Autosampler, and LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA), equipped with an ESI source, isocratic LC-20AT pump (Shimadzu, Kyoto, Japan) and column-switching valve (Shimadzu, Kyoto, Japan). RAM (reversed phase Hisep column, Supelco, 50 mm × 4.0 mm; particle size 5 μm) column was used in first dimension. The first dimension was used for on-line sample clean-up while second dimension for chromatographic separation of the drugs under study. It consisted of an Inertsil ODS (250 mm × 4.6 mm I.D.; particle size 5 μm) analytical column at a room temperature. The final mobile phase comprised of acetonitrile: 0.02 M aqueous ammonium acetate for both the dimensions. Isocratic elution mode was used for first dimension using acetonitrile: 0.02 M aqueous ammonium acetate (5:95, v/v) as mobile phase at a flow rate of 0.4 ml/min. For second dimension, gradient elution mode at flow rate of 1.0 ml/min was opted. The total run time was 20 min. The data acquisition was under the control of a Xcalibur software (Thermo Electron Corporation, USA). The mass spectrometer was operated in positive ion mode. Spray voltage was optimized at 5 kV, transfer capillary temperature at 300 °C, sheath gas and auxiliary gas (nitrogen) pressure at 30 and 8 arbitrary unites (set by the LCQ software, Thermo Electron Corporation), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr and collision energies used were 20–40 eV throughout the experimental work.

2.3. Animals

Ten Wistar rats (180–200 g) were housed under standard conditions and had ad libitum access to water and standard laboratory diet throughout the experiments. After a single dose by oral administration of 20 mg/kg each analyte to healthy Wistar rats ($n = 6$), blood samples (1 ml) were collected for determination of analyte concentrations. Serial blood samples were collected into the processed test tubes at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h post-dose. Specimens were thawed and allowed to reach room temperature, and the concentrations of analytes were determined from the calibration curve on the same day.

2.4. Preparation of stock solutions, calibration standards and quality controls

Two 1000 μg/ml stock solutions were prepared for each analyte, one used for calibration standards and one for the preparation of quality controls (QC). The stock solutions were prepared in water and stored in polypropylene tubes at −20 °C. Working standards were prepared by subsequent dilution of the stock solution to produce solution of 10 μg/ml concentration of all three analytes.

Calibration standards were prepared by appropriate dilutions of the intermediate solution in water followed by addition of appropriate amounts of those dilutions to blank serum. The final concentration of analytes in serum was 50, 40, 30, 20, 10, and 5.0 ng/ml. Precision and accuracy standards having the concentrations of 100 (high), 20 (medium) and 5.0 (ABC and NVP) or 6 ng/ml (low) for (IDV) were also prepared. Stock solutions were kept refrigerated when not in use at −20 °C and replaced by fresh stock solution on a bi-weekly basis. Fresh working standards were prepared on every day of analysis.

2.5. Sample analysis

Control serum used for calibration curve and validation of the assay was obtained from Wistar rats (Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad, India). The rat blood samples were collected at a regular time intervals of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 h after a single 20 mg/kg oral dose of analytes to rats. Collected samples were immediately kept at −20 °C till analysis. The blood samples were centrifuged for 5 min (2500 × *g*) and serum was directly injected into HPLC system.

2.6. Calibration curves

Blank serum sample was collected from untreated animals. Calibration points were constructed by spiking 200 μl of the biological matrices with known concentrations of ABC, NVP and IDV to obtain a series of concentrations. The calibration curves of all three analytes were in the range of 4–50 ng/ml.

2.7. Precision and accuracy

The method was validated using five QC points for each calibration curve. Five replicates of each QC point were analyzed every day to determine the intra-day accuracy and precision. The process was repeated three times over 3 days in order to determine the inter-day accuracy and precision.

2.8. Stability

The stability of ABC, NVP and IDV in serum was evaluated by comparing the assays in fortified samples at three different concentrations and by analyzing aliquots of the same samples after storage at −20 °C. The samples were stable at least 3 months. The quality control samples had been frozen and thawed three times were compared with freshly prepared quality control samples and found stable. The reinjection reproducibility after storage of the samples in glass vials in the auto sampler for 24 h was also determined. Stability experiments were performed on at least two concentrations (10 and 50 ng/ml) in triplicate. ABC, NVP and IDV were considered to be stable in stock and working solutions having the mean recoveries 93.2–103.2% of the original concentration.

3. Results and discussion

3.1. Optimization of chromatographic conditions

In a preliminary study, LC–MS/MS system was evaluated using an Inertsil ODS column. In off-line sample clean-up, liquid–liquid and solid-phase extractions were tried. Solid-phase extraction was carried out by C_{18}, Oasis HLB (Waters, USA) and DSC (Supelco, USA) cartridges. But none of them offered acceptable recoveries at low concentration of the drugs. Further, the procedures were time consuming and tedious. Thus 2D-LC–MS/MS was tried. In first

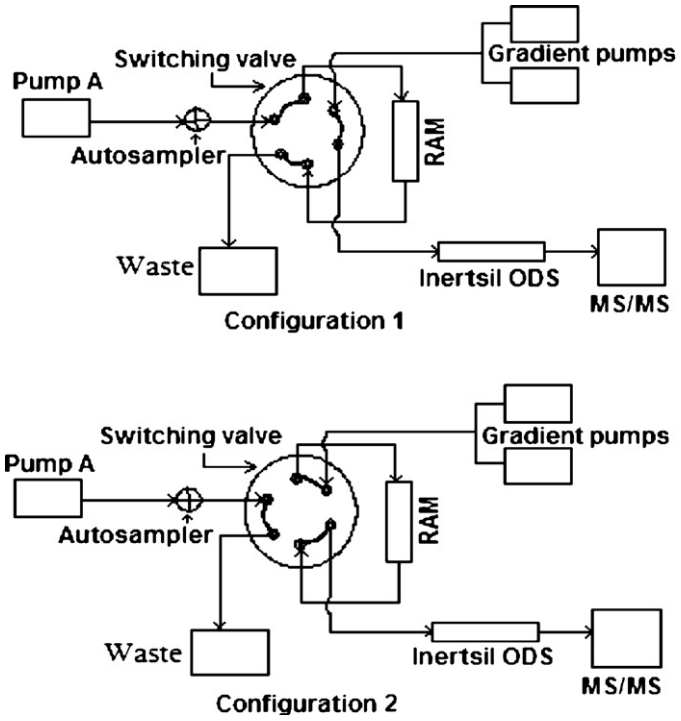

Fig. 2. A schematic representation of the 2D-LC/MS/MS system.

dimension, a RAM column was used for the removal of proteineous part of biological matrix and trapping of drug analytes. The high pressure pump in 1D was used to deliver the mobile phase through the autosampler and the RAM column. The two different configurations in 2D-LC–MS/MS were assembled by means of a six port switching valve. The schematic diagram of the on-line 2D-LC–MS/MS system used in the study is shown in Fig. 2. In configuration 1, the RAM and Inertsil ODS columns were acted in two different chromatographic runs, where as in configuration 2, both the columns were connected in series. The sample was injected into the RAM column, which was conditioned by mobile phase 0.02 M ammonium acetate: ACN (95:5, v/v; flow rate 0.4 ml/min) in isocratic elution mode with the switching valve in position 1 (configuration 1). Simultaneously the Inertsil ODS column was conditioned with the same mobile phase (flow rate 1.0 ml/min) used for configuration 1. After 3 min of injection of the sample in RAM column, the valve was switched to position 2 thus the RAM column got assembled with Inertsil ODS column in configuration 2. Thus two columns were connected together and the analytes were transferred from RAM column to Inertsil ODS column within 5 min. At 5th min the valve was again switched to position 1. The time program of the system and gradient program for 2D is shown in Table 1. The analytes were separated further on 2nd column prior to MS/MS.

Table 1
Gradient program.

Time (min)	%A	%B
0.01	95	5
7	95	5
10	30	70
15	15	85
16	95	5
25	95	5

A = 0.02 M ammonium acetate; B = acetonitrile.

Table 2
Linearity, LOD and LOQ for abacavir (ABC), indinavir (IDV) and nevirapine (NVP) in rat serum and urine.

Sample	Compound	Regression equation	r^2	LOD (ng/ml)	LOQ (ng/ml)
Serum	ABC	$y = 38{,}228x - 11{,}013$	0.9995	1.4	4.3
	IDV	$y = 15{,}427x - 1840.5$	0.9993	1.7	5.1
	NVP	$y = 39{,}941x - 13{,}169$	0.9996	1.4	4.3
Urine	ABC	$y = 25{,}243x - 9{,}965$	0.9997	1.2	3.7
	IDV	$y = 13{,}562x - 1{,}558$	0.9995	1.5	4.7
	NVP	$y = 41{,}254x - 10{,}981$	0.9998	1.3	4.0

$n = 5$.

3.2. Validation

3.2.1. Linearity

The linearity of peak area versus concentration was studied using solutions of six different concentrations ranging from 4 to 50 ng/ml for ABC, NVP and IDV. The calibration curves were linear for all the analytes. The regression equations and correlation coefficients obtained using statistical data analysis are listed in Table 2.

3.2.2. Accuracy and precision

The intra- and inter-day precision and accuracy of the method were determined by analyzing six replicates of three QC samples of each analyte. This analysis represents the entire range of the calibration curves viz., low, medium and high concentrations on 3 consecutive days. Precision was expressed as the relative standard deviation (RSD) at each concentration level for six repeated measurements evaluated. Accuracy was expressed as percent (%) error. The results are given in Table 3. The overall mean precision (RSD) ranged from 0.93 to 11.51% while the accuracy (% error) ranged from 1.3 to 14.0%.

3.2.3. Recovery

The extraction recovery for all the analytes in serum was determined by comparing the peak areas of the ABC, NVP and IDV in standard solutions at three different levels with those spiked serum samples at corresponding concentrations. The extraction recoveries for each concentration level are given in Table 4. The recoveries were ranged from 93.2 ± 1.2 to 103.2 ± 1.8% for the LOQs and from 93.2 ± 1.9 to 102.1 ± 2.3% for the other concentrations.

3.2.4. Selectivity

A drug free rat serum sample was analyzed in optimized conditions and no interfering peaks were observed at the retention times of ABC, NVP and IDV (Fig. 3). The selectivity was also assessed using a blank serum sample collected prior to administration of the drugs.

Table 3
Intra-day ($n = 5$) and inter-day ($n = 15$) precision (% R.S.D.) and accuracy (% error) measured for quality control samples from rat serum.

T.C.	Day 1			Day 2			Day 3			Inter-day		
	E.C.	R.S.D.	Error	E.C.	R.S.D.	Error	E.C.	R.S.D.	Error	E.C.	R.S.D.	Error
Serum, ABC												
50	49.35	0.93	1.3	48.8	1.74	2.4	48.89	1.60	2.2	48.37	2.38	3.2
20	20.25	3.37	5.0	18.67	4.99	6.6	19.12	1.66	2.4	18.75	3.72	5.1
5	4.6	2.95	4.0	4.8	6.15	8.0	4.5	6.15	8.0	4.5	3.37	5.0
IND												
50	47.3	4.11	5.5	46.9	4.69	6.2	46.5	5.24	6.9	48.7	1.90	2.6
20	18.5	7.68	9.8	19.5	7.34	9.4	18.8	3.10	4.2	18.71	4.35	5.8
6	5.8	7.64	8.5	6.3	9.51	11.0	5.2	8.74	10.4	5.1	5.32	7.0
NVP												
50	48.9	1.47	2.0	48.7	1.84	2.5	49.2	1.13	1.6	48.4	5.44	3.2
20	18.21	2.85	4.2	19.86	2.03	2.8	18.71	4.74	6.1	18.11	3.21	3.4
5	4.5	6.06	4.5	4.7	4.18	6.4	5.2	4.42	7.4	5.1	5.01	6.5
Urine, ABC												
50	48.0	3.32	2.3	48.5	3.14	2.9	47.92	2.12	1.9	48.6	2.38	3.2
20	18.86	2.98	4.5	18.67	4.99	6.6	19.12	1.66	2.4	18.36	3.72	5.1
5	5.2	2.02	3.5	5.1	4.21	8.0	5.4	6.15	8.0	4.5	3.37	5.0
IND												
50	47.2	4.11	5.5	46.89	4.69	6.2	46.55	5.24	6.9	48.69	1.90	2.6
20	18.74	5.25	4.7	18.87	5.14	7.8	17.59	6.80	5.7	18.84	4.01	2.5
6	6.2	6.75	9.7	5.2	11.01	11.1	5.7	7.89	9.54	5.5	7.31	6.4
NVP												
50	48.7	4.74	4.5	49.1	3.25	3.2	48.4	5.56	3.2	47.97	3.97	3.1
20	19.34	4.13	5.3	20.53	3.24	4.2	18.52	4.35	4.7	19.26	3.21	4.1
5	6.1	7.35	4.5	4.6	3.45	4.3	4.7	8.10	8.5	4.7	7.72	7.7

T.C. denotes theoretical concentration; E.C. denotes experimental concentration.

Table 4
Absolute recoveries (±S.D.) of ABC, IDV and NVP from serum.

Concentration (ng/ml)	Recovery (%)					
	ABC		IDV		NVP	
	Serum	Urine	Serum	Urine	Serum	Urine
LOQ	93.2 ± 1.2	94.2 ± 2.7	92.6 ± 2.2	94.5 ± 2.1	103.2 ± 1.8	94.0 ± 3.0
10	94.7 ± 2.3	93.2 ± 1.9	95.3 ± 2.5	102.1 ± 2.3	93.2 ± 1.9	95.3 ± 2.5
20	95.1 ± 2.5	94.5 ± 2.5	96.7 ± 2.1	93.7 ± 2.0	93.1 ± 2.2	95.1 ± 3.1
50	100.7 ± 2.7	94.7 ± 3.1	97.1 ± 2.3	95.1 ± 2.1	94.5 ± 3.5	98.4 ± 2.2

($n = 10$).

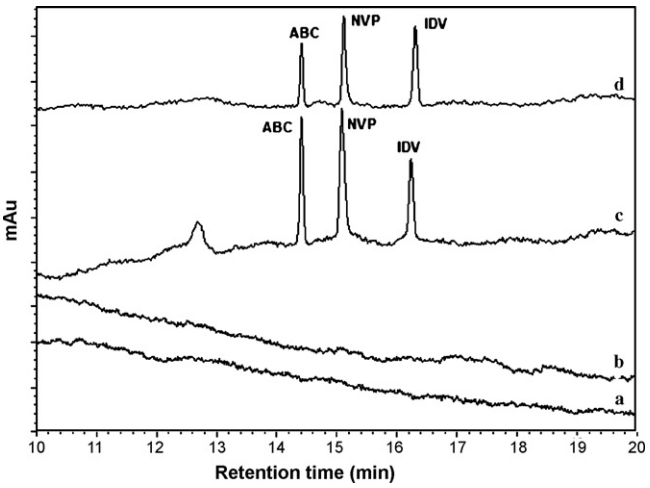

Fig. 3. Chromatograms of (a) blank serum, (b) blank urine, (c) rat serum and (d) rat urine drawn at 5th hour.

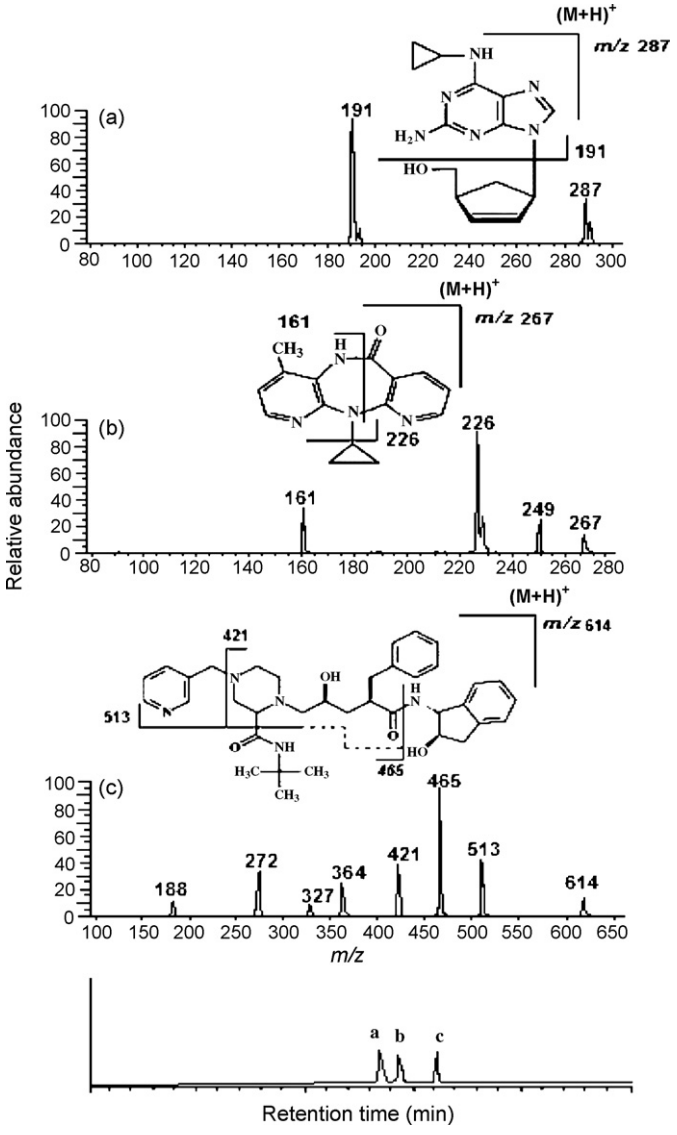

Fig. 4. LC/MS chromatograms of (a) ABC, (b) NVP and (c) IDV.

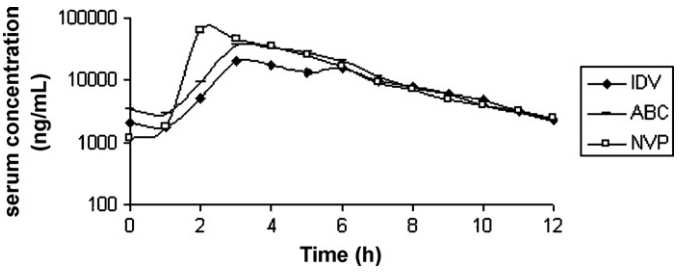

Fig. 5. Concentration verses time profiles of ABC, NVP and IDV in rat serum after single oral dose of 20 mg/kg.

3.2.5. Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection (LOD) and quantitation (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ were determined by measuring the magnitude of analytical background by injecting blank samples and calculating the signal-to-noise ratio for each compound by injecting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ. The LOD, were 1.2 (ABC), 1.3 (NVP) and 1.5 ng/ml for IDV. The LOQ was 3.7 (ABC), 4.0 ng/ml (NVP), whereas for IDV it was 4.7 ng/ml.

3.3. Electrospray ionization ion-trap tandem mass spectrometry

By operating ESI in a positive ionization mode, IDV yielded a protonated molecule at m/z 614. The precursor ions were subjected to collision-induced dissociation (CID) to determine the resulting fragment ions. The product ion spectrum for IDV is shown in Fig. 4. Major fragments for IDV occurred at m/z ratios of 513, 465 and 421, which attributed to the loss of *t*-butylaminocarbonyl, amino-2-hydroxyindanyl and methyl pyridine, respectively. The product ion spectrum of ABC shows a prominent ion at m/z ratio 191 after loss of cyclopent-2-enyl-methanol while NVP (Fig. 4) shows daughter ions at m/z ratios 249 and 226 corresponding to the loss of H_2O and cyclopropane. The daughter ion m/z 226 produced a fragment at m/z at 161 due to loss of (–N–CH=CH=CCH$_3$).

3.4. Pharmacokinetics of ABC, IDV and NVP in rats

The optimized HPLC method was applied to monitor the concentrations of ABC, NVP and IDV in serum and urine of rats. After a single dose by oral administration of 20 mg/kg of each drug to healthy Wistar rats, blood and urine samples were collected at regular time intervals. Serum was separated by centrifugation at 4500 rpm for 5 min and stored at −20 °C until analysis. Serum and urine samples were filtered before direct injection. Fig. 5 shows concentration versus time profiles over 12 h of ABC, NVP and IDV in serum. Cumulative excretion profile of the drugs in urine of rats receiving a single 20 mg/kg dose of each drug is shown in Fig. 6. The pharmacokinetic parameters were calculated and recorded in Table 5.

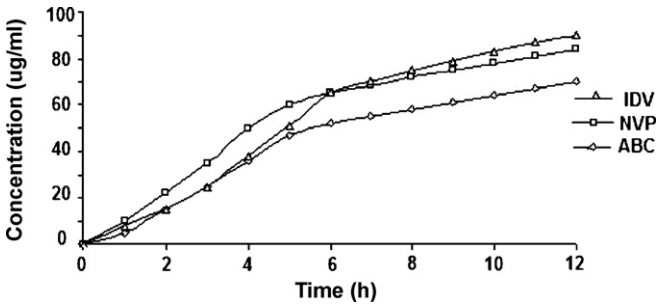

Fig. 6. Cumulative excretion of ABC, NVP and IDV in rat urine.

Table 5
Pharmacokinetic parameters of ABC, NVP and IDV in rat serum ($n = 5$) after oral administration.

Parameter	ABC	IDV	NVP
t_{max} (h)	4	4	3
C_{max} (μg/ml)	37.08	20.51	62.77
AUC (μg/ml/h)	168.11	120.10	225.21
$t_{1/2}$ (h)	2.07	3.11	2.05

C_{max} (μg/ml): maximum serum concentration; t_{max}: time to C_{max}; *AUC*: area under serum concentration–time curve; $t_{1/2}$: half-life.

4. Conclusion

The development and validation of on-line two dimensional-liquid chromatography–tandem mass spectrometric (2D-LC–MS/MS) method was described. The method was sensitive enough to determine the low concentrations of antiretrovirals ranging from 3.7 to 4.7 ng/ml. The developed method was also applied to study pharmacokinetics of antiretrovirals in rats.

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