Analysis of Nevirapine and Lamivudine in human plasma of HIV infected patients by high-performance liquid chromatographic-mass spectrometric (LC-MS-MS)

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Abstract: High pressure liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method was developed and validated for the determination of lamivudine and nevirapine in human plasma. The method was validated over the linearity range of 10–500 ng/ml ($R^2 > 0.99$) using zalcitabine as an internal standard. Blood samples were obtained from HIV patients at two different collecting times which were first blood samples collected when the questionnaire was administered and second blood sample after one month from the collection of the first blood sample. The plasma extraction was performed using protein precipitation of plasma, followed by centrifugation. The lower limit of quantification (LOQ) was 10 ng/ml. Chromatographic separation was achieved on a Zorbax-C18 and the flow rate was kept constant at 0.8ml/min. Mobile phase A was formic acid in water and mobile phase B was 10mM ammonium formate in acetonitrile with pH 5.8. LC-MS-MS in positive mode used pairs of ions of 230.20/111.90, 267.08/226.10, 212.08/112.00 for lamivudine, nevirapine and zalcitabine, respectively. Excellent precision and accuracy were observed. The recovery achieved was 85%. A second sample was collected from the same patients after a period of one month to validate the method. Higher sensitivity and accuracy achieved for this method make it suitable to measure low concentration of lamivudine and nevirapine in plasma of healthy and diseased subjects.

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Keywords: Nevirapine; Lamivudine; LC-MS-MS; Validation

1. Introduction

The AIDS epidemic is one of the most destructive epidemics in the history of mankind. In Malaysia, it is estimated 105000 people living with HIV/AIDS and over 14000 AIDS related death has been reported as of December 2009 (Kamarulzaman, 2009; Muchini, 2011). The introduction of highly active antiretroviral treatment and the increase in its availability has improved survival rate and decrease mortality and morbidity among HIV positive patients who adhere to their treatment (Fabbiani, 2009). World Health Organization (WHO) recommends first-line ART to include a combination of nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI) and require 95% adherence level to prevent developing drugs resistance, treatment failure and keeps the patients on first line treatment which is cheap compared to the second line of treatment (Becquet, 2009). There are many ways for measuring adherence level, but Therapeutic Drug Monitoring (TDM) is the best objective method for measuring adherence by indicating the concentration of drugs in the serum (Ribera, 2005; Aarnoutse, 2003). There are no published studies on measuring adherence to ART by therapeutic drug monitoring in Malaysia and whether it is at the required 95% level or not, however an analytical methods for HAART determination in blood may represent a useful clinical tool indicating the concentration of drug level (Vervoort, 2009; Ensoli, 2010). In this study we have developed and validated a simple, fast and sensitive LC-MS/MS method to measure the levels of first generation NNRTI (nevirapine) and an NRTI (lamivudine) in human plasma. The simplicity in the extraction method and rapid running time by using short LC column gave an advantage that this method was used to analyse large number of samples in this study.

In the past, there were many published studies that described antiretroviral drugs assay methods in human plasma mostly using HPLC with UV detection (Nageswara Rao, 2009; Sarkar, 2006) and have been

reported to quantify HIV drugs level in human biological fluids (Yuen, 2004; Sudha, 2010). These methods had either long running time or lower sensitivity in addition to labour- intensive and time consuming sample preparation either by liquid-liquid extraction or solid phase extraction. Recently a more sensitive, rapid and specific high performance liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method has been used to determine the HAART drug concentration in the plasma by plasma protein precipitation (Mistri, 2007; Elens, 2009; Droste, 2003; Chi, 2003). The advantage of this method was in the running time which was more rapid and also faster and cheaper in sample preparation technique as compared to the previous HPLC with UV detection method (Mistri, 2007; Poirier, 2002; Le Saux, 2008).

In this study we have developed and validated a simple, fast and sensitive LC-MS/MS method to measure the levels of first generation NNRTI (nevirapine) and an NRTI (lamivudine) in human plasma. The simplicity in the extraction method and rapid running time by using short LC column gave an advantage that this method was used to analyse large number of samples in this study.

2. Material and Methods

2.1. Experimental

a) Chemicals

All chemicals were HPLC grade or reagent grade. Nevirapine, lamivudine and zalcitabine (USP-grade) were obtained from LABCHEM SDN BHD, Kuala Lumpur, Malaysia. Acetonitrile, ammonium formate and formic acid were purchased from Fisher Scientific UK, Loughborough and Leicestershire, UK. Drug free human plasma was obtained from the University of Malaya Medical Centre blood bank.

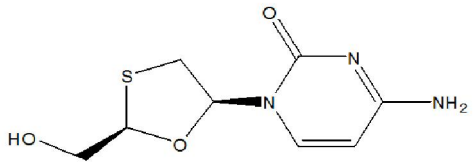
b) Apparatus

The LC-MS/MS system consists of an LC-10A UFLC system with a SIL-HT automatic sample injector (Shimadzu, Kyoto Japan) and an API 3200 Q-Trap LC-MS/MS system (Applied Bio systems, Lincoln Centre Drive, Foster City, CA, USA). The LC-MS/MS system was controlled by analyst 1.42 software (Applied Bio systems).

2.2. Chromatographic System

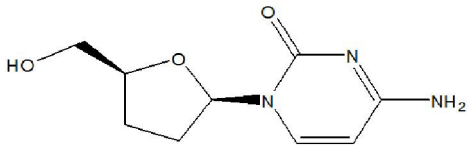
The column used was a Zorbax C18 reversed phase column, 4.6 mm ID 100 mm length with 3.5 µm particle sizes packing and Gemini-NX C18 4mm ID x 2.0mm length guard column. The flow rate was kept constant at 0.8mL/min. Mobile phase A was 0.05% formic acid in water and mobile phase B was 10mM Ammonium formate in acetonitrile with pH 5.8. The gradient flow was initially 80:20 v/v of A: B for 0.10 minute and ramped to 35 % B over 0.50minute then held at this level for 0.80min. The percentage of was ramped to 95% until 2.00min and held for 0.50min. The gradient then return to 20% B at 2.51minutes and this condition was held for further 3.50 minutes.

Scheme 1: Structures of the drugs



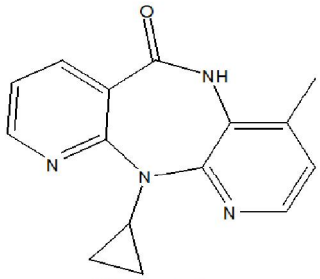
(Lamivudine)

4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl] -1,2-dihydropyrimidin-2-one



(Zalcitabine)

4-amino-1-((2R,5S)-5-(hydroxymethyl)tetrahydrofuran-2-yl) pyrimidin-2(1H)-one



(Nevirapine)

11-cyclopropyl-4-methyl-5,11-dihydro-6H- dipyrido [3,2-b:2',3'-e][1,4]diazepin-6-one

2.3. Preparation of Mobile Phase

Mobile phase A was 0.05% v/v solution of formic acid in deionised water. Mobile phase B was prepared by dissolving 770mg ammonium formate in acetonitrile and adjusted the pH to 5.8 with formic acid prior to filtration through a 0.45µm membrane.

2.3. Assay Procedures

a) Preparation of stock solution and control

200 µg/mL of lamivudine, nevirapine and zalcitabine (internal standard) stock solution were prepared in methanol. All the calibrators and quality controls (QC) samples were prepared by appropriate dilution of stock solution with analyte free plasma. All the stock solutions, calibrators and QC samples were stored at approximately -20^{0}C.

b) Preparation of calibration standard

The calibration curve covered the range from 10 to 500ng/mL with seven calibrators. Five batches of calibration curves were prepared for validation purposes. The calibration curve was plotted using the area ratio of nevirapine and lamivudine to zalcitabine

versus known concentrations of nevirapine and lamivudine. All the results were calculated using y=Ax+B linear regression. The regression coefficient for all the calibration curves were greater than 0.99.

a) Extraction procedures

Frozen plasma samples from study patients, QC samples and calibration standards were thawed as needed. The same procedure was followed for all samples. Five hundred microliters of IS in Acetonitrile solution was added to aliquots of 100 uL of plasma from calibrators and QC in 1.5 mL micro centrifuge tubes and vortexed for 20s at high speed. The tube was centrifuged at 14800 rpm for 10 min. to pellet the precipitated proteins and give a clear supernatant. Five hundred microliters supernatants was filtered using PHENEX RC 0.25µm syringe filter and transferred to vial inserts and placed in the auto sampler tray for injection onto the LC column.

2.4. Method Validation

All the validation procedures and the acceptance criteria used in this study were adapted from the European Agency for the Evaluation of Medicinal Products (EMEA) guideline for method validation (Milton) and USFDA guideline (Mistri, Jangid et al. 2007).

a) Specificity

Assay specificity was determined by analyzing double blank (plasma sample without analyte and internal standard), blank (plasma sample spike with internal standard only), LLOQ (Lower Limit of Quantification) and ULOQ (Upper Limit of Quantification) sample.

Table 1. Within assay imprecision and inaccuracy of lamivudine and nevirapine in plasma

Drug name	Batch	Nominal (ng/mL)	Mean (ng/mL)	SD	CV (%)	Mean accuracy
Nevirapine	1	10	10.62	0.342053	4.617497	100.74
		30	28.62	2.148721	5.014131	101.8667
		240	222	15.01666	3.216278	98.91667
		400	385.8	15.73849	4.0594	106.9
		500	516.6	47.65816	3.92217	107.12
Lamivudine	1	10	10.074	0.465167	4.617497	100.74
		30	30.56	1.532319	5.014131	101.8667
		240	237.4	7.635444	3.216278	98.91667
		400	427.6	17.358	4.0594	106.9
		500	535.6	21.00714	3.92217	107.12
Nevirapine	2	10	12.42	0.725948	5.844989	124.2
		30	29.62	1.30269	4.398007	98.73333
		240	199.4	8.619745	4.322841	83.08333
		400	332.6	19.33391	5.812961	83.15
		500	493.2	50.30606	10.19993	98.64
Lamivudine	2	10	10.77	1.174521	10.90549	107.7
		30	29.96	2.12791	7.102503	99.86667
		240	233	7.905694	3.393002	97.08333
		400	377.6	15.1096	4.001483	94.4
		500	499	30.11644	6.035359	99.8
Nevirapine	3	10	10.64	0.750333	7.052004	106.4
		30	29.84	0.835464	2.799812	99.46667
		240	246.8	4.438468	1.798407	102.8333
		400	407.6	4.97996	1.221776	101.9
		500	541.8	16.6343	3.070192	108.36
Lamivudine	3	10	11.008	0.786333	7.143289	110.08
		30	34.58	0.460435	1.331505	115.2667
		240	264.6	11.58879	4.379738	110.25
		400	452	6.442049	1.425232	113
		500	540.4	33.50821	6.200631	108.08

b) Linearity

The calibration consists of seven non zero, calibrators assayed in duplicate (nominal values 10, 25, 50, 100,250, 350 and 500ng/mL. Two analyte free samples were analysed, one with the internal standard and one without the internal standard; neither being included when fitting the calibration line. The correlation coefficient (r) between concentration and

peak area ratio should be equivalent to, or better than, 0.98. The simplest mathematical model that adequately describes the concentration-response relationship was used.

2.5. Imprecision and inaccuracy

Imprecision and inaccuracy were assessed using three quality control samples with nominal lamivudine and nevirapine concentrations at of 200ug/mL, LLOQ (lower limit of quantification) of 10 ng/mL and ULOQ (upper limit of quantification) of 500 ng/mL.

a) Within-assay reproducibility

The three quality control samples, LLOQ and ULOQ were initially extracted six times in one batch each. Subsequently, they extracted six times in two additional batches. On each occasion a separate calibration curve was extracted. The calibration curve and the percentage for imprecision and inaccuracy including LOQ and ULOQ were all within the accepted range (Table 1).

Table 2. Between assay imprecision and inaccuracy of lamivudine and nevirapine in plasma

Drug name	Nconc (ng/mL)	Mean (n=15) (ng/mL)	SD	CV (%)	Mean inaccuracy (%)
Lamivudine	10	10.61733	0.894926	8.428914	106.1733
	30	31.7	2.555945	8.062919	105.6667
	240	245	16.79711	6.855963	102.0833
	400	419.0667	34.50562	8.233922	104.7667
	500	525	32.74577	6.23729	105
Nevirapine	10	10.64	0.750333	7.052004	106.4
	30	29.84	0.835464	2.799812	99.46667
	240	246.8	4.438468	1.798407	102.8333
	400	407.6	4.97996	1.221776	101.9
	500	541.8	16.6343	3.070192	108.36

b) Between-assay repeatability

The mean concentration from each assay was used to calculate the between assay reproducibility. The CV and the percentage for between assay precision and accuracy including LLOQ and ULOQ were within the accepted range. The within and between-batch imprecision and the percentage inaccuracy were obtained during validation assay. All the results were contained within the accepted range as outlined in the Bioanalytical Method Validation, US Food and Drug Administration (2001) (Shah, 2000) (Table 2).

2.6. Recovery

Recovery of both lamivudine and nevirapine from plasma following sample preparation was assessed by comparing the concentration of the analyte from plasma sample to the concentration from drugs spiked into mobile phase at the same concentration as in the plasma samples. Peak area measurements obtained from extracted samples were compared to the peak area measurements obtained from direct solvent injection of the test compounds. Mean and standard deviations were calculated from at least three measurements at each level.

2.7. Stability

Stability of lamivudine and nevirapine in plasma were assessed for freeze and thaw (three cycles), after 48 hours at room temperature, after 48 hours at 4ºC, and the stability of the sample extract in autosampler for the period of 24 hours.

3. Results

A high-performance liquid chromatographic mass spectrometric method for the estimation of nevirapine and lamivudine in human plasma has been developed and validated according to the principles of EMEA, Milton and USFDA guideline. The pairs of ions 230.20/111.90, 267. 08/226.10 and 212.08/112.00 were monitored for lamivudine, nevirapine and zalcitabine, respectively. The mass spectrum scans for the above two analytes are shown in Fig (1) and Fig (2). Fig (3) shows the chromatogram of zalcitabine, lamivudine and nevirapine with the retention of 1.0, 1.03 and 2.7 min, respectively with a total run time of 3.4 min.

3.1. Validation parameters

No significat interfering peaks were found at the retension time of lamivudene, nevirapine and zalcitabine. The signal to noise ratio for the drugs at the lower limit of quantitation was greater than 10. Chromatograms obtained from blank plasma spiked with 10 ng/mL (lamivudine and nevirapine) and 1.0 ng/mL zalcitabine are shown in Figure (4), Fig 5 and Fig 6 below. The linearity of the method developed in this study was determined by a none-weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 0.5 to 48.5 ng/ml. Best-fit calibration lines of the ratio of for the drugs examined in this study to IS peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$.

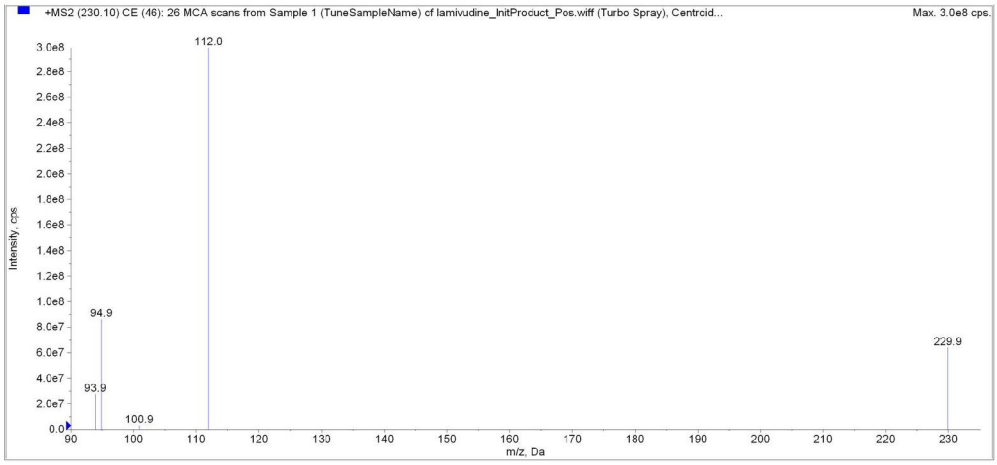


Fig 1 Spectra for lamivudine

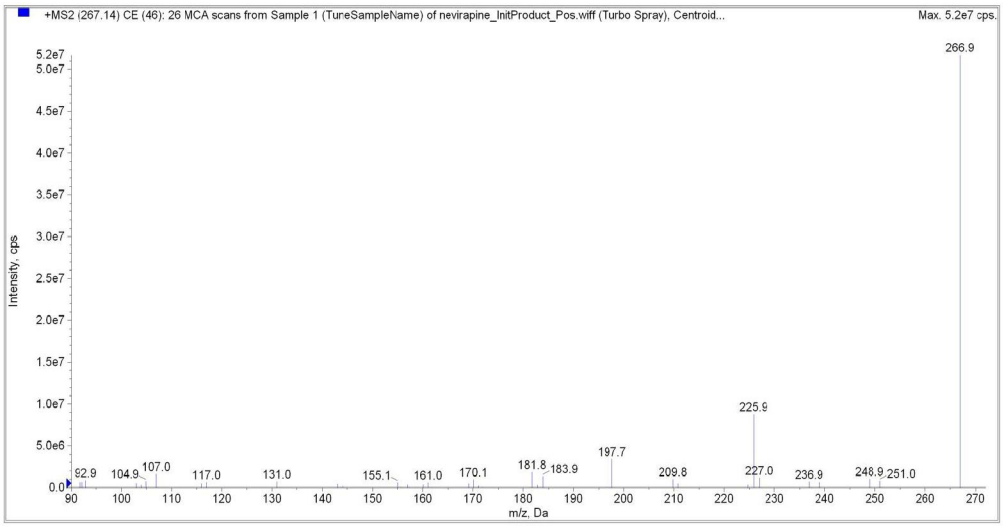


Fig 2 Spectra for nevirapine

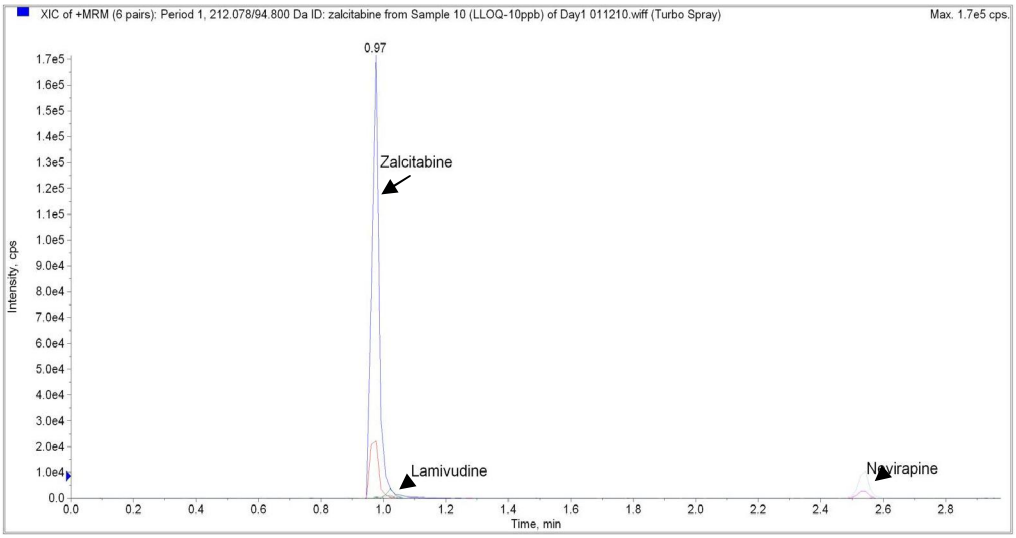


Fig 3 Chromatogram of zalcitabine, lamivudine and nevirapine

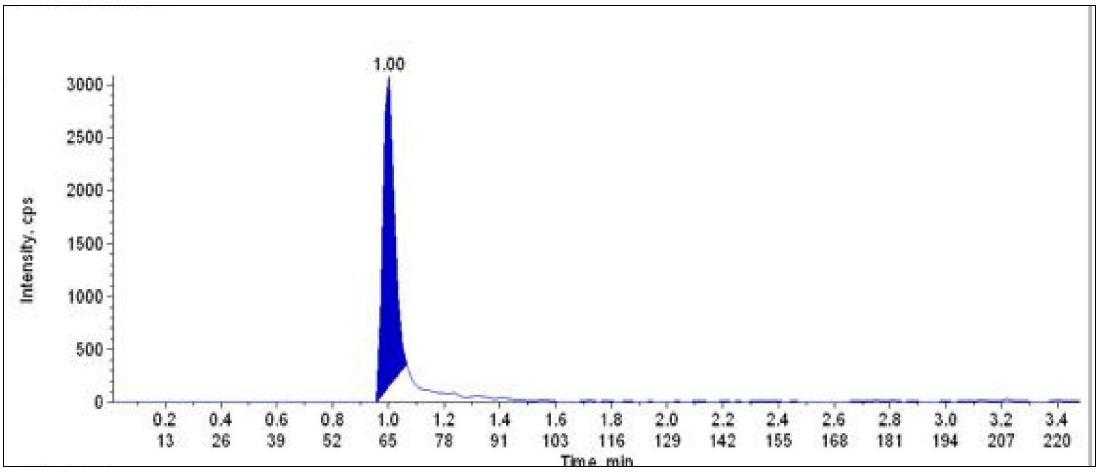


Figure 4. Representative chromatograms of zalcitabine

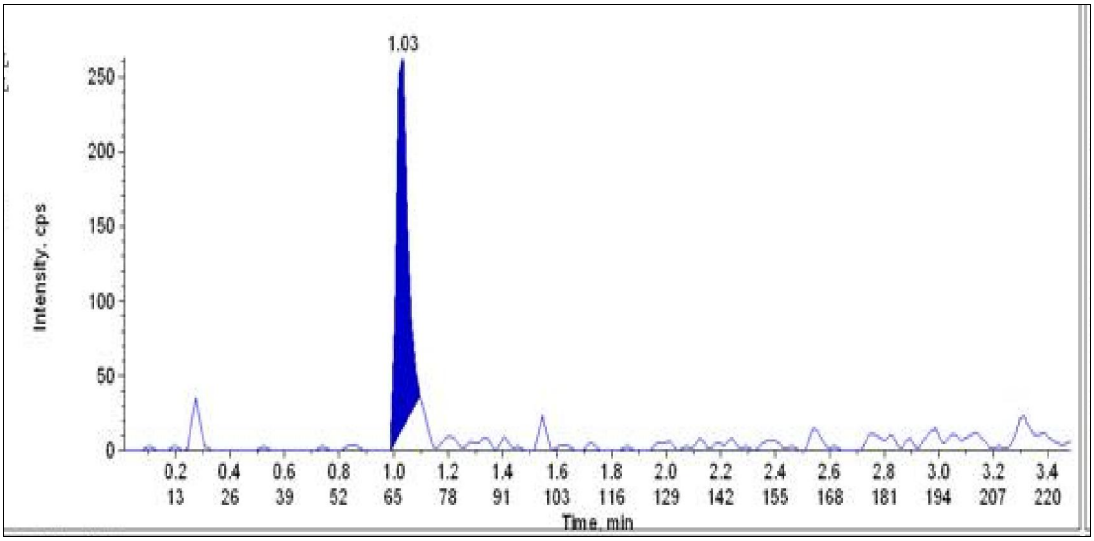


Figure 5 Representative chromatogram of lamivudine

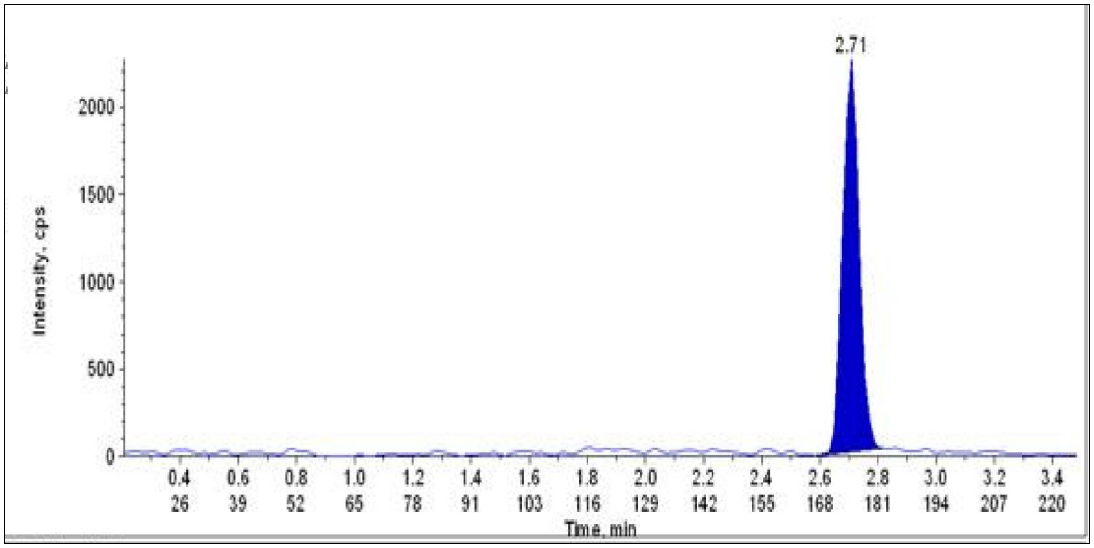


Figure 6. Representative chromatograms of nevirapine

The R-squared values were consistently 0.99 during the course of validation. The imprecision and inaccuracy for intra-assay and inter-assay for both nevirapine and lamivudine were all less than 15%. The absolute recovery of lamivudine, nevirapine and zalcitabine was above 80%. Nevirapine and lamivudine also found to be stable in plasma after three freeze and thaw cycles, after 48 hours at room temperature, after 48 hours at 4ºC, and within 24 hours of the sample extract when put in autosampler.

Table 3 below shows the results of nevirapine and lamivudine as analysed in the first and second plasma samples obtained from HIV positive patients. Out of 925 samples analysed 269 first blood samples had nevirapine detected while the remaining 656 samples were negative. The results of lamivudine analysis in the first and second blood samples obtained from HIV positive patients are shown in Table 3. Whereby, out of 925 samples analysed 243 first blood samples had lamivudine detected while the remaining 682 samples were lamivudine free. Fifty two second blood samples were analyzed, the remaining 873 participants did not provide second blood samples for analysis.

Table 3. Analysis of lamivudine and nevirapine in HIV positive plasma samples using (LC-MS/MS)

Drugs	Plasma concentration (ng/ml)			Detection of the drugs in the plasma samples	
	Minimum	Maximum	Mean	Positive	Negative
Nevirapine					
1st sample	11±1.2	11650±12	2734.17±121	269	656
2nd sample	44±2.3	12566±40	3853.3±29	82	13
Lamivudine					
1st sample	12±1.1	43029±308	1534.3±31	243	682
2nd sample	38±1.7	21057±423	4917.1±27	38	14

A second samples were collected from the same patients after a period of one month to be compared with results of the first blood samples. Table 4 below presents the different concentrations of 269 first blood samples and 82 second blood samples containing nevirapine as detected by LC-MS/MS method. Thirty six participants' first blood samples had nevirapine concentrations greater than 6001 ng/ml while 15 participants' second blood samples had concentrations greater than 6001. Ninety five or 35.3% of the participant's first blood sample had concentrations ranged from 1 -1000 ng/mL, while only 12 or 14.6 % of the second blood samples contains nevirapine concentrations ranged between 1 ng/mL to 1000 ng/mL.

Table 4 also shows the different concentrations of 243 first blood samples and 38 second blood samples containing lamivudine as detected by LC-MS/MS machine. Thirty one participants' first blood samples had lamivudine concentrations greater than 6001 ng/mL while 12 participants' second blood samples had concentrations greater than 6001. Ninety two or 37.9% of the participant's first blood sample had concentrations ranged from 1 -1000 ng/mL.

Table 4 Detected lamivudine and nevirapine concentrations in human plasma using LC-MS/MS

Concentration (ng/ml)	Nevirapine				Lamivudine			
	First sample		Second sample		First sample		Second sample	
	N	(%)	N	(%)	N	(%)	N	(%)
1--1000	95	35.3	12	14.6	92	37.9	6	15.8
1001-- 2000	34	12.6	7	8.5	35	14.4	4	10.5
2001--3000	41	15.2	13	15.9	33	13.6	6	15.8
3001--4000	24	08.9	15	18.3	19	7.80	3	7.9
4001--5000	22	08.2	13	15.9	14	5.80	3	7.9
5001--6000	17	06.3	7	8.5	19	7.80	4	10.5
> 6001	36	13.4	15	18.3	31	12.8	12	31.6
Total	269	100	82	100	243	100	38	100

N = Number of patient. LC-MS/MS = Liquid Chromatography Mass-Spectro-photometry Conc = Concentration

4. Discussion

The development of the LC-MS-MS method for the detection of drugs from human plasma has received substantial attention in recent years because of its importance in bioavailability and bioequivalence (Solans, 1995). The sensitive and selective analytical method developed here has been used to analyze the concentration of lamivudine and nevirapine in plasma obtained from HIV/AIDS positive patients receiving their treatment and followed up at the Infectious Disease Unit, Sungai Buloh Hospital, Malaysia. In total 151 patients received the two drugs lamivudine and nevirapine for their treatment. Blood samples were collected into heparinized tubes at least 10 hours post dose. Two blood samples were obtained from each patient at one month interval. Monitoring intra-cellular drug concentrations is useful to ensure efficacious antiretroviral levels in target cells especially for HIV patients on HAART treatment. Consequently, this may help to improve the management of HIV patients.

Since nevirapine is weakly basic in nature (ionizes in acidic medium) so LC-MS-MS was thought to the best choice. C18 column was favored for the separation of the drugs because other column was known to decrease retention of drug and plasma impurities were getting coeluted with drug peak (van Heeswijk, 1998). There was no other interfering peak around the retention time of nevirapine. Blank sample did not show any significant peak at the retention time of the drug. Sufficient resolution between the drugs and internal standard peak was also obtained. The mobile phase was selected after numerous trials with other solvent combinations. Mobile phase choice was based on peak parameters (symmetry, tailing), run time, ease of preparation and cost (Fawzia, 2011; Fan, 2002; Villani, 2001).

A second samples were collected from the same patients after a period of one months to validate the method. The assay described in this article combines a very short run time of 8 minutes per sample with quick and simple sample pre-treatment procedures and is therefore suitable for Therapeutic Drug Monitoring (TDM) purposes whereby very large numbers of samples can be processed quickly and efficiently. This method is also suitable for the analysis of plasma samples for pharmacokinetic studies.

5. Conclusion

A simple proteins precipitation method for the analysis of lamivudine and nevirapine in human plasma using LC/MS/MS has been developed and validated. The method also successfully applied for the analysis of large number of HIV infected plasma samples for the therapeutic drug monitoring. Besides greater precision and sensitivity attained using this LC/MS/MS method, the specificity offered is undoubtedly another advantage compared to the other costlier methods/techniques of analysis.

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