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Development and Validation of HPLC-UV Method for Simultaneous Determination of Nevirapine, 2-OH Nevirapine and 3-OH Nevirapine in Human Plasma.

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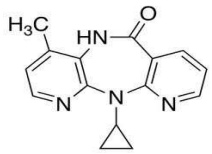
Abstract: A simple, selective, precise, and accurate HPLC method has been developed and validated for analysis of nevirapine, 2-hydroxy nevirapine and 3-hydroxy nevirapine. Reversed-phase chromatography was performed on a C_8 column with ammonium acetate–acetonitrile, 80:20 (%, v/v) as mobile phase at a gradient flow rate of 1ml/min for 17 minutes and increased to 2ml/min with analysis time of 30 min. Detection was performed at 280 nm and peaks were quantified at 5.87 min for 2-hydroxy nevirapine, 7.42 min for 3-hydroxy nevirapine, 12.88 min for nevirapine and 26 min for carbamazepine as an internal standard. Linear regression analysis data for the calibration plot showed there was a good linear relationship between response and concentration for nevirapine and the oxidative metabolites with regression coefficient value, r^2 were > 0.99. The method was validated in accordance with ICH guidelines. In this paper, we used UV detection for the simultaneous detection of nevirapine and two of its major oxidative metabolites and the results were satisfactory. UV detection is simple and available to most analytical laboratories. In addition, this method involves a simple liquid-liquid extraction with good reproducibility, which makes it suitable for pharmaco - kinetic study.
Keywords : Nevirapine, 2-hydroxy nevirapine, 3-hydroxy nevirapine.

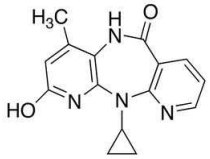
1. INTRODUCTION

Nevirapine (NVP) is the first non-nucleoside reverse transcriptase inhibitors (NNRTIs) developed for the treatment of human immunodeficiency viral infection (HIV-1). It is an active drug and mainly excreted in the urine as glucoronide conjugates of hydroxylated metabolites. Nevirapine is extensively metabolized via cytochrome P450 isoenzymes CYP2B6 and CYP3A [1]. CYP2B6 is mainly responsible for the oxidation to 3-hydroxy nevirapine (3-OH NVP) while CYP3A4 to 2-hydroxy nevirapine (2-OH NVP). A single nucleotide polymorphism (SNP) in CYP2B6 516G>T is associated with a significant reduction in enzyme catalytic activity in the liver[2] and has been reported to influence nevirapine pharmacokinetics during treatment [3,4]. Polymorphism of CYP enzymes involved would affect the steady-state metabolic elimination of nevirapine and contribute to a changed in systematic exposure as well as shift in the metabolic profile generated.

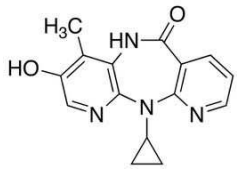
There are several methods available for determination of nevirapine in human plasma using HPLC with UV detection [5-8]. Literature survey for simultaneous determination of nevirapine and its metabolites using HPLC with UV detection shows no result. However, there was a report on HPLC with mass detection (or LCMS method) for simultaneous determination of nevirapine and its oxidative metabolites in human plasma[9]. LCMS assay was considered necessary because the nevirapine oxidative metabolites are region specific isomers having the same molecular weight [9]. HPLC with mass detection is more costly and not available in most analytical laboratories in the developing countries.



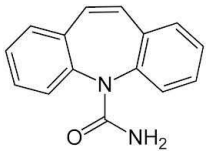
Nevirapine (MW 265)



2-hydroxy nevirapine (MW 281)



3-hydroxy nevirapine (MW 281)



Carbamazepine (MW 236.28

Fig. 1. The chemical structures of nevirapine, 2-hydroxy nevirapine, 3-hydroxy nevirapine and carbamazepine (internal standar

2. EXPERIMENTAL

2.1 Chemicals and Reagents

The working standard of nevirapine was procured from USP Rockville. Carbamazepine as internal standard was procured from Sigma Aldrich. Nevirapine oxidative metabolites; 2-hydroxy and 3-hydroxy nevirapine were purchased from Chemtron Biotechnology. HPLC grade acetonitrile, methanol (purity 99.9 %), diethyl ether were purchased from Merck (Darmstadt, Germany), hexane and acetic acid were procured from Fisher Scientific. The de-ionized water was obtained from water deionizer Pure Lab Ultra ELGA ANMK 2. Blank, drug-free plasma was obtained from Blood Bank, Hospital University Science of Malaysia.

2.2 Equipment

The Liquid chromatographic system is consisted of these following components: Gilson HPLC model containing Gilson 305 model pump, Gilson 152 Variable UV Detector and Gilson – 234 autoinjector with 20 µl fixed loop. Chromatographic analysis was performed using Gilson UniPoint System software on a Zorbax SB - C-8 column having 150 × 4.6 mm ID and 5 µm particle size.

2.3 Preparation of Standard Solution

A stock solution of nevirapine (1 mg/ ml) was prepared in methanol. Standard solution was prepared by dilution of the stock solution with methanol to give solutions in of 100 µg /ml. Similarly, dilutions were made for 2-hydroxy nevirapine and 3-hydroxy nevirapine, and carbamazepine which were used as internal standard (I.S)

2.4 Preparation of Calibrators and Quality Control samples in human plasma

Calibrators ranging from 20–1000 ng /ml for 3-hydroxy nevirapine, 0.05 – 2.5 µg /ml for 2-hydroxy nevirapine and 1.0 – 10.0 µg /ml for nevirapine were prepared by subsequent dilution of the above stock solution with methanol. Suggested quality control (QC) levels were 30, 200, 600 and 900 ng /ml for 3-hydroxy nevirapine,

0.2, 0.6, 1.0 and 2.0 µg /ml for 2-hydroxy nevirapine and 2.0, 4.0, 6.0 and 8.0 µg /ml for nevirapine. These QC samples were stored at 4^0C in clear glass bottles.

2.5 Preparation of Mobile Phase

Ammonium acetate buffer was weighed at 0.771g and dissolved in 1000 ml of water to get 10 mM solution. 400ml of the 10mM ammonium acetate was diluted with 1600 ml of water to get 2mM solution. The pH of the solution was then adjusted to 4.0 with acetic acid and filtered using Whatman filter Paper No.1. The final volume was adjusted by adding this 400 ml of acetonitrile to 1600 ml of buffer. The final solution was mixed well and sonicated for 30 min.

2.6 Chromatography

The chromatographic analysis was performed at room temperatures on a Zorbax SB-C8 analytical column with a mobile phase composed of 2 mM ammonium acetate – acetonitrile (80:20 v/v) pH 4.0. Absorbance was measured at 280 nm. The active principles were eluted at a gradient flow rate of 1ml/min for 17 minutes and increased to 2ml/min with analysis time of 30 min. Aliquots of 20 µl were injected using a 20 µl fixed loop.

2.7 Extraction Procedure from plasma

20 µl of I.S (internal standard–Carbamazepine 100 µg /ml) was added to 1.0 ml of drug spiked plasma in centrifudge tube and vortexed for 30 seconds. Then 5 ml of extraction solvent (4 ml diethyl ether : 1 ml hexane) was added to it, vortexed for 30 seconds and centrifuged at 3000g for 10 min. Organic layer was then aspirated and transferred to V-tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with 100 µl of mobile phase and 20 µl was injected on to the HPLC.

3.0 VALIDATION OF HPLC METHOD

3.1 Recovery

The extraction recovery of analytes was determined by measuring the peak areas of the drug from the prepared plasma quality control samples. The percentage recovery was obtained for 4 QC (5 replicates).

3.2 Calibration curves

A six point calibration curve for the concentration of 0.05, 0.1, 0.4, 0.8, 1.5 and 2.5 µg /ml for 2-hydroxy nevirapine, 20, 60, 100, 300, 500 and 1000 ng/ml for 3-hyroxy nevirapine and 1.0, 3.0, 5.0, 7.0, 9.0 and 10.0 µg/ml for nevirapine were constructed daily for 3 consecutive days. Calibrations were made by plotting peak area ratios of nevirapine and its metabolites against the spike concentrations.

3.3 Accuracy and Precision

Spiked controls at 4 QC concentrations for nevirapine and its metabolites were prepared and were analyzed in triplicates for three days.

3.4 Stability

Stability of analytes was evaluated in four conditions; in human plasma over three month storage of -20^0C, 24 hours room temperature and freeze-thaw for three cycles

3.5 Specificity

Interference from blank plasma, compounds spiked in plasma and plasma taken from six HIV patients on HAART were determined. One ml of plasma was extracted using method described.

3.6 Reproducibility

The reproducibility of the method was checked by determining precision on different instrument at four different concentrations.

4.0 RESULTS AND DISCUSSION

Nevirapine is insoluble in water, slightly soluble in methanol, soluble in dichloromethane, dimethylsulphoxide and dimethylformamide, Calibrator solutions were prepared in methanol. Since nevirapine has a 'weak' basic in nature (pKa of 2.8), it ionizes in acidic medium.

4.1 Selection of mobile phase

The mobile phase was chosen after several trials with other solvent combinations. It was selected based on separation of metabolites, peak parameters and run time. Ammonium acetate : acetonitrile was optimize after several trials. Proportion of acetonitrile was altered to get full separation of nevirapine, 2-hydroxy and 3-hydroxy nevirapine. With proportion of 40%, retention times were decreased (3.17 min, 2.29 min and 2.27 min respectively) and peak of 2-hydroxy nevirapine was co-eluted with 3-hydroxy nevirapine. However, the problem was solved when acetonitrile was reduced to 20%. Good resolutions were obtained and the peaks were symmetry without showing any tailing. Nevirapine, 2-hydroxy nevirapine and 3-hydroxy nevirapine were adequately separated on our chromatographic run. The retention times were 12.88 min, 5.87 min and 7.42 min respectively. Retention time for internal standard was 26 min. No significant changes in peak shape and response observed in pH range of 3.8 – 4.1. In the acidic pH, probability of drug remaining in ionized form is more, which in turn has an impact on peak shape and retention time.

4.2 Selection of extraction method for Quality Control Samples

Nevirapine and its oxidative metabolites were extracted from an aqueous medium at high pH into organic solvent. Various solvents like ethyl acetate, diethyl ether, dichloromethane and hexane were tried as extracting solvent, but gave very poor recovery of nevirapine oxidative metabolites (< 20% recovery) ; hence decreased response. However extraction with solution of 4 ml ethyl acetate and 1 ml hexane gave 81% recovery of nevirapine, 66% recovery of 2-hydroxy nevirapine and 74% recovery of 3-hydroxy nevirapine. The extraction procedure produced clean and clear supernatants from plasma as there was no interference from endogenous compounds.

4.3 Recovery

The recovery for nevirapine and its metabolites at 4 concentrations were shown in table 1. The recovery ranges from 87 – 126% for the analytes with various concentrations.

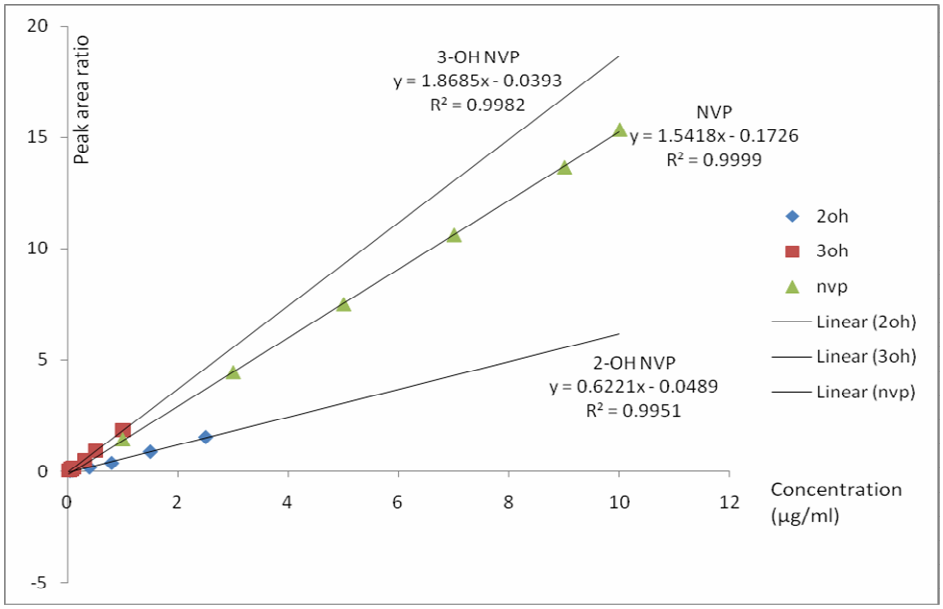
4.4 Linearity

The calibration curves were linear within the working range for nevirapine and its metabolites. Limit of detection was 0.5 µg/ml, 0.02 µg/ml and 15ng/ml for nevirapine, 2-hydroxy nevirapine and 3-hydroxy nevirapine respectively. The curves for three days were constructed and combined to give composite curves in extracted samples as shown in figures below.

4.5 Accuracy and precision

Accuracy and precision were determined by the analysis of QC pools. Accuracy was measured as the percent difference from theoretical while precision was expressed as the % of CV by each pool. Intra-assay accuracy and precision were evaluated by multiple analyses of the QC pools during one of the validation runs (presented in table 2) Inter-assay accuracy and precision were evaluated by multiple analyses of the QC pools during each validation run for three days consecutively (presented in table 3). The largest variability was observed in the lowest concentration of each analytes. The largest deviation from theoretical was observed in the lowest concentration for 3-OH nevirapine. The results indicate that the method developed has a good degree of precision.

Fig 2: Calibration curve for peak area ratio of 2-hydroxy nevirapine/carbamazepine, 3-hydroxy nevirapine/carbamazepine and nevirapine/carbamazepine Vs concentrations in human plasma



4.6 Stability of spiked plasma and extracts

Nevirapine and its metabolites were stable in human plasma over three month storage of -20^{0}C, 24 hours room temperature and freeze-thaw for three cycles. No degradation was seen in the extracts during the injection duration of 24 hours (presented in table 4).

Table 1: Recovery for nevirapine and its metabolites at 4 concentrations

	2-OH Nevirapine			3-OH Nevirapine			Nevirapine		
Concentration	Average recovery (%)	SD	CV (%)	Average recovery (%)	SD	CV (%)	Average recovery (%)	SD	CV (%)
QC 1	107.045	0.020	9.140	126.293	0.002	4.630	98.648	0.101	5.114
QC 2	89.545	0.022	4.004	96.628	0.019	9.953	102.915	0.247	5.999
QC 3	86.681	0.030	3.442	114.663	0.045	6.483	104.046	0.422	6.760
QC 4	103.392	0.048	2.317	107.832	0.085	8.804	100.726	0.179	2.229

Table 2 : Intraday accuracy and precision for nevirapine and its metabolites at 4 concentrations

		2-OH Nevirapine				3-OH Nevirapine				Nevirapine			
		0.2 (µg/ml)	0.6 (µg/ml)	1.0 (µg/ml)	2.0 (µg/ml)	0.03 (µg/ml)	0.2 (µg/ml)	0.6 (µg/ml)	0.9 (µg/ml)	2.0 (µg/ml)	4.0 (µg/ml)	6.0 (µg/ml)	8.0 (µg/ml)
Day 1	Mean (SD)	0.192 (0.011)	0.534 (0.037)	0.837 (0.021)	2.098 (0.108)	0.039 (0.0036)	0.189 (0.056)	0.632 (0.031)	0.934 (0.032)	1.928 (0.136)	3.881 (0.183)	5.782 (0.067)	7.901 (0.428)
	CV (%)	5.839	7.004	2.477	5.168	5.854	6.209	4.969	3.427	7.077	4.706	1.159	5.415
	Accuracy (%)	-4.105	-10.941	-16.282	4.082	31.96	-5.833	5.424	3.807	-3.59	-2.97	-3.64	-1.23
Day 2	Mean (SD)	0.222 (0.006)	0.517 (0.015)	0.897 (0.093)	2.013 (0.141)	0.037 (0.0004)	0.175 (0.014)	0.699 (0.062)	0.905 (0.116)	1.902 (0.248)	4.095 (0.168)	6.610 (0.541)	8.019 (0.361)
	CV (%)	2.85	2.83	10.32	7.00	1.098	7.889	8.921	12.78	13.01	4.9197	8.184	4.497
	Accuracy (%)	11.049	-13.775	-10.315	0.630	22.48	-12.40	16.54	0.630	-4.89	2.352	10.167	0.2336
Day 3	Mean (SD)	0.228 (0.004)	0.560 (0.059)	0.866 (0.043)	2.093 (0.131)	0.0399 (0.0013)	0.191 (0.032)	0.639 (0.035)	0.938 (0.071)	2.089 (0.044)	4.374 (0.607)	6.336 (0.288))	8.254 (0.501)
	CV (%)	1.668	10.45	4.913	6.257	3.35	16.51	5.54	7.53	2.108	13.88	4.55	6.073
	Accuracy (%)	14.19	-6.65	-13.36	4.668	33.03	-4.45	6.47	4.28	4.425	9.35	5.608	3.18

Table 3 : Interday accuracy and precision for nevirapine and its metabolites at 4 concentrations

	2-OH Nevirapine				3-OH Nevirapine				Nevirapine			
	0.2 (µg/ml)	0.6 (µg/ml)	1.0 (µg/ml)	2.0 (µg/ml)	0.03 (µg/ml)	0.2 (µg/ml)	0.6 (µg/ml)	0.9 (µg/ml)	2.0 (µg/ml)	4.0 (µg/ml)	6.0 (µg/ml)	8.0 (µg/ml)
Mean (SD)	0.214 (0.02)	0.537 (0.022)	0.867 (0.03)	2.068 (0.048)	0.038 (0.002)	0.184 (0.009)	0.657 (0.037)	0.926 (0.018)	1.97 (0.101)	4.117 (0.247)	6.243 (0.422)	8.058 (0.180)
CV (%)	9.14	4.00	3.442	2.32	4.63	4.60	5.60	1.90	5.114	5.999	6.760	2.229
Accuracy (%)	7.05	-10.46	-13.32	3.39	26.29	-7.56	9.48	2.90	-1.352	2.915	4.046	0.726

Table 4 : Stability assessment of nevirapine and its metabolites at 4 concentrations

Parameter	2-OH Nevirapine				3-OH Nevirapine				Nevirapine			
	0.2 (µg/ml)	0.6 (µg/ml)	1.0 (µg/ml)	2.0 (µg/ml)	0.03 (µg/ml)	0.2 (µg/ml)	0.6 (µg/ml)	0.9 (µg/ml)	2.0 (µg/ml)	4.0 (µg/ml)	6.0 (µg/ml)	8.0 (µg/ml)
One month in -20°C (% Δ from freshly prepared)	7.9	3.3	9.4	9.3	3.63	-2.53	-0.98	9.46	-0.07	-5.98	1.8	1.12
24 hours in room temperature(% Δ from freshly prepared)	7.18	-4.19	-3.78	-9.44	-10.2	8.19	-7.92	-10.3	3.64	2.25	-1.44	2.21
3 cycle freeze-thaw (% Δ from freshly prepared)	9.9	-0.71	2.37	0.78	-10	2.9	-3.11	1.48	6.22	-1.4	1.07	-1.8
Injection duration 24 hours (% Δ from freshly prepared)	-6.77	-7.49	3.1	-2.96	2.43	-5.82	0.63	-1.92	0.16	1.75	1.18	-3.42

Table 5: Reproducibility of nevirapine and its metabolites at 4 concentrations

	2-OH Nevirapine				3-OH Nevirapine				Nevirapine			
	0.2 (µg/ml)	0.6 (µg/ml)	1.0 (µg/ml)	2.0 (µg/ml)	0.03 (µg/ml)	0.2 (µg/ml)	0.6 (µg/ml)	0.9 (µg/ml)	2.0 (µg/ml)	4.0 (µg/ml)	6.0 (µg/ml)	8.0 (µg/ml)
Mean	0.212	0.541	0.9	2.1	0.037	0.247	0.65	0.996	1.988	4.183	5.982	7.801
SD	0.0066	0.0094	0.098	0.119	0.0037	0.0007	0.0073	0.0563	0.0338	0.1187	0.1505	0.484
CV (%)	3.13	1.74	10.91	5.68	10.15	0.28	1.123	5.651	1.699	2.837	2.516	6.205

4.7 Specificity

No interference from endogenous components in plasma was seen to correspond to the retention times of the peaks of interest. In addition, no peak interferences were seen with the following antiviral drugs; abacavir, didanosine, emcitarabine, efavirenz, lamivudine, lopinavir, ritonavir, tenofovir and zidovudine, bactrim, pyridoxine, methadone, ketoconazole and antituberkulosis isoniazide, ethambutol and pyrazinamide (data not shown).

4.8 Reproducibility with intra instrument

The reproducibility of the validated method was checked by determining precision at four different concentrations in triplicate with Agilent 1200 series HPLC system with DAD detector (presented in table 5).

5.0 CONCLUSION

The HPLC-UV method developed provides a sensitive, precise, and specific means for the quantification of nevirapine, 2-hydroxy nevirapine and 3-hydroxy nevirapine in human plasma. The method is also simple, reproducible, cost-effective and stable under variety of conditions anticipated during process of collecting samples. This validated method will be used to analyze association of CYP 2B6 polymorphism with pharmacokinetic profiles of NVP in 230 subjects.

ACKNOWLEDGEMENT

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