

Original Research Article

Development and Validation of Liquid Chromatography-Mass Spectroscopy/Mass Spectroscopy Method for Quantitative Analysis of Naproxen in Human Plasma after Liquid-Liquid Extraction

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Abstract

Purpose: To determine naproxen levels in human plasma using a new liquid chromatography-Mass spectroscopy/Mass spectroscopy (LC-MS/MS) method that involves a simple and single step extraction procedure using low-cost reagents.

Method: A novel liquid chromatography-tandem mass spectrometry method for the quantitative determination of naproxen in human K₂-EDTA plasma in negative ion mode was employed and validated using zidovudine as internal standard (IS). Sample preparation was accomplished by liquid-liquid extraction technique. The eluted samples were chromatographed on Zorbax Eclipse XDB phenyl 4.6 × 75 mm, 3.5 μm column (Agilent Technologies) using a mobile phase consisting of acetonitrile: 20 mM ammonium acetate (90:10 v/v). The injection volume was 15 μL and the total run time was 3.0 min. The method was validated for all parameters for naproxen.

Results: The method showed selectivity and linearity over a concentration range of 500.1 ng/mL to 100028.5 ng/mL. The validation data indicate precision and accuracy of 90 - 110 % and < 15 %, respectively, as well as recovery (80.63 %), stability (mostly stable) and carryover (0 %).

Conclusion: A rapid and selective LC-MS/MS method for the quantification of naproxen in human plasma has been developed and can be used in therapeutic drug monitoring of this drug as well as in bioequivalence studies of the drug.

Keywords: Naproxen, Therapeutic drug monitoring, Mass spectrometry, Human plasma

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INTRODUCTION

Naproxen (+)-2-(6-methoxy-2-naphthyl) propionic acid is a non-steroidal anti-inflammatory drug with anti-inflammatory, analgesic and antipyretic properties often preferred to acetylsalicylic acid (aspirin) because of its better absorption following oral administration and fewer adverse effects [1,2-5]. Anti-inflammatory effects of naproxen are generally thought to be related to

its inhibition of cyclo-oxygenase and consequent decrease in prostaglandin concentrations in various fluids and tissues [2]. Most of its therapeutic activity is probably mediated through prostaglandin synthesis inhibition [6-8]. Several chromatographic methods have been reported for determination of naproxen in raw material [9], tablets [10-12], plasma [13-15], urine [16], intestinal perfusion samples [17] and pharmaceutical preparations [18-20].

The coupling of HPLC with mass spectrometry (LC-MS/MS) is now generally accepted as the preferred technique for quantitating small molecule drugs and metabolites in biological matrices, since this technique is highly selective and sensitive [21,22]. LC-MS/MS technique was successfully employed to provide a satisfactory sensitivity and selectivity in a desirable time of chromatographic run.

EXPERIMENTAL

Chemicals

Naproxen [Fig.1 (a)] was purchased from Dr. Reddy's Laboratory (Hyderabad, India) and zidovudine, the internal standard [Fig.1 (b)], from Hetero Labs Limited (Hyderabad, India). HPLC grade acetonitrile, methanol and TBME (Methyl tertiary butyl ether) were obtained from J.T. Baker. HPLC grade water was procured from Rankem pharma. Ammonium acetate was procured from Fisher scientific.

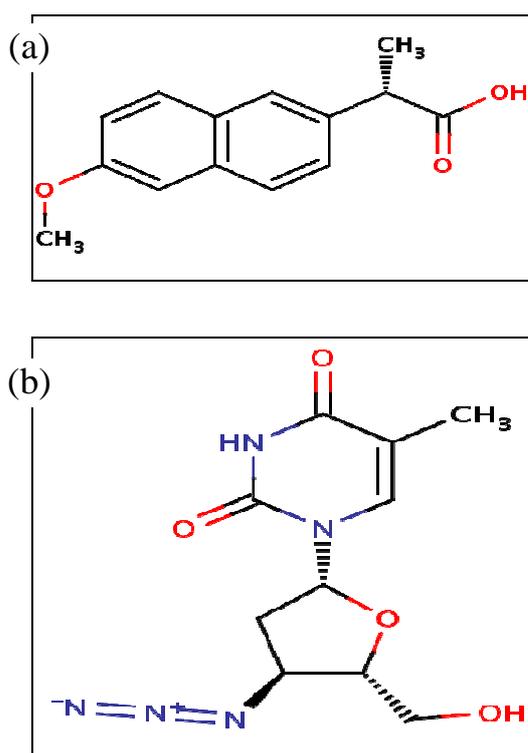


Figure 1: Naproxen (a) and Zidovudine (b)

Instrumentation

The liquid chromatographic system consist of LC Shimadzu LC10 from Shimadzu, an auto sampler of Shimadzu (SIL-HTc) coupled with an applied Biosystems SCIEX a triple quadrupole mass spectrometer (API 3000) with electrospray

ionization (ESI) used for analysis. Date of acquisition and processing were controlled by Applied Biosystems/MDS SCIEX Analyst software (version 1.4.2) with Zorbax Eclipse XDB Phenyl column (4.6 × 75 mm, 3.5 μm).

Bio-analytical conditions

The chromatographic analysis was performed by using a mobile phase of HPLC grade acetonitrile: 20 mM Ammonium acetate buffer (90:10, v/v) with flow rate 0.5 ml/min by negative ion mode (API 3000). Detection is performed by atmospheric pressure electro spray ionization (ESI) tandem mass spectrometry in negative ion mode. The chromatograms were recorded. Detection of the ions were performed by multiple reaction monitoring (MRM) of the transitions m/z 229.0–185 for naproxen and m/z 266.0–222.0 for the internal standard.

Preparation of zidovudine stock solution

A stock solution of Internal Standard (IS) was prepared by dissolving 5.00 mg of zidovudine in HPLC grade methanol and made up the volume with the same in a 5 mL volumetric flask to produce a solution of 1000000.0000 ng/mL. This solution was kept in refrigerator at 2 °C – 8 °C. Working IS solutions were prepared by suitably diluting the above mentioned stock solution afresh before use.

Preparation of naproxen stock standard and working solutions

The naproxen stock solution was prepared by dissolving 40.00 mg of naproxen in HPLC grade methanol and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 4000000.0000 ng/mL. This solution was kept in refrigerator at 2 °C – 8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into plasma to obtain calibration curve (CC) standards, quality control (QC) samples for further use.

Preparation of calibration curve standards and quality control (QC) samples

Calibration curve standard consisting of a set of ten non-zero concentrations ranging from 500.1 ng/mL to 100028.5 ng/mL of naproxen was prepared. Prepared quality control samples consisted of concentrations of 500.4 ng/mL (lower limit of quantification QC sample), 1500.1 ng/mL (lower quality control sample), 15000.7 ng/mL (middle quality control sample-1), 50002.2 ng/mL (middle quality control sample-2) and 85008.8 ng/mL (higher quality control sample) for

naproxen. These samples were stored at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$ until use. Twelve sets of LQC and HQC were stored at $-20\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ deep freezer to check $-20\text{ }^{\circ}\text{C}$ stability.

Sample preparation procedure

After bulk spiking, aliquots of 200 μL for CCs and 200 μL for QCs of spiked plasma samples were pipetted out into a pre-labelled polypropylene micro centrifuge tubes and then all the bulk spiked samples were stored to deep freezer at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$, except twelve replicates each of LQC and HQC, which were stored in deep freezer at $-20\text{ }^{\circ}\text{C} \pm 5\text{ }^{\circ}\text{C}$ for generation of stability data at $-20\text{ }^{\circ}\text{C}$. The thawed samples were vortexed to ensure complete mixing of the contents. 100 μL of the plasma sample was pipetted into 15 mL glass stoppered tubes, 25 μL (201592.2 ng/mL of zidovudine) internal standard spiking solution was added to it and vortexed, except in blank plasma samples where 25 μL diluent was added to it and vortexed. Then 100 μL of 1 % formic acid in water was added to it and vortexed. 5 mL of methyl tert butyl ether was added and shaken for 20 min on reciprocating shaker at 200 rpm. Samples were centrifuged at 4000 rpm for 10 min at $4\text{ }^{\circ}\text{C}$. Then supernatant organic layer (4.0 mL) was transferred to pre-labelled glass dry test tubes and evaporated to dryness under gentle stream of nitrogen at $45\text{ }^{\circ}\text{C}$. The samples were reconstituted in 4000 μL of mobile phase and 15 μL sample was injected to HPLC with MS-MS detection.

Method validation

The method was validated for selectivity, linearity, accuracy, precision, recovery, stability, and carry over test according to the principles of the FDA industry guidance.

Selectivity

At least 6 different lots of plasma were screened for the interference at the retention times of analyte(s) and internal standard using proposed extraction procedure and chromatography. One LLOQ or ULOQ extracted sample was processed from each blank. The percent interference was calculated.

Linearity

The linearity of calibration curve for naproxen was assessed at ten concentration levels in the range of 500.1 ng/mL to 100028.5 ng/mL in plasma samples. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was

obtained from the least-squares linear regression presented with their correlation coefficient.

Extraction recovery

Processed 18 blank plasma samples and reconstituted with six sets each of 4.000 mL of LQC, MQC2 and HQC final dilutions along with internal standard, which represent 100 % extraction of analyte and internal standard (non-extracted samples). Six sets of LQC, MQC2 and HQC (7-12) samples were processed and injected (extracted samples). The extracted samples of naproxen were compared with the non-extracted samples of LQC, MQC2 and HQC. The extracted samples of internal standard were compared with the response of internal standard in the entire non-extracted eighteen quality control samples LQC, MQC2 and HQC levels. The extraction recovery at low, medium and high levels of QC samples were obtained.

Accuracy and precision

Intra assay precision and accuracy were determined by analyzing six replicates at five different QC levels in two runs on the same day. Inter-assay precision and accuracy were determined by analyzing six replicates at five different QC levels on five different runs. The acceptance criteria included accuracy within $\leq 15\%$ deviation (SD) from the nominal values, except LLOQ QC, where it should be $\leq 20\%$, and a precision of $\leq 15\%$ relative standard deviation (RSD), except for LLOQ QC, where it should be $\leq 20\%$.

Stability

Stability of naproxen in plasma was performed using six replicates of two QC samples at low and high levels. Samples were prepared by spiking drug-free plasma with appropriate volumes of naproxen standard solutions. The stability was evaluated with different studies such as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, freeze-thaw, short term stability, bench top stability, etc. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions ($2 - 8\text{ }^{\circ}\text{C}$) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (9 hrs), processed sample stability (auto sampler stability for 49 h, freeze thaw stability (four cycles) and

short term stability (-20 °C for 6 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy ($\leq 15\%$ SD) and precision ($\leq 15\%$ RSD).

Carryover test

To check the carryover effect of analyte and internal standard, a suitable high concentration of calibration curve (ULOQ) of the analyte along with working concentration of the internal standard, extracted matrix blank and extracted LLOQ samples were prepared and injected.

Statistical analysis

Data analysis was carried out using ANOVA using Prism 6 software. The level of significance was set at $p < 0.05$.

RESULTS

Optimization of the method

In optimizing the chromatographic conditions, the ammonium acetate buffer solution was adopted in the mobile phase of the HPLC in order to suppress the tailing phenomena of chromatographic peaks of naproxen and zidovudine. Besides, the concentration of the ammonium acetate buffer was investigated and the concentration of 20 mM ammonium acetate

made the chromatographic peaks sharp and symmetric.

Furthermore, experimental results showed that acidifying the mobile phase with formic acid also contributed to improve peak shapes of naproxen and zidovudine. Therefore, a concentration of 1% formic acid was used in mobile phase. The acceptable retention and separation of naproxen and zidovudine was obtained by using an elution system of acetonitrile: 20 mM ammonium acetate 90:10, v/v) as the mobile phase. The chromatogram for aqueous standard with internal standard mixture is shown in Figure 2.

The LC/MS/MS method described here satisfies the requirement of routine analyses since it has a short run time (3.0 min), which has advantages over other methods described in the literature. Naproxen and zidovudine were retained at 1.98 and 1.74 min respectively in both aqueous standard and human plasma samples. Representative chromatogram of MQC2 plasma sample spiked with naproxen and IS is shown in Figure 3.

The mass parameters were optimized to obtain better ionization of naproxen and zidovudine molecules. The full scan spectrum was dominated by protonated molecules $[M+H]^+$ m/z 229 and 266 for naproxen and zidovudine and the major fragment ions observed in each product spectrum were at m/z 185.0 and 222.9 respectively.

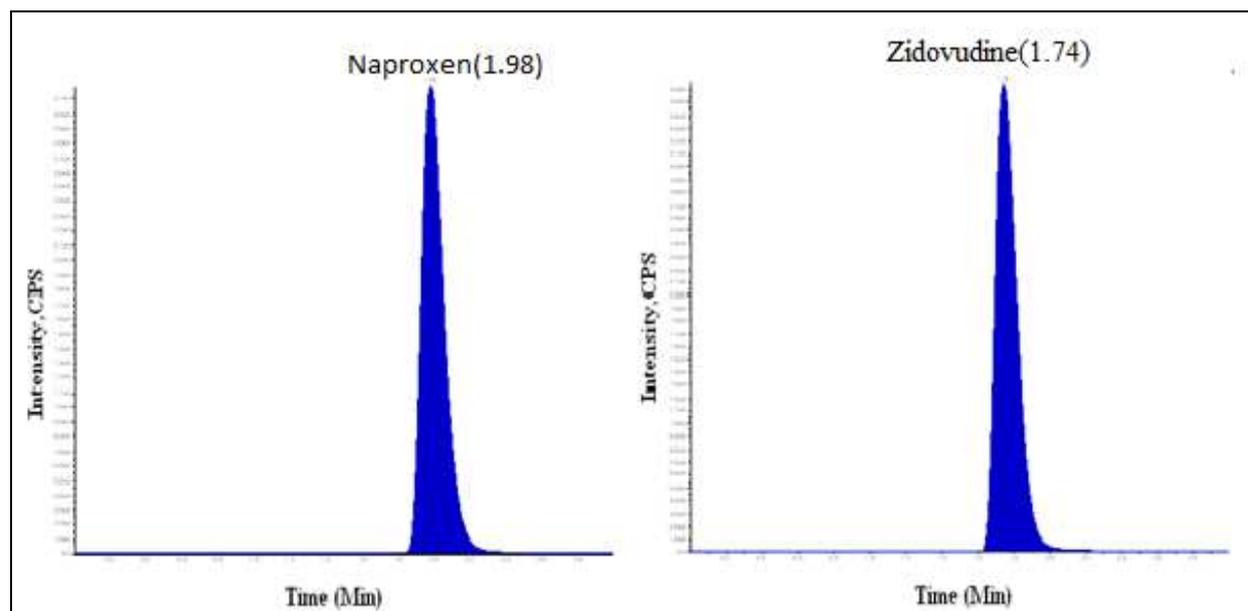


Figure 2: Representative chromatogram of an aqueous standard and internal standard mixture of naproxen

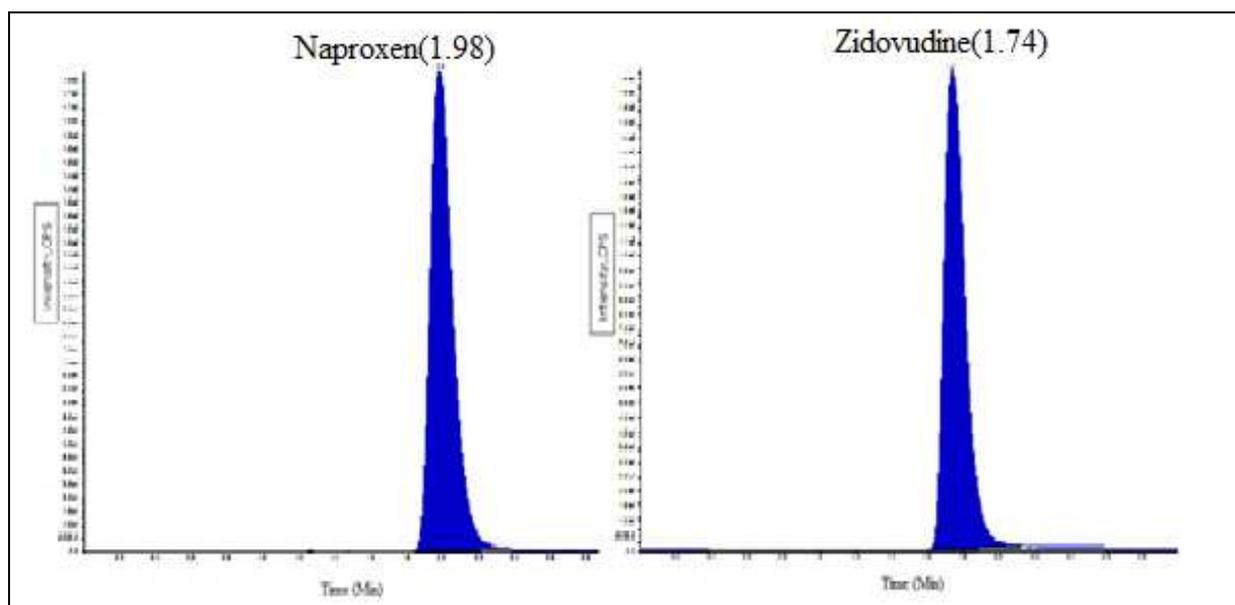


Figure 3: A representative chromatogram of MQC2 sample of naproxen with internal standard

Linearity

The correlation coefficient for naproxen over the concentration range of 500.1 ng/mL to 100028.5ng/mL was 0.9991. The average slope and intercept of regression equations were 0.0001 and 0.00196666 respectively. Linearity was found to be quite satisfactory and regression equation was found to be $Y = 0.00001, X - 0.0017$.

Selectivity

There was no significant interference from endogenous components observed at the mass transitions of naproxen and internal standard. For analyte selectivity, there was no significant interference observed at the retention time of analyte in six blank with internal standard sample as well as during the retention time of internal standard in all six ULOQ samples.

Extraction recovery

The percent recoveries of naproxen and zidovudine are shown in Table 1. The extraction

Table 1: Recovery of naproxen from human plasma

Parameter	LQC Response		MQC2 Response		HQC Response	
	Extracted QC	Unextracted QC	Extracted QC	UnExtracted QC	Extracted QC	Unextracted QC
CV (%)	6.62	2.68	3.79	2.13	3.87	1.19
Recovery (%)		73.52		80.75		87.62
Overall recovery				80.63 %		

recoveries determined were found to be between 80.63 % with a precision ranging from 1.19 % to 6.62 % for naproxen and 81.93 % with a precision ranging from 2.12 % to 6.20 % (Data not shown) for zidovudine respectively. The results are well within the limits.

Accuracy and precision

The intraday and inter day precision and accuracy for QCs are summarized in Table 2. The international acceptance criteria were met in each case [19,23].

Stability

Stability studies play an important role in a bio-analytical method development. In this study, the stability was assessed by considering different studies such as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, freeze-thaw, short term stability, bench top stability, etc. The results for all stability studies were presented in Table 3.

Table 2: Intraday and inter day precision and accuracy results for naproxen

Parameter	<i>Intraday precision and accuracy for naproxen</i>				
	LLOQ QC	LQC	MQC1	MQC2	HQC
C.V.%	12.73	11.58	10.63	7.19	7.24
% Nominal	102.08	98.16	100.78	105.81	105.55
Parameter	<i>Inter day precision and accuracy for naproxen</i>				
	LLOQ QC	LQC	MQC1	MQC2	HQC
C.V.%	10.40	10.61	9.63	6.16	
% Nominal	102.00	100.79	98.22	104.74	

Table 3: Stability results of naproxen and zidovudine

Validation parameter	Naproxen		Zidovudine	
	% Nominal / % Stability	Precision	% Nominal/% Stability	Precision
Room temperature stock Solution stability (9 h)	99.38 %	1.78 % - 2.13 %	97.93 %	3.18 % - 3.84 %
Room temperature spiking Solution stability (9 h)	98.77 %	1.97 % - 2.13 %	99.64 %	3.84 % - 3.86 %
Refrigerated Stock Solution Stability (7 days)	102.13 %	1.83 % - 2.27 %	104.14 %	2.18 % - 3.46 %
Refrigerated spiking Solution stability (7 days)	103.70 %	0.66 % - 2.27 %	106.55 %	1.80 % - 2.18 %
Auto sampler stability (49 h)	99.87 % - 110.17 %	5.38 % - 9.79 %	-	-
Freeze thaw stability (4 Cycle)	108.03 % - 109.72 %	6.43 % - 8.94%	-	-
Bench top stability (9 h)	96.74 % - 111.39 %	4.53 % - 6.60 %	-	-
Short term -20 °C stability (6 days)	108.63 % - 110.02 %	3.25 % - 5.74 %	-	-

Carryover test

The results demonstrate that there was no carryover effect of analyte and internal standard as the % carry over was found to be 0 %.

DISCUSSION

The acceptable retention and separation of naproxen and zidovudine was obtained by using mobile phase of acetonitrile: 20 mM ammonium acetate (90:10, v/v). The LC/MS/ MS method described here fulfils the requirement of routine analysis as it has a short run time (3.0 min). Representative chromatograms of an aqueous standard with internal standard mixture and plasma samples spiked with naproxen and IS were shown in Figures 2 and 3. Calibration curve of naproxen was linear over the concentration range of 500.1 ng/mL to 100028.5ng/mL for plasma which is as good as or superior to the previous methods reported [13,15].

There was no significant interference from endogenous components observed at the mass transitions of naproxen and internal standard.

Naproxen was extracted from plasma by a liquid-liquid extraction procedure. This method is also the most comprehensive method which can extract naproxen in a single extraction procedure. The mean recovery is better for plasma than those of the studies from literature review.

Intra- and inter-day precision values were lower than 15 %. These results indicate good accuracy, precision and reproducibility of the present method.

The results presented in Table 3 show that naproxen is stable under the studied conditions, since in all cases the international acceptance criteria (variation values for area smaller than 15 %) were met [24].

The results demonstrate there was no carryover effect of analyte and internal standard.

CONCLUSION

An alternative HPLC/ESI/MS/MS method for quantification of naproxen in human plasma has been successfully developed and validated. A

simple and inexpensive liquid-liquid extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real time analysis. The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, stability and carry over test. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for therapeutic drug monitoring of naproxen.

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