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Research Article

Bioanalytical Method Development and Validation of Naproxen: Application to Bioequivalence Studies

Senthil Rajan Dharmalingam[1*], Srinivasan Ramamurthy[2], Sai Siddhardh[1], M D Basheerudhin[1]

[1]*Pharmaceutical Engineering, Padmasri Dr. B V Raju Institute of Technology, Narsapur, Medak, Telangana Telagana, India*
[2]*School of Pharmacy, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia*

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ABSTRACT

A new selective and sensitive high-performance liquid chromatography method was developed for the quantification of Naproxen in human plasma using diclofenac sodium asinternal standard (IS). Chromatographic separation was achieved on aPhenomenex GEMINI C_{18} (150 x 4.6 mm, 5 mm) column. The mobile phase consists of a mixture of Acetonitrile: 0.5% Triethylamine buffer (50:50; v/v) and the pH of the mobile phase was adjusted to 3.5 by 85 % orthophosphoric acid. Flow rate of mobile phase was 1 mL/min.Detection was performed at 230nm. The calibration curve was linear over the concentration range from 10 to 120µg/mL. The detection (LOD) and quantification (LOQ) limits were 10 ng/mL and 25 ng/Ml respectively. The method was validated for accuracy, precision, specificity, robustness, and detection and quantification limits, in accordance with ICH guidelines.The developed method for the determination of Naproxen from human plasma has been found accurate, precise, selective, and suitable for the bioequivalence and pharmacokinetic studies.

Keywords*:* Naproxen, bioequivalence studies, method development, bioanalytical.

INTRODUCTION

Naproxen is chemically 2-Naphthaleneacetic acid, 6-methoxy-α-methyl-,(s)-(+)-(s)-6-Methoxy-α-methyl-2-naphthaleneacetic acid, is a non-steroidal anti-inflammatory drug with analgesic, antipyretic and certain non-rheumatic conditions with fewer adverse effects. It works by inhibiting both the COX-1 and COX-2 enzyme and consequent decrease in prostaglandin concentrations in various fluids and tissues[1].

Naproxen is rapidly and completely absorbed from the gastrointestinal tract with an in vivo bioavailability of 95%. The extent and rate of oral absorption is influenced by the presence of food in the stomach. After administration of naproxen and naproxen sodium tablets, peak plasma levels are attained in 2 to 4 hours and 1 to 2 hours, respectively. These differences between naproxen products are related to both the chemical form of naproxen used and its formulation.

Many analysis methods of Naproxen in commercial formulations have been reported in coulometry[2], UV spectrophotometry[3,4,5], HPTLC[6], heavy atom-induced room temperature phosphorescence[7], HPLC[8,9], LC-MS/MS[10].

Since 30 years, there have been quite a few chromatographic analysis reported for the quantitative determination of naproxen and its metabolites in biological samples[11,12,13,14,15,16].

The previous reported HPLC methods do not show the usage of internal standard comparison with analyte which is most important as per ICH bioanalytical method development. These methods may not be widely accessible due to their long run time, complex extraction procedure, cost, ion suppression effects, which may require expensive sample and clean-up procedures.

The purpose of this study was to develop a rapid, simple, sensitive, and selective HPLC method for the quantitative estimation of naproxen in less volume of human plasma using internal standard. It is also expected that this method would provide an efficient solution for bioequivalence, pharmacokinetic and bioavailability studies of naproxen.

MATERIAL AND METHOD

Chemicals and Reagents

Naproxen was obtained from RPG Life science, India. Acetonitrile of HPLC grade by Merck, Triethylamine and O- phosphoric acid AR grade obtained from Qualigens fine chemicals and Water HPLC grade from Milli-Q RO system were used. Diclofenac sodium was purchased from Sigma Chemical (St Louis, MO, USA).All reagents and solutions used were analytical grade except methanol and acetonitrile which were HPLC grades.

Instrumentation and Chromatographic Conditions

A Shimadzu® LC - 10AT HPLC / Waters HPLC systems were used for the analysis.The data was recorded by using the software Class VP data station/ Breeze data station.Chromatographic separations were carried out at room temperature using a reversed phase Phenomenex GEMINI C_{18}(150 x 4.6 mm, 5 mm) column.

**Author for Correspondence: senthil.rajan@bvrit.ac.in*

The mobile phase consists of a mixture of Acetonitrile: 0.5% Triethylamine buffer (50:50; v/v) and the pH of the mobile phase was adjusted to 3.5 by 85 % orthophosphoric acid. Flow rate of mobile phase was 1 mL/min.The detection wavelength, 230 nm was determined by scanning the maximum absorbance wavelength of naproxen in the mobile phase. The injection volume was 50µl using Rheodyne 7725i injector.The mobile phase was filtered through a 0.22µ membrane and degassed using ultrasonicator. The experiments were carried out at room temperature of about 20^0C.

Preparation of Naproxen standard stock solution

100 mg of Naproxen working standard was accurately transfer into a 100 ml volumetric flask and dissolved in acetonitrile and the final volume was made up and acetonitrile was labelled and stored to give 1.0 mg/mL solution of naproxen.

Standard solution for Quality control (QC)

10 ml each of 20.0, 120.0 and 240.0 µg $ml^{-1}$of Naproxenstandard solutions were preparedusing the Naproxen standard stock solution and mobile phase and stored at –20 ± 2^0C until analysis.

Preparation of stock and Calibration curve (CC) samples

10.0 ml each of 10.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 µg/mLof NaproxenCalibration curve sampleswere preparedusing 0.5ml of Naproxen standard stock solution and made up the volume with blank plasma, transferred in to different 2ml centrifuge tubes and stored at –70 ± 2^0C until processing.

Preparation of Quality control (QC) Samples

10.0 ml each of 20, 60.0 and 120.0 µg/mLof NaproxenCalibration curve sampleswere preparedusing 0.5ml of Naproxen standard stock solution and made up to the volume with blank plasma, transferred in to different 2ml centrifuge tubes and stored at –70 ± 2^0C until processing.

Plasma sample processing

At the time of analysis, the samples were removed from the deep freezer and in the room temperature and allowed to thaw. A volume of 0.5 ml of the sample was pipetted into 2.0 ml centrifuge tube and to 50 µl of internal standard solution (200µg/mL) and 0.5ml of precipitating agent (acetonitrile) was added. The resulting solution was vortexed for 5 minutes and centrifuged at 4000 rpm for 10 min. Supernatants from the above solutions were separated and used for the analysis.

Bioanalytical Method Validation

The developed method was intensively validated according to USFDA guidelines. Bioanalytical method validation required the determination of sensitivity (LOD, LOQ), linearity, range, accuracy, precision, recovery and stability respectively.

Selectivity and sensitivity

The selectivity of method was assessed by analyzing six different drug free human control plasma. Chromatograms were compared for any interference from the matrix or any of the assay reagents. The lowest standard 10 ng/mL on the calibration curve was identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20 %.

System Suitability

System suitability test is used to verify that the resolution and reproducibility of the Chromatographic systems are adequate for the analysis to be done. The tests are based on the fact that the equipment, electronics, samples to be analyzed constitutes an integral system that can be evaluated as such. The limits for system suitability were set for theoretical plates, resolution, and asymmetry.

Linearity

The calibration curve was prepared from seven calibration spiked plasma samples within the range of 10-120µg ml^{-1}, including LOQ. Calibration curve standard samples and QC samples were prepared in replicates (n = 6) for analysis. Correlation coefficients (r^2) were obtained by using quadratic regression model in whole range of tested concentrations. The acceptance criteria of back calculated standard concentration was 15 % deviation from nominal value except the LOQ (for LOQ less than or equal to 20 % deviation was applied). The calibration curve was obtainedby plotting the area ratios of Naproxen and IS as a function of the Naproxen concentrationusing least squares linear regression analysis. The LOQ was defined as a reproducible lowest concentration with signal to noise ratio greater than 10.

Recovery

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (10, 60, 120 µg ml^{-1}). The recoveries of Naproxen and IS were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

Accuracy and precision

Intra-day accuracy and precision were determined by analysis of three replicates of 3 concentrations including low, medium and high concentration QC samples. The intraday precision was evaluated by analysis of plasma samples containing naproxen at three different concentrations containing internal standard using nine replicate determinations for three occasions. The inter-day precision was similarly evaluated over two-week period. Precision studies were carried out for three levels at nine times and three occasions. The overall precision of the method was expressed as percentage of coefficient of variation and the accuracy of the method was expressed in terms of standard deviation.

Limit of Detection and Lower limit of Quantification

Plasma samples were spiked with decreasing, concentrations of the analytes and analyzed. The lowest concentration solution that shows characteristics similar to the reference chromatogram was considered to be the lowest detectable dilution. The LOQ was estimated in accordance with the baseline noise method at a signal-to noiseratio (S/N) of 5.

It was experimentally determined by injecting six samples with naproxen at the LLOQ concentration.The acceptance criterion for S/N was ≥5 and calculated by selecting the noise region as close as possible to the signal peak, which was at least 8 times of the signal peak width at half height.

Table 1: System Suitability Studies.

Parameters	Naproxen	Diclofenac sodium (I.S)
Theoretical Plate	22651	24571
Resolution factor	1.26	
Asymmetric factor	0.98	1.02
LOD(ng/mL)	10.0	5.0
LOQ(ng/mL)	25.0	25.0

Table 2: Linearity and Range.

Drug Concentration (µg ml^{-1})	Internal Standard Concentration (µg ml^{-1})	Response Factor (RSD)
10.0	200.0	0.041
20.0	200.0	0.085
40.0	200.0	0.187
60.0	200.0	0.332
80.0	200.0	0.396
100.0	200.0	0.499
120.0	200.0	0.615

Stock solution stability

The working solution (1.0 mg/ml) of Naproxen was repeatedly (n=3) injected into the chromatograph immediately after bench top storage at room temperature for 8hrs. This injection protocol was repeated after 1, 8, 15 and 30 days storage of this solution between 4-8°C.

Bench Top Stability

The bench top stability was examined by keeping replicates of the low, medium and high plasma quality control samples at room temperature for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Long term Stability of Naproxenin human plasma was tested after storage at approximately -70±2°C for 30 days. For each concentration and each storage condition, three replicates were analysed in one analytical batch. The concentration of Naproxen after each storage period was related to the initial concentration as determined for the samples. The precision and accuracy for the stability samples must be within ≤ 15 and ± 15 % respectively of their nominal concentrations.

RESULT AND DISCUSSION

Sensitivity and selectivity

Chromatographic separation of analyte and internal standard wasoptimized to provide acceptable resolution, good peak shape and intensityof the response. Mobile phase, Acetonitrile: 0.5% Triethylamine buffer (50:50; v/v) with the pH: 3.5 provided good resolution for Naproxen and diclofenac sodium (IS). No endogenous interference and matrix effect werefound at the retention times of Naproxen and IS. Figure 1.shows representativefor Naproxen and IS in plasma. The LOQ was defined as a reproduciblelowest possible concentration linear with the calibration curve. The LOQwas found to be 25 ng/ml. The intra and inter day CV were found to be 5.3 and 12.0 respectively (Table 1). This method showed sufficientselectivity and sensitivity for analyzing naproxen from plasma for pharmacokineticand bioequivalence studies.

System Suitability

The suitability of the system was studied by performing the experiment and looking for changes in separation, retention times, and asymmetry of the peaks.

Five injections of the standard and two injections of the sample were injected for this purpose. The resolution, theoretical plate values and peak asymmetry were calculated for standard and sample solutions. Resultsobtained are given in Table 1

Linearity

The standard calibration curves was linear over the concentration range from 10-120 µg/mL with mean r^2=0.9961, n=6. The LOQ was 25 ng/mL. The calibration curve had a regression equation of y=0.0052x + 0.0077, where y is the peak area ratio of Naproxen to IS and x is the plasma concentration of Naproxen.

Accuracy and precision

The percent coefficient of variation (CV) of intra-day precision ranged between 0.086% and 0.724%, whereas that of inter-day precision were between 0.025% and 0.613%.The percent intra-day accuracy ranged between 92.86 and 99.73%, whereas those of inter-day accuracy were between 91.66 and 102.10%, which is within the acceptable criteria of not more than 15%. The percent coefficient of variation (%CV) in both intra-day and inter-day precision was less than the range of 15% for lowest and 20% for highest concentration of US FDA criteria for biological fluids.

Recovery

The recoveries for Naproxen at low (10µg/mL), medium (60µg/mL) and high (120 µg/mL) plasma concentrations with six replicate injections each showed 93.40, 99.79 and 99.52%. The overall extraction relative recovery of Naproxen was found to be 99.51%.

Protein precipitation was employed for the extraction of Naproxen and I.S. from human plasma. The precipitating method using acetonitrile as the precipitating solvent was able to extract above 93.40% recovery and also there were

Table 3: Summary of the accuracy and precision of Naproxen.

Nominal conc. (µg/mL)	Mean concentration of Naproxen (µg/mL)					
	Intra day			Inter-day		
	Mean±SD	%CV	% Accuracy	Mean±SD	%CV	% Accuracy
10 (LQC)	9.18±0.06	0.724	91.86	9.16±0.05	0.613	92.66
60(MQC)	59.02±0.30	0.510	98.37	59.22±0.015	0.025	98.71
120(LQC)	129.28±0.112	0.086	99.73	118.92±0.525	0.441	102.10

Table 4: Extraction recovery of Naproxen from plasma (n = 6).

Analyte	Concentration added (µg/mL)	Mean recovery ± SD (%)	Recovery (%)	CV%
Naproxen	10.0	9.34 ± 0.132	93.40	1.41
	60.0	59.64 ± 0.534	99.79	0.89
	120.0	119.29 ± 0.997	98.52	0.84
Diclofenac sodium (I.S)	200.0	198.38± 0.67	99.04	1.76

Table 5: Stability studies of Naproxen.

Nominal conc. (µg/mL)	Mean found concentration (µg/mL)	CV (%)	Bias (%)
Stock solution stability at 9 h (n=3)			
10.0	9.36±0.07	0.831	-0.079
60.0	59.48±0.17	0.297	-0.011
120.0	129.07±2.0	0.159	0.080
Bench top stability for 24 h (n=3) in plasma			
10.0	9.53±0.035	0.371	-0.047
60.0	59.53±0.246	0.413	-0.008
120.0	129.42±0.141	0.109	0.079
Three freeze and thaw cycles (n=3)			
10.0	9.21±0.035	0.384	-0.079
60.0	59.37±0.260	0.438	-0.011
120.0	129.6±0.049	0.038	0.080
Long term 30-days stability at -70 ± 5 °C (n=3)			
10.0	9.195±0.035	0.385	-0.081
60.0	59.16±0.106	0.179	-0.014
120.0	129.13±0.170	0.131	0.076

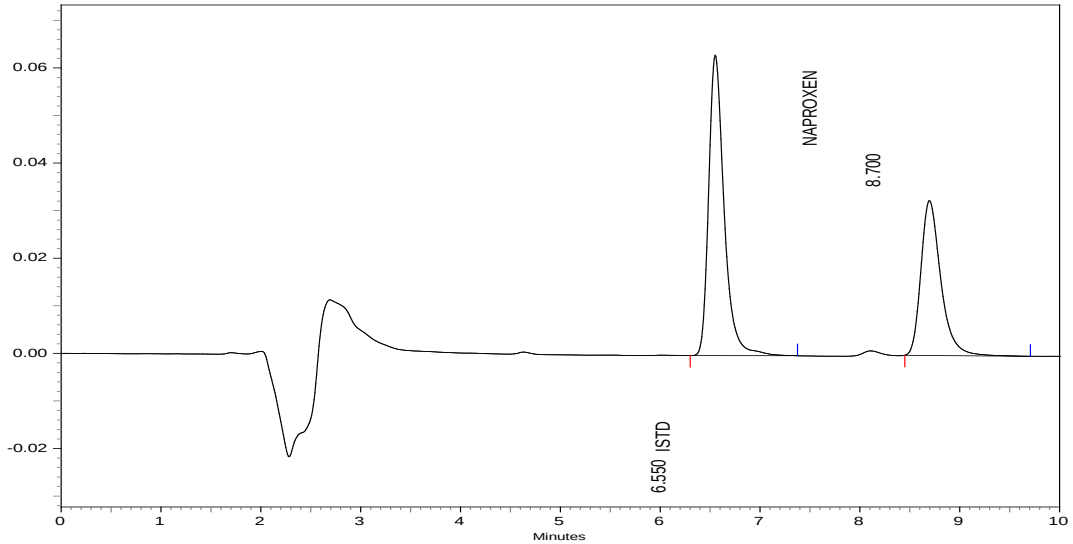

Figure 1: Representative chromatogram of Naproxen in plasma.

no interferences at the retention time of Naproxen hydrochloride and I.S. Recoveries of Naproxen are high, precise, and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

Bench Top Stability

The stability tests of the QC samples were designed to cover anticipated conditions that clinical samples may experience. Twelve hour room temperature storage and freeze–thaw cycles for low, mid and high quality controls samples indicated that naproxen was stable in rat plasma under experimental condition. Stability test samples were stable for 30 days, which are frozen at approximately -70 ± 5°C. The stability results from the current method demonstrated that spiked plasma samples were found stable without significant degradability of drugs after three freeze-thaw cycles and long term storage. Twenty-four-hour bench top stabilityand freeze-thaw cycles for LQC, MQC and HQC samples indicated that Naproxen was stable in human plasma under experimental condition.

CONCLUSION

The bioanalytical method developed of naproxen in human plasma is simple and shows good accuracy, specificity, reproducible and linear in the concentration range 10-120

µg/mL. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of naproxen without any interference. The developed method could be applied in bioequivalence, pharmacokinetic and toxicokinetic studies.

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