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Bioanalytical Method Validation for the Determination of Enalapril in Human Serum by Lc/Ms/Ms Detection

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ABSTRACT

*Enalapril was extracted from an aliquot of human serum using liquid liquid extraction method, then injected into a liquid chromatograph, equipped with mass spectrometry detector. Internal standard method was used for quantitation of Enalapril. A common liquid-liquid extraction procedure for the isolation of drug was developed from serum samples. The samples were analysed on API 3200 Triple quadrapole mass spectrometer using Phenomenex C18 , 5µm, 50*4.6 mm column in atmospheric pressure electrospray ionization. The mobile phase composition was a isocratic mixture of Methanol :Acetonitrile: Water: Formic acid (70:20 :10: 0.01 %v/v). The method was validated over a linear range of 0.25 ng/ml to 50.0 ng/ml and the limit of quantification were 0.25 ng/ml. Recoveries was observed above 35% for the analyte. The storage stability of Quality control samples was investigated under various conditions*

Keywords: Enalapril , lc-ms-ms , Pharmacokinetic studies

Introduction:

Enalapril N-[(1S)-1-(ethoxycarbonyl)-3phenylpropyl]-L-alanyl-L-proline, is a prodrug. Enalapril is a pro-drug; following oral administration, it is bioactivated by hydrolysis of the ethyl ester to Enalaprilat, which is the active angiotensin converting enzyme inhibitor. Enalapril Maleate is the maleate salt of Enalapril, the ethyl ester of a long-acting angiotensin converting enzyme inhibitor. Enalaprilat has been shown to be effective in the treatment of hypertension and congestive heart failure. Various analytical methods for the estimation of Enalapril in the given dosage form were reported in literature which includes high performance liquid chromatography with Ultra violet detection , capillary electrophorosis and flow injection analysis based on the formation of ternary complex .

Recently, HPLC coupled with mass spectroscopic detection has been extensively used for pharmaceutical analysis. For example , A high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method using liquid liquid extraction for the simultaneous determination of plasma concentrations of Enalapril and Enalaprilat in hypertensive patients treated with different pharmaceutical formulations . Along with the advancement in LCMS techniques for estimation of drugs in biological matrix different techniques of extractions were also applied like liquid- liquid extraction , protein precipitation , and liquid liquid extraction to extract Enalapril and Enalaprilat from human plasma samples.

In this article, we have validated a simple and selective high performance liquid chromatography couples with mass spectrometry method for the detection of Enalapril, from human serum rather than plasma as per USFDA guidelines . Fluoxetine was used as internal standard.

Enalapril, was extracted from an aliquot of human serum using liquid liquid extraction method and then injected into a liquid chromatograph, equipped with mass spectrometry detector. Internal standard method was used for quantitation of Enalapril. Linear regression with 1/X2 weighting was performed to determine the concentration of the drug from serum samples. All regressions and figures presented in this validation report were generated by analyst software version 1.4.1.

Materials and methods

Enalapril, and Fluoxetine were purchased from Synfine Research, Canada or Sigma Aldrich, USA. Methanol, n-Hexane, Ethyl acetate, Ammonia, and Formic acid were obtained from Qualigens (Worli, Mumbai) India. De-ionized water was prepared on Milliq Laboratory Plant (Millipore, Bedford. USA). Organic solvents and reagents used were of analytical grade. Serum was prepared from the blood obtained from suraktam blood bank, Vadodara.

Equipments and chromatographic conditions

The chromatographic system consist of LC-2010HT (Shimadzu,Japan) equipped with SIL-HTc autosampler. Mass Spectrometric analysis were conducted using API 3200 Q-trap Triple quadrapole instrument (Applied Biosystem, Sciex, Concord, Canada), equipped with a pneumatically assisted APCI(heated nebulizer) and ESI (electrospray) ionization source, which was operated in negative mode. The whole system was controlled using Analyst software version 1.4.1(Applied-Biosystem-Sciex, Concord, Canada).

Chromatographic separation was achieved on Phenomenex C18 , 5µm, 50*4.6 mm column in atmospheric pressure electrospray ionization. The mobile phase composition was a isocratic mixture of Methanol : Acetonitrile: Water: Formic acid (70:20 :10: 0.01 %v/v). Flow rate was maintained at 1.0 ml/min.

MS tuning

Tuning of mass spectrometer involves optimizing voltages, currents, flow rates and optimization of ion source parameters to achieve the maximum mass spectral sensitivity and proper resolution. Mobile phase was introduced in to the mass spectrometer via the ESI source operating in the negative ion mode under multiple reaction monitoring conditions (MRM). Quantitation was performed using selective ion Monitoring (SIM) mode at m/z 375.07, and 310.20 for Enalapril and Fluoxetine respectively.

Preparation of calibration standard

Stock solution of Enalapril, was prepared by accurately weighing and dissolving respective reference standards in methanol to give the final concentration of 100 µg/ml of each. Stock solution of internal standard i.e Fluoxetine was obtained in methanol at a concentration of 500 ng/ml and was used directly for serum sample preparation. Stock solution of Enalapril was further diluted with methanol to give serial concentrations of 2.00, 10.00, 20.00, 40.00, 100.00, 200.00, 400.00, 1000.00 ng/ml to form working solution of calibration standards. Quality control standard solutions of Enalapril was prepared in methanol at concentration of 15.00, 150.00 and 800.00 ng/ml. Working solution of analytes as well as internal standard were stored at 5°C .

Sample Preparation

A common procedure for the isolation of Enalapril from serum samples prior to LC/MS/MS was developed. The calibration standards and QCs samples were prepared by spiking of 25µl of working solutions of Enalapril to 475µl of Enalapril free control serum, Then added 25µl of working solution of Fluoxetine and vortexed it. Added 475 µl of 1% ammonia solution and mixed by vortexing and samples were extracted using liquid-liquid Extraction procedure. Added 3.0ml of extraction solvent to each sample preparation and vortexed for 2 min. Centrifuged the samples at 4000 rpm for 3 min. Transferred 2.1 ml aliquot of organic solvent to drying test tube and dried under a gentle stream of nitrogen at 40°C for 20 minute in Zymark nitrogen evaporator. Reconstituted dried residues with 500µl of mobile phase.

Method validation

The method was validated for specificity, linearity, precision, accuracy, recovery and stability.

Results:

Limits of Quantitation

The lower limit of quantitation i.e., lowest standard level with a coefficient of variation less than 20 % was 0.25 ng/ml, for Enalapril with between-batch coefficient of variation 2.90 % and accuracy 99.12 %. The Upper limit of Quantitation for Enalapril was 50 ng/ml with between-batch coefficient of variation of 0.37 % and accuracy were 98.71%.

Linearity and Sensitivity

Good linearity was achieved over the concentrations in the range of 0.25 to 50 ng/ml for Enalapril. The data of linearity are listed in Table 1. The limit of quantification (LOQ) was 0.50 ng/ml using 500 µL of serum for enalapril, with accuracy, precision ≤ 20%. Back calculations were made from the calibration curves to determine Enalapril, concentration of each calibration standard. For Enalapril The co-efficient of correlation were found to be better than 0.9987.

Table No 1: Intermediate precision ,accuracy and linear regression parameters of Enalapril determination in human serum by LC-MS-MS detection.

Added Concentration (ng /ml)	Mean measured Concentration (n = 5)(ng/ml)	Precision (RSD,%)	Accuracy (%)a
Enalapril			
0.25	0.25	2.90	99.12
0.50	0.51	6.56	102.64
1.00	0.99	1.79	98.98
2.00	1.94	2.64	97.12
5.00	5.21	2.60	104.14
10.00	9.59	3.82	95.93
20.00	20.67	3.12	103.33
50.00	49.35	0.37	98.71
Calibration curve			
Slope 0.0490			
Intercept : 0.0005 Correlation Coefficient : 0.9987			

Specificity

Presence of any interference from endogenous substances was estimated by analyzing human serum from six different lots of analyte (s) free human serum including hemolised and lipemic serum used for analysis .No significant interference was observed at the retention times of both analyte (s) and internal standard.

Precision , Accuracy and Recovery of Method

A good precision and accuracy was observed in this method. The intra and inter-day precision and accuracies are summarized in Table 2. The intra-day CV (%) were less than 7.36 % and inter-day CV (%) were less than 5.80 % for Enalapril. The Intra- day accuracies was found between 2.16 to 7.01% and the inter-day accuracies was between 3.85 to 5.88% for Enalapril. The recovery of the method was found between 39.04 to 49.81% for Enalapril. These datas were found satisfactory for pharmacokinetic studies.

Table No 2 : Intra- day and Interday precision and accuracy for Enalapril of QC.

Intra- day precision and accuracy for Enalapril of QC (n = 5)				
Added Concentration (ng/ml)	Mean measured Concentration (ng/ml)	standard deviation	CV (%)	Accuracy (Mean relative error)
0.75	0.71	0.05	7.36	7.01
7.50	7.39	0.23	3.17	3.12
40.00	37.07	0.86	2.33	2.16
Inter- day precision and accuracy for Enalapril of QC (n = 30)				
Nominal Concentration (ng/ml)	Mean found Concentration (ng/ml)	standard deviation	CV (%)	% Recovery
0.75	0.76	0.04	5.80	39.04
7.50	7.64	0.29	3.78	44.08
40.00	39.66	1.85	4.66	45.81

Samples Stability

Enalapril, showed a good stability under the conditions used for storage and processing. Enalapril was highly stable under the influence of autosampler stability, short term stability as well as three freeze/thaw cycles. Stability data of analytes under various storage conditions were mentioned in Table 3.

Table No 3 : Stability data of Enalapril under various storage conditions

Nominal Concentration (ng/ml)	Short term stability at about 25± 5°C for 24 hours .		Autosampler stability at about -5°C for 24 hrs.		Three freeze/ thaw cycles.	
	Lower Quality Control	Higher Quality control	Lower Quality Control	Higher Quality control	Lower Quality Control	Higher Quality control
	0.75 ng /ml	40 ng /ml	0.75 ng /ml	40 ng / ml	0.75 ng /ml	40 ng /ml
Mean found Concentration (ng /ml)	0.79	41.87	0.76	39.98	0.79	38.59
standard deviation	0.04	0.53	0.03	0.64	0.03	1.38
CV(%)	4.60	1.28	3.41	1.61	3.94	3.58
% change(bias)	2.20	2.47	-1.42	-2.15	3.22	-2.83

Discussion

A convenient method for the determination of Enalapril in human serum has been developed. The analytical method was validated as per the well defined standard operation procedure of Bioanalytical laboratory. The calibration curve for the standard was linear over the range from 0.25 to 50.0 ng/ml .

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