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Protein Precipitation Extraction Technique for High Performance Thin-layer Chromatographic Determination of Ambroxol HCl in Human Plasma

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ABSTRACT

A sensitive and selective High Performance Thin Layer Chromatographic (HPTLC), method was developed for the quantification of ambroxol HCl in human plasma. Six parameters from validation guidelines linearity, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), selectivity, accuracy and 3 parameters from stability including extraction efficiency, Matrix factor, Benchtop stability, Freeze-thaw stability were determined. Extracted sample were spotted on 20 × 10 cm, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany, aluminum backed silica gel 60 F_{254} Thin Layer Chromatography (TLC) plates. HPTLC mobile phase proportion used was Acetonitrile: Methanol: Triethylamine (8.2:1:0.8). Ambroxol HCl was separated at 0.6 Rf value. Ambroxol HCl gives linear response in concentration range 200-1000 ng/spot with r^2 0.997. %RSD for precision is 1.7 and 1 for two concentration level. %RSD of accuracy were found to be 10.80, 14.89, 5.1% for three concentration level. LOD obtained is 0.0011 ng/spot and LOQ is 0.0033 ng/spot. Extraction efficiency of ambroxol HCl is 51.83% with 4.7% RSD; Coefficients of variance for Matrix factor are 2.1 and 0.35 ng/spot for two concentration level. Coefficients of variance for Benchtop stability are 1.7, 1, 1.3 and 0.9 for two concentration level. Coefficients of variance for Freeze-thaw stability are 2.0, 2.7, 0.35 and 1.1 for two concentration level. All %RSD values were found to be in acceptable limit as per guidelines for bioanalytical method development and validation. The method is simple; sensitive for HPTLC determination of ambroxol HCl in human plasma can be useful for quality control of ambroxol HCl.

Keywords: Ambroxol HCl, Matrix factor, Benchtop stability, Freeze-thaw stability

INTRODUCTION

Ambroxol HCl (Figure 1) trans-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol hydrochloride an active metabolite of bromhexine is used as an expectorant and bronchodecretolytic in the treatment of bronchial asthma and chronic bronchitis. Ambroxol is also used in Pulmonary Alveolar Proteinosis (PAP) and infant respiratory distress syndrome. It is a pharmacologically active metabolite of bromhexine N-cyclohexyl-Nmethyl-(2-amino-3,5-dibromobenzyl)-amino hydrochloride. Ambroxol stimulates the transportation of viscous secretion in the respiratory organs and reduces secretion stagnation [1,2]. The literature survey revealed that there are High Performance Liquid Chromatography (HPLC), Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) and Liquid Chromatography/Mass Spectrometry (LC/MS) method for estimation of ambroxol HCl from human plasma. The proposed method uses High Performance Thin Layer Chromatography (HPTLC) method for estimation of ambroxol HCl from human plasma, is unique method for it bioanalysis.

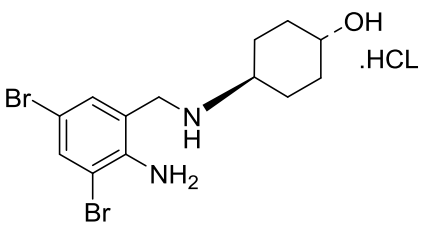

Figure 1: Ambroxol HCl

EXPERIMENTAL ANALYSIS

Chemicals and solutions

Human plasma brought from Arpan blood bank, Nashik and harvested. Protein precipitation was performed by using diethyl ether. Methanol was used as solvent for reconstitution of analyte. Various solvents system reported in literature for TLC analysis of ambroxol HCl trial an error experiments were carried out and lastly Acetonitrile: Methanol: Triethylamine (8.2:1:0.8) was selected as mobile phase for HPTLC separation. Separation and identification of ambroxol HCl was performed on (20 × 10 cm, layer thickness 0.2 mm, E-Merck, Darmstadt, Germany) aluminum backed silica gel 60 F_{254} TLC plates.

Instrumental conditions

Camag-HPTLC system was used for separation and identification of ambroxol HCL. Consisting Linomat V applicator (Camag, Muttenz, Switzerland), Camag, twin trough chamber, Camag-TLC scanner 3 equipped with Wincats software (version 1.4.2), Camag syringe of 100 µl capacity. The chamber saturation time optimized and kept 20 min. migration distance was 70 mm band with was kept at 6 mm slit, dimensions were 6.00 × 0.45 mm. Deuterium lamp was radiation source and 254 nm was scanning wavelength, distance between bands was kept at 10 mm. Remi Cyclo mixer CM 101 DX (1 min.) used for Vortex mixing and Remi research Centrifuge used for centrifugation of matrix containing ambroxol HCl.

Preparation of standard stock solution

Standard stock solution was prepared by dissolving 10 mg of 10 ml volumetric flask in methanol, and volume was adjusted to 10 ml with the same, to give a solution containing 1000 µg/ml of ambroxol HCl.

Extraction of ambroxol from human plasma

Analysis was performed on plasma containing known amounts of the drug and drug-free plasma. A 1 ml sample of plasma was transferred to a 10 ml centrifuge tube. The tubes were vortexes on a cyclomixer for 1 min. Appropriate blank was prepared simultaneously. The plasma proteins were separated with diethyl ether. The samples were centrifuged for 5 min at 5000 rpm. This combined diethyl extract was dried completely at 40°C in a water bath. The sample residue was reconstituted with 1 ml methanol and spotted to obtain ambroxol HCl in concentration range of 200-1000 ng.

Extraction efficiency

Extraction efficiency of ambroxol HCl from sample by the isolation procedure was demonstrated by external standardization. To each tubes of 1 ml drug free plasma 100 µg, 200 µg, 300 µg, drug from 1000 µg/ml was added. All protein precipitation and reconstitution process was performed. The supernatant from each of the nine tubes was poured into empty tubes. The content of all the nine tubes were evaporated to dryness at 40°C on water bath. The residue was reconstituted in 1 ml methanol, 1 µl, 2 µl, 3 µl of which was spotted. Simultaneously the standards of same concentration were spotted. The ratio of the mean area under the curves from tubes one to nine divided by the area under curves of standard spotted were calculated and multiplied by 100 expressed the percentage recovery of ambroxol HCl or extraction efficiency. The results of extraction efficiency were summarized in Table 1.

Table 1: Results of extraction efficiency

Concentration (ng/spot)	Standard (area)	Extracted from plasma (area)	%Recovery
100	1594.57	801.00	50.49
100	1716.09	768.08	
100	1598.60	938.41	
200	3358.30	1410.95	50.33
200	3505.32	1895.15	
200	3368.10	1776.59	
300	5043.51	3024.55	54.67
300	5003.22	2732.49	
300	5053.45	2924.54	
%RSD			4.7

Validation parameters

Linearity

Linearity was performed by applying the stock solution on plate by microliter syringe with the applicator to give concentrations of 200-1000 ng/spot ambroxol HCl. Each concentration was spotted three times on the plate. Calibration curve was established by plotting peak area on ordinate and corresponding concentration on abscissa (Tables 2 and 3; Figure 2).

Table 2: Linearity

Concentration (ng/spot)	Area	%RSD (n=3)
200	824.12	1.53
400	1317.7	0.37
600	1945.94	1.29
800	2342.73	1.31
1000	2840.39	1.44

Table 3: Linearity parameters

Parameter	Ambroxol HCl
Linear equation	y=58537 x -92979
Correlation coefficient	R^2=0.997
Linearity range	200-1000 ng/spot
LOD	0.0011 ng/spot
LOQ	0.0033 ng/spot

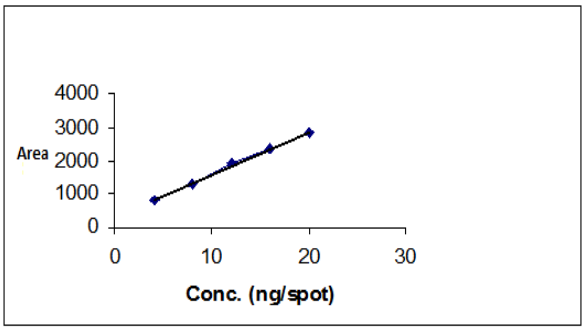

Figure 2: Calibration curve for ambroxol HCl

Precision

Precision f method was determined by carrying out repeatability by taking triplicates of 100 ng and 300 ng. The complete protein precipitation and reconstitution process was carried out and areas were determined. The results are summarized in Table 4.

Table 4: Results of precision

Concentration of drug added (ng/spot)	Area	CV
100	1120.30	1.7
100	1096.02	
100	1081.68	
300	4861.55	1.0
300	4946.22	
300	4947.98	

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined by using linear equation slope and SD of precision and formula available to calculate the same. The results are shown in Table 3.

Accuracy

Accuracy was determined at three concentration level that was 100 ng/spot, 200 ng/spot, 300 ng/spot. The results of which are given in Table 5.

Table 5 Accuracy

S. No.	Concentration of drug spiked (n=3) (ng/Spot)	%RSD
1	100	10.80
2	200	14.89
3	300	501

Selectivity

Selectivity was assessed to show that the intended analyte was measured and that their quantitation was not affected by the presence of biological matrix that is human plasma (Figure 3).

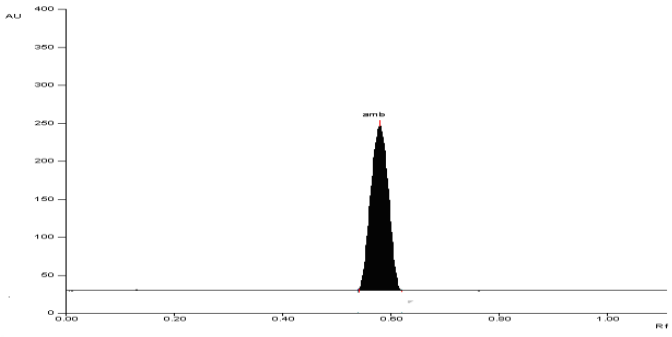

Figure 3: Chromatogram of ambroxol HCl (Extracted from plasma)

Matrix factor

The matrix factor was calculated for 100 ng and 300 ng level. The low coefficient of variation values indicative of acceptable of the assay. The results were summarized in Table 6.

Table 6: Results of matrix factor

S. No.	Concentration of drug spiked (ng/spot)	Matrix factor	Concentration of drug spiked (ng/spot)	Matrix factor
1	100	0.449	300	0.567
2	100	0.434	300	0.565
3	100	0.432	300	0.563
CV	-	2.1	-	0.35

Stability parameters

Among the various parameters of Benchtop stability and Freeze-thaw stability were determined for ambroxol HCl.

Benchtop stability

The Benchtop stability was tested 100 ng and 300 ng level (n=3). The low coefficient of variation values indicative of acceptable of assay. The comparison of areas of Benchtop samples values and nominal sample areas were significant. The results of stability study are summarized in Table 7.

Table 7: Results of Benchtop stability

S. No.	Concentration of drug (ng/spot)	Freshly prepared and extracted drug (area)	Thaw for 24 h and extracted drug (area)
1	100	1120.30	1341.30
2	100	1096.02	1377.96
3	100	1081.68	1360.34
CV		1.7	1.0
4	300	4861.55	4257.53
5	300	4946.22	4283.70
6	300	4947.98	4204.94
CV		1.3	0.9

Freeze-thaw stability

The Freeze-thaw stability was tested for 100 ng and 300 ng level (n=3). The low coefficient of variation values indicative of acceptable of assay. The comparison of areas of Freeze-thaw cycle samples and nominal sample areas were significant. The results were summarized in Table 8 [3-8].

Table 8: Results of freeze-thaw study

S. No.	Concentration of drug (ng/spot)	Freshly prepared and extracted drug (area)	Three days freeze-thaw cycle and extracted drug (area)
1	100	765.30	880.08
2	100	740.26	923.73
3	100	737.17	922.80
CV	-	2.0	2.7
4	300	2554.96	3068.63
5	300	2543.74	3056.53
6	300	2534.13	3003.36
CV	-	0.35	1.1

RESULTS AND DISCUSSION

Ambroxol HCl was separated at 0.6 Rf value. Ambroxol HCL gives linear response in concentration range 200-1000 ng/spot with r^2 0.997. Extraction efficiency of ambroxol HCl was carried out by taking 100, 200, 300 ng spotting on HPTLC plates. The extraction efficiency was found to be 51.83% with 4.7% RSD. The precision of method was determined at 100 ng and 300 ng/sopt, the coefficients of variance are 1.7 and 1.0 respectively. Coefficients of variance for matrix factor are 2.1 and 0.35 ng/spot for 100 ng/spot and 300 ng/spot. Coefficients of variance for Benchtop stability are 1.7, 1, 1.3, 0.9 for 100 ng/spot and 300 ng/spot. Coefficients of variance for Freeze-thaw stability are 2.0, 2.7, 0.35, 1.1 for 100 ng/spot and 300 ng/spot. LOD and LOQ were obtained from SD and slope of calibration curve. The %RSD values for accuracy were within acceptable limits of bioanalytical guidelines. Chromatogram of ambroxol HCl and extraction efficiency indicates the method was selective as there was no interference of matrix in ambroxol HCl estimation. All coefficients of variance values of extraction efficiency, matrix factor, Benchtop stability, Freeze-thaw stability, linearity, precision indicates that the method is suitable for estimation of ambroxol HCl in human plasma as per International Conference on Harmonisation (ICH) and other guidelines for bioanalytical method development and validation.

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