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Rapid, Selective and Rugged Method Development and Validation of Diltiazem and its Metabolites, N-Desmethyl Diltiazem, Desacetyl Diltiazem in Human plasma using Liquid Chromatography coupled with Tandem Mass Spectrometry.

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ABSTRACT

A simple, sensitive, selective and rugged liquid chromatography coupled with mass spectrometry (LC/MS/MS) method for quantification of Diltiazem and its metabolites, N-desmethyl Diltiazem, desacetyl Diltiazem in human plasma was developed and validated. The chromatography was developed using Luna 5 µ, C18, 100×4.60 mm column having a mobile phase of Acetonitrile: 0.1 % formic acid (85:15 % v/v). The flow rate was 0.5 ml/min at a column temperature of 50 ± 5º C. Electron spray ionization technique in positive mode was selected to improve the selectivity and sensitivity required for this application. The retention times of Diltiazem, desmethyl Diltiazem, desacetyl Diltiazem were 2.5, 2.0 and 2.5 minutes respectively. The method was validated for linearity, precision, accuracy, specificity, sensitivity, matrix effect, dilution integrity, ruggedness, injection reproducibility and stability. Calibration curves during the course of validation were found to be linear for Diltiazem, desmethyl Diltiazem, desacetyl Diltiazem in the ranges of 0.604-603.902, 0.303-303.274 and 0.299-299.489 ng/mL with correlation coefficient ≥ 0.9969, 0.9958 and 0.9970 respectively and by using a 1/x2 weighted least square regression analysis of standard plots associated with ten point calibration standards. The precision and mean accuracy were within the acceptable limits.

Keywords: Diltiazem; desmethyl Diltiazem, desacetyl Diltiazem; LC/MS/MS; Validation.

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INTRODUCTION

Diltiazem, a benzothiazepine calcium-channel blocker, is used alone or with an angiotensin-converting enzyme inhibitor, to treat hypertension, chronic stable angina pectoris, and Prinzmetal's variant angina. Diltiazem is similar to other peripheral vasodilators. Diltiazem inhibits the influx of extra cellular calcium across the myocardial and vascular smooth muscle cell membranes possibly by deforming the channel, inhibiting ion-control gating mechanisms, and/or interfering with the release of calcium from the sarcoplasmic reticulum. The decrease in intracellular calcium inhibits the contractile processes of the myocardial smooth muscle cells, causing dilation of the coronary and systemic arteries, increased oxygen delivery to the myocardial tissue, decreased total peripheral resistance, decreased systemic blood pressure, and decreased afterload. Diltiazem is well absorbed from the gastrointestinal tract but undergoes substantial hepatic first-pass effect. Diltiazem is metabolized by and acts as an inhibitor of the CYP3A4 enzyme.

Several analytical methods such as high performance liquid chromatography coupled with ultra violet (UV) detection and liquid chromatography-mass spectrometry (LC-MS) and ultra-high performance liquid chromatography- tandem mass spectrometric detection (UPLC-MS/MS) have been reported for the analysis of Diltiazem alone and its metabolites in biological matrix. High performance liquid chromatography coupled with ultra violet (UV) detection methods which were published were proved to be time consuming and have higher limit of quantification. Most of the reported LC-MS, UPLC-MS/MS and LC-MS/MS methods utilize electrospray ionization interface in positive ion mode towards the quantification of Diltiazem and its metabolites. These methods are tedious and require time consuming extraction procedures. They are less sensitive for the estimation of active metabolites concentration for pharmacokinetic study. The current method was sensitive and rapid, selective. Some of the reported analytical methods as follows, Dasandi, B et al., reported "development and validation of a high throughput and robust LC-MS/MS with electrospray ionization method for simultaneous quantitation of diltiazem and its two metabolites in human plasma[1]. Georgita, C. et al., reported nonlinear calibrations on the assay of dilitiazem and two of its metabolites from plasma samples by means of liquid chromatography and ESI/MS2 detection[2]. Li, J. L. et al., reported rapid and simultaneous determination of tacrolimus and diltiazem in human whole blood by liquid chromatography-tandem mass spectrometry [3]. Li, K. et al., reported HPLC determination of diltiazem in human plasma and its application to pharmacokinetics in humans [4]. Ghandour, M et al., reported adsorptive stripping voltammetric determination of antihypertensive agent[5]. Christensen et al., reported a simple and sensitive high-

performance liquid chromatography assay of diltiazem and main metabolites in renal transplanted patients [6]. Scully et al., reported high-performance liquid chromatographic assay for diltiazem in small-volume blood specimens and application to pharmacokinetic studies in rats [7]. Carignan, G. et al., reported simultaneous determination of diltiazem and quinidine in human plasma by liquid chromatography[8]. Alebic-Kolbah et al., reported determination of serum diltiazem concentrations in a pharmacokinetic study using gas chromatography with electron capture detection[9]. Zendelovska, et al., reported high-performance liquid chromatographic determination of diltiazem in human plasma after solid-phase and liquid–liquid extraction [10].

MATERIALS AND METHOD

Reagents

Diltiazem calcium, and Diltiazem D_4 reference standards were purchaged from Vardha Biotech, Desmethyl Diltiazem, desacetyl Diltiazem acetonitrile from Fischer Scientific, acetic acid from Merck, GR grade Formic acid Merck, HPLC grade Tertiary butyl methyl ether from Lab-scan and HPLC grade water was used.

Standard solutions

Stock solutions of Diltiazem, desmethyl Diltiazem, desacetyl Diltiazem of 1.0 mg/ml concentration were prepared in methanol. Intermediate stock solutions of each of the above solutions were prepared by diluting with acetonitrile: water (80:20% v/v) and the solutions were stored at 5 ± 3ºC. Internal standard solutions of Diltiazem D_4, with concentration of 1.0 mg/ml were prepared in methanol. Internal standard working stock solution was prepared by diluting stock solution 1.0 mg/ml in acetonitrile: water (80:20% v/v) and the solution was stored at 5 ± 3ºC.

Extraction Method

Selection of Extraction buffer

The 0.1% formic acid in water gave reproducible recoveries among different buffering agents compared to 5mM ammonium acetate and 5nM ammonium formate.

Selection of Extraction Solvent:

Methyl tertiary butylether was found to be suitable solvent for the extraction of analytes and internal standards from plasma.

Chromatographic and Mass Spectrometric conditions

The liquid chromatography separation was peformed using a waters Quattropremier XE (Waters, Canada) consisted of an ESI interface was operated in positive ion mode. Quantification was carried out using multiple reaction monitoring (MRM) mode. The source/gas and compound

parameters were optimized. Chromatographic method was optimized using different C18 columns like Synergy polar, Gemini-NX C18(50×4.6mm), Kromasil (100×4.6mm, 5µ), Luna C18 5µ 100x4.6mm and Zorbax Eclips. Luna 5µ 100x4.6 mm was found to be gave good peak shape, resolution, sensitivity and reproducibility and all optimum operation conditions were represented in Table 2. The mobile phase was Acetonitrile: 0.1% formic acid (85:15% v/v) with flow rate 0.500 mL/min.

Validation

To demonstrate the suitability of the method, validation was done as per US FDA guide lines.[11] As a first step of method validation, specificity was done. Specificity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The degree of interference by endogenous substances was assessed by inspection of chromatograms. It was established by screening the standard blanks of different lots of commercially available human plasma. Six plasma lots were screened for the experiment.

The linearity of the method was determined by using a 1/x2 weighted least square regression analysis of standard plots associated with a ten-point standard curve. All the four calibration curves analyzed during the course of validation were found to be linear. Sensitivity of the method is the smallest concentration of a substance that can be reliably measured by a given analytical method.

Within and between run the precision of the method was expressed as coefficient of variation (% CV). It was evaluated by the % CV at different concentration levels corresponding to lower limit of quantification (LLOQ), lower quality control (LQC), Medium quality control 1 (MQC1), medium quality control 2 (MQC2) and higher quality control (HQC) during the course of validation. Within batch accuracy and between batch accuracy was calculated as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values and expressed as percentage.

The % mean recoveries for Diltiazem, desmethyl Diltiazem, desacetyl Diltiazem were determined by measuring the area ratios of the extracted plasma quality control samples against un-extracted quality control samples at HQC, MQC1, MQC2 and LQC levels. The % mean recoveries for internal standards were determined by measuring the area ratios of internal standards in the extracted samples against un-extracted samples respectively. Dilution integrity during validation ensures that a known "sample concentration" above the ULOQ can be successfully diluted, analyzed and produce an original concentration result of acceptable accuracy. The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate

variations in operating conditions. It was performed by using a different column. injection reproducibility was performed by re-injecting the passed precision and accuracy batch after a period of 45 hours 49 minutes.

The stability of analytes in plasma was investigated in order to characterize each operation. i.e. freeze and thaw stability, bench top stability, autosampler stability, wet extract stability at room temperature, wet extract stability at refrigerated temperature, dry extract stability were performed. Long term aqueous stock stability was also performed. Stability studies were conducted in various conditions using six replicates of LQC and HQC samples. All the stability study testing conditions were represented in table 3.

Matrix effect

The matrix effect for the method was assessed by using six different lots of chromatographically screened human plasma. The results were presented in table 6.

RESULTS AND DISCUSSION

All the investigated human plasma lots were found to be free of significant interferences at the retention time of Diltiazem, and metabolites (i.e. area of the peak at the retention time of drug in standard blank samples was ≤ 20.00 % of the area of the drug in the extracted LLOQ sample; area of the peak at the retention time of ISTD in standard blank samples was ≤ 5.00 % of the area of the ISTD in the extracted LLOQ sample). No interference was observed.

Representative chromatograms of Diltiazem, Desmethyl Diltiazem and desacetyl Diltiazem were represented in Figure 2. Sensitivity of the method was evaluated at LLOQ and results were represented for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem respectively in table 4. The precision and accuracy results for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem at LLOQ level were shown in Table 5.

All the four calibration curves analyzed during the course of validation were found to be linear for the standards concentration ranging from 0.604-603.902, 0.303-303.274 and 0.299-299.489 ng/mL for Diltiazem, Desmethyl Diltiazem and desacetyl Diltiazem respectively. The correlation coefficient (r) was observed to be ≥ 0.9993, 0.9993 and 0.9994 for Diltiazem, Desmethyl Diltiazem and desacetyl Diltiazem respectively. The overall % mean accuracy for the calibration curve standards were found to be in between 95.39-104.19, 97.04-104.23 and 97.32-103.31 % for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem respectively. The overall precision was ≤ 1.89, 6.14 and 3.21 % for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem respectively.

The % mean recoveries for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem were determined by measuring the area ratios of the extracted plasma quality control samples against unextracted quality control samples at HQC, MQC1, MQC2 and LQC levels. The % mean recovery for Diltiazem at HQC, MQC1, MQC2 and LQC levels were found to be 70.79, 81.87, 69.01 and 65.03 respectively. The % mean recovery for desmethyl Diltiazem at HQC, MQC1, MQC2 and LQC levels were found to be 64.06, 72.43, 64.39 and 62.64 respectively. The % mean recovery for desacetyl Diltiazem at HQC, MQC1, MQC2 and LQC levels were found to be 73.90, 82.91, 72.05 and 70.80 respectively. The % mean recoveries for Internal Standards were determined by measuring the area ratios of internal standards in the extracted samples against unextracted samples respectively. The % mean recovery for Internal Standard was found to be 74.52.

The dilution integrity of the method was evaluated by diluting the stock solution to the concentration of 1507.001, 757.941 and 754.320 ng/mL in the screened plasma for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem respectively. The precision and accuracy for dilution integrity standards at 1/5 and 1/10 dilution were determined by analyzing the samples against calibration curve standards. The precision for dilution integrity of 1/5 and 1/10 for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem were found to be 1.56, 3.00 and 1.38 and 2.26, 2.53 and 1.49 % respectively. The % mean accuracy for dilution integrity of 1/5 and 1/10 for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem were found to be and 96.57, 97.14 & 101.13 and 101.13, 101.04 and 100.12 respectively which is within acceptance limit 85.00 - 115.00 %.

Ruggedness was performed by using different column. The precision for the quality control samples at LQC, MQC2, MQC1 and HQC concentration levels for different column ranged from 1.02-2.35, 2.89-3.30 and 2.83-3.32 for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem which is within acceptance limit ±15.00 %. The % CV of back calculated concentrations for all the samples of LLOQ QC was found to be 5.26, 12.41 and 12.36 for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which is within the acceptance limit ±20.00 %. The % mean accuracy for the quality control samples at LQC, MQC1, MQC2 and HQC concentration levels for a different column ranged from 95.38 to 98.82, 97.02 to 100.15 and 97.23 to 100.53 respectively for analyte, metabolite-1 and metabolite-2, which is within the acceptance limit 85.00 to 115.00 % except of LLOQ QC accuracy which was found to be 92.67, 94.53 and 94.52 respectively for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which is within the acceptance limit of 80.00 to 120.00 %.

injection reproducibility was performed by reinjecting the passed precision and accuracy batch after a period of 86 hours 30 minutes. The % CV of back calculated concentrations for all quality control samples of LQC, MQC2, MQC1 and HQC concentration levels ranged from 1.84-3.86, 3.51-9.22 and 2.08-5.35 respectively for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which are within the acceptance limit of 15.00 %. The % CV of back calculated concentrations for all LLOQ QC samples were found to be 6.81, 7.21 and 5.87 respectively, for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which is within the acceptance limit of ±20.00 %. The % mean accuracy of back calculated concentrations for all quality control samples at LQC, MQC2, MQC1 and HQC concentration levels were ranged from 96.52 - 100.17, 96.31 - 98.68 and 94.34 – 100.61 respectively, for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which is within acceptance limit 85.00 - 115.00 %. The % mean accuracy of back calculated concentrations for all the samples of LLOQ QC was found to be 99.53, 98.03 and 100.11 respectively, for Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem, which is within the acceptance limit of 80.00 - 120.00 %.

Stability studies in plasma were conducted under various conditions using six replicates of LQC and HQC sample (Table 3). Freeze and thaw stability of the quality control samples was determined after four freeze thaw cycles stored at -28 ± 5 °C. Stability was assessed by comparing them against the freshly prepared calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 98.61, 101.94 & 95.58 and 99.84, 97.31 and 96.91 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Bench top stability of the quality control samples was determined for a period of 16 hours 30 minutes stored at room temperature. Stability was assessed by comparing them against freshly spiked calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 97.85 and 102.29, 96.88 and 97.53, 106.08 and 95.18 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Autosampler stability of the processed quality control samples was determined for a period of 100 hours 40 minutes by storing them in autosampler maintained at temperature 5 ± 3 °C. Stability was assessed by comparing against the fresh calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 96.43 and 99.03, 94.46 and 101.05, 106.49 and 102.35 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Wet extract stability of quality control samples was determined for a period of 06 hours 30 minutes by storing them at room temperature. Stability was assessed by comparing them against the fresh

calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 100.72 and 103.04, 98.48 and 98.44, 97.23 and 98.32 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Wet extract stability of quality control samples was determined for a period of 46 hours 20 min by storing them at 5 ± 3 °C. Stability was assessed by comparing them against the fresh calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 97.48 and 99.99, 95.34 and 99.38, 100.20 and 96.92 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Dry extract stability of the spiked quality control samples was determined for a period of 60 hours 10 minutes by storing them at -20 ± 5 °C. Stability was assessed by comparing them against the freshly spiked calibration standards and quality control samples. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 98.58 and 97.78, 96.09 and 96.98, 100.32 and 94.13 respectively, which is within the acceptance limit of 85.00 - 115.00 %. Stability in blood was determined at room temperature for a period of 06 hours 12 minutes. Stability was assessed by comparing them against the freshly prepared samples in blood. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC was found to be 99.78 and 103.71, 103.83 and 104.48, 102.78 and 96.30 respectively at room temperature, which is within the acceptance limit of 85.00 - 115.00 %. Stability of analyte in blood at refrigerated temperature was determined at 5 ± 3 °C for a period of 05 hours 30 minutes. Stability was assessed by comparing them against the freshly prepared samples in blood. The % mean stability of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem for HQC and LQC were found to be 98.19 and 101.43, 100.28 and 95.52, 98.77 and 94.81 respectively, at 5 ± 3 °C, which is the acceptance limit of 85.00 - 115.00 %. The matrix effect for the method was assessed by using six different lots of chromatographically screened human plasma and results were represented in Table 6.

Table 1: Optimized mass parameters for Diltiazem, N-DesmethylDiltiazem, DesacetylDiltiazem and their corresponding internal standard

Parameter	MS Function (MRM)	Dwell time	Cone Voltage	Collision Energy
Diltiazem	415.31/178.01	0.1	28	23
N-DesmethylDiltiazem	401.28/178.01	0.1	26	24
DesacetylDiltiazem	373.29/178.01	0.1	25	25
Diltiazem-d4	419.31/182.01	0.1	28	24

Table 2: Optimized Chromatographic parameters for Diltiazem, N-desmethylDiltiazem, desacetyl Diltiazem

Column	**Luna 3µ, C18 (2), 100A, 100 x 4.60 mm**
Mobile Phase	Mobile Phase buffer /Acetonitrile (20/80, V/V)
Flow rate	1.0 mL/min, 75 % flow splitting.
Column oven temperature	40 ± 3 ºC
Autosampler temperature	5 ± 3 ºC
Volume of injection	10 µL
Detector	Mass detector
Retention time of Analyte	2.5, 2.0 and 2.5 min
Run time	3.0 min

Table 3: Stability study conditions and % mean stability results

Stability Study	Condition	N	% Mean stability					
			Diltiazem		Metabolite 1		Metabolite 2	
			HQC	LQC	HQC	LQC	HQC	LQC
Freeze thaw stability	Four freeze thaw stored at -28±5ºC	6	97.85	102.29	96.88	97.53	106.08	95.18
Bench top stability	16 hours 30 minutes storage at room temperature		97.85	97.53	102.29	106.30	96.88	95.18
Wet extract stability at room temperature	6 hours 30 minutes at room temperature		100.72	103.04	98.48	98.44	97.23	98.32
Wet extract stability at refrigerated temperature	46 hours 30 minutes storage at 5±3ºC		97.48	99.99	95.34	99.18	100.20	96.92
Dry extract stability	Storage at -20 ±5 ºC for a period of 60 hours 10 minutes		98.58	97.78	96.09	96.98	100.32	94.13
Auto sampler stability	Storage for 100 hours 40 minutes at 5 ±3 ºC		96.43	99.03	94.46	101.05	106.49	102.35
Stability of analyte in blood at room temperature	Storage at room temperature for 6 hours 12 minutes		99.78	103.71	103.83	104.48	102.78	96.30
Stability of analyte in blood at refrigerated	Storage at 5±3 ºC for 5 hours 30 miunutes		98.19	101.43	100.28	95.32	98.77	94.81

Table 4: Sensitivity of Diltiazem, Metabolite 1, Metabolite 2

Diltiazem (Nominal Concentration, ng/mL)	0.604
Mean	0.5718
SD	0.05990
% CV	10.48
% Mean accuracy	94.67
Metabolite 1 (Nominal Concentration)	0.303
Mean	0.2930
SD	0.04137
% CV	14.12
% Mean accuracy	96.70
Metabolite 2 (Nominal Concentration)	0.299
Mean	0.2685
SD	0.01840
% CV	6.85
% Mean accuracy	89.80

Table 5 Intraday and Inter day Precision and Accuracy

Sample	**Intraday (*n*=6)**			**Inter day (*n*=24)**		
	Mean conc. found (ng/mL)	**% Mean accuracy**	**% CV**	**Mean conc. found (ng/mL)**	**% Mean accuracy**	**% CV**
Diltiazem						
LLOQ	0.5983	98.90	2.58	0.5847	96.65	5.70
LQC	1.8460	103,13	3.46	1.8207	101.71	4.53
MQC 2	56.3778	100.21	1.09	56.1617	99.82	2.43
MQC 1	272.5163	96.88	1.45	273.4143	97.19	1.44
HQC	496.8647	98.91	2.47	492.9851	98.14	2.17
Metabolite 1						
LLOQ	0.2898	95.34	8.13	0.038	99.95	14.17
LQC	0.9048	100.43	3.79	0.9063	100.59	6.35
MQC 2	28.2560	99.86	3.38	27.9795	98.88	3.87
MQC1	134.8642	95.32	1.94	134.2369	94.88	2.32
HQC	246.0748	97.40	2.60	247.0444	97.78	3.93
Metabolite 2						
LLOQ	0.2568	84.76	5.45	0.2693	88.87	7.69
LQC	0.8995	100.39	3.91	0.9182	102.48	6.17
MQC 1	27.3933	97.27	2.02	27.0017	95.85	3.00
MQC 2	134.4110	95.46	1.88	134.6850	95.65	1.55
HQC	250.3608	99.57	2.03	246.4571	98.02	2.78

Table 6: Matrix effect evaluation for Diltiazem, Metabolite 1, and Metabolite 2

	Peak area ratios	
	HQC	LQC
Diltiazem (Nominal Concentration)	502.334	1.790
Mean	495.8055	1.8660
S.D	8.86973	0.07126
% C.V	1.79	3.82
% Mean accuracy	98.70	104.24
Metabolite 1(Nominal Concentration)	252.647	0.901

Mean	242.9134	0.9389
S.D	10.22019	0.05078
% C.V	4.21	5.41
% Mean accuracy	96.15	104.21
Metabolite 2 (Nominal Concentration)	251.440	0.896
Mean	242.0465	0.9062
S.D	5.60407	0.05617
% C.V	2.32	6.20
% Mean accuracy	96.26	101.14

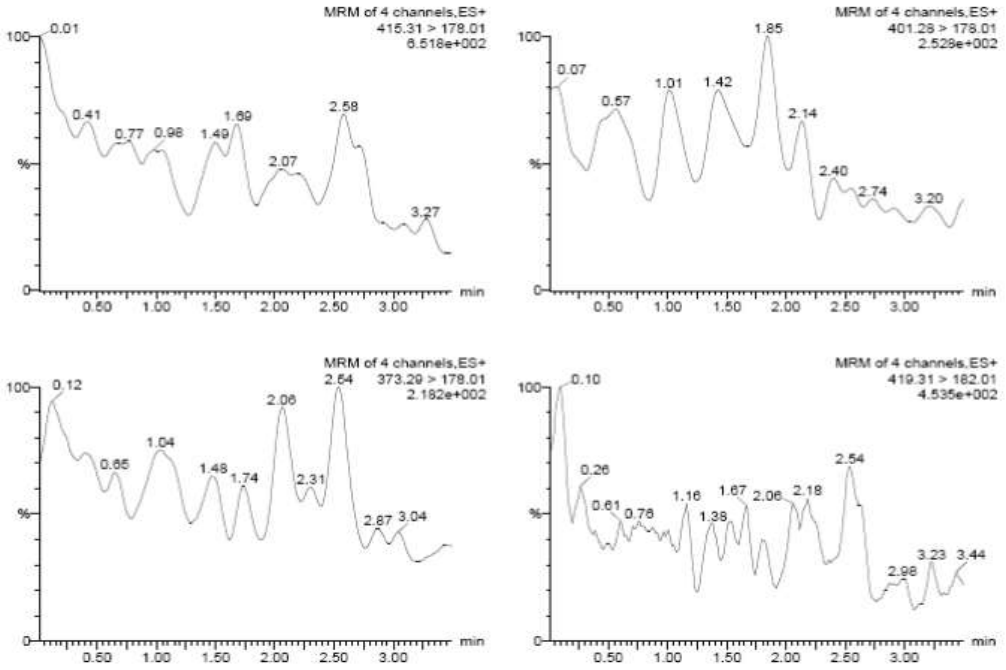

Figure 1: Standard blank

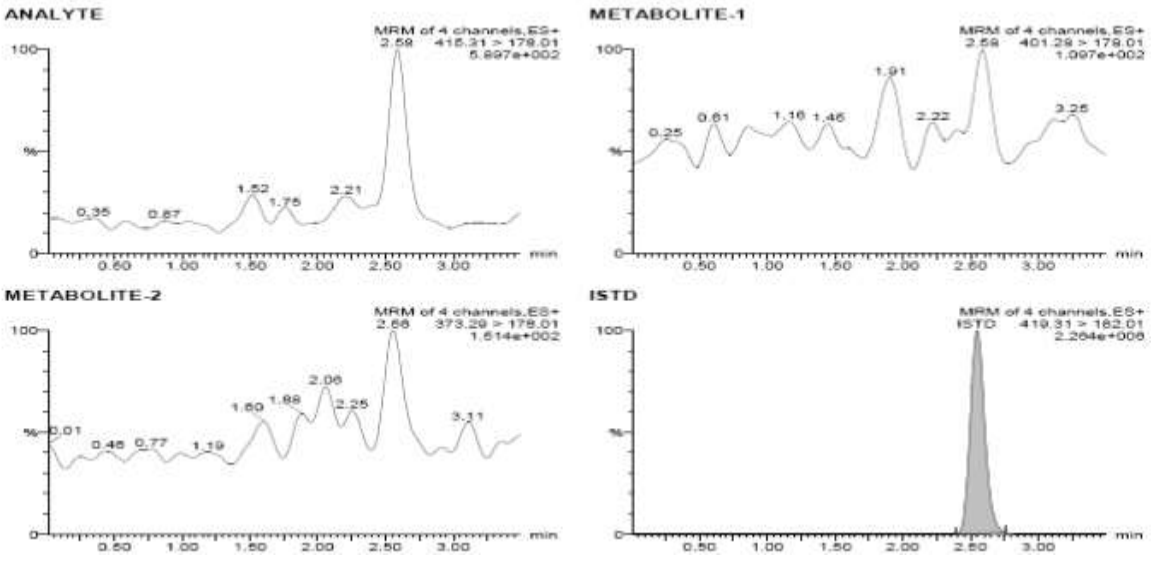

Figure 2: Standard zero

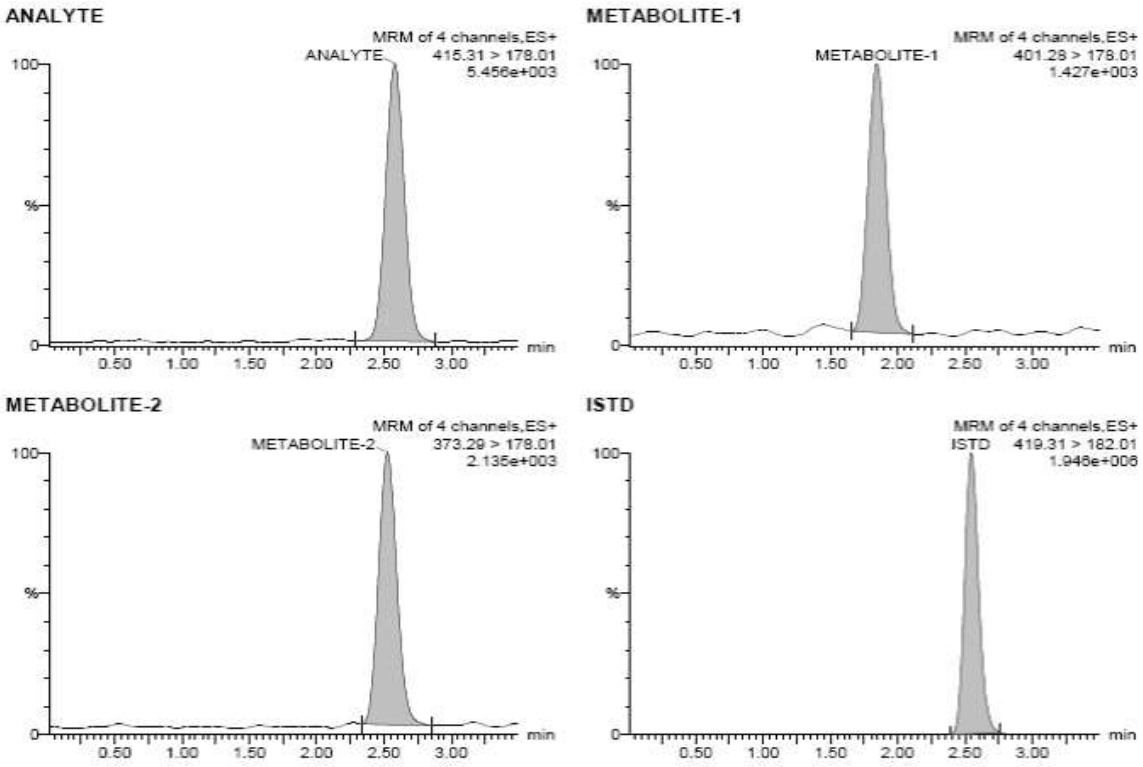

Figure 3: LLOQ

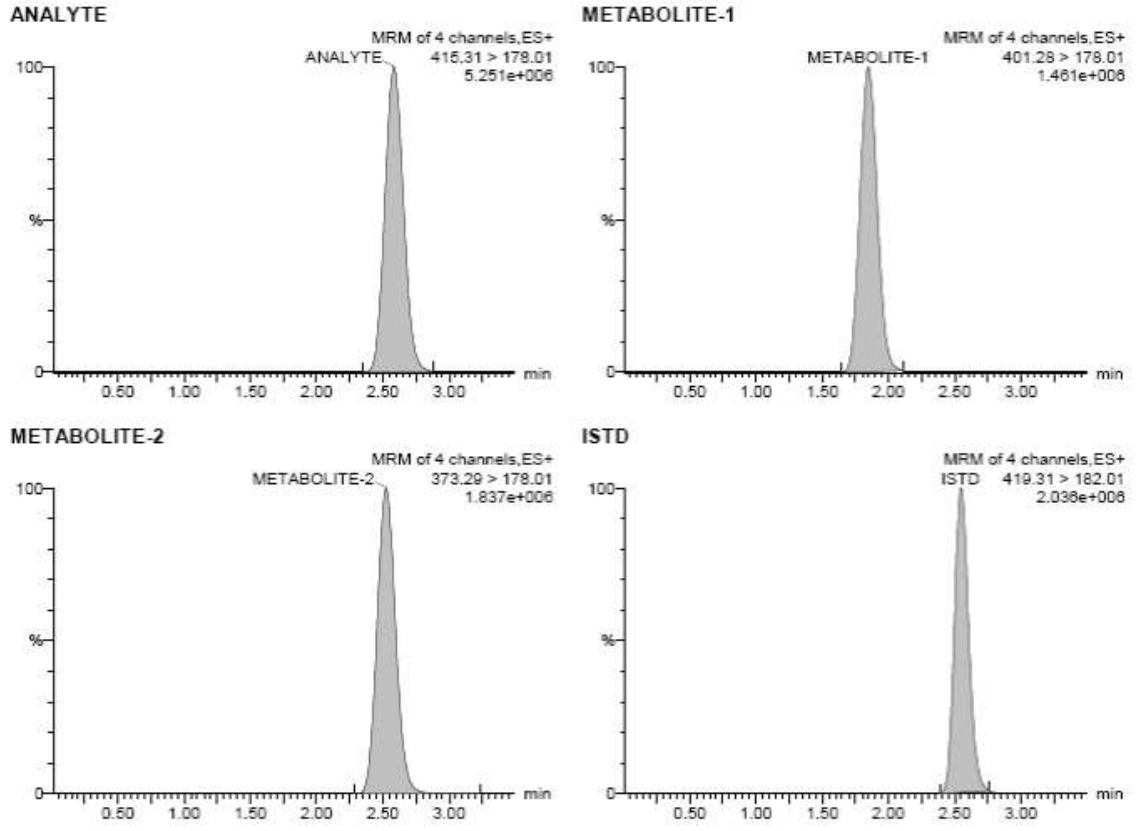

Figure 4: ULOQ

CONCLUSION

The experiments performed during the validation, concluded that the method is validated for the simultaneous quantitation of Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem human plasma over the concentration range of 0.604-603.902, 0.303-303.274 and 0.299-299.489 ng/mL respectively, using Diltiazem d_4, as internal standard. The precision and mean accuracy are within the acceptable limits. Consistent recoveries were observed for LQC, MQC2, MQC1 and HQC. The method is specific enough in the presence of K_2EDTA anticoagulant. The method is precise and accurate enough to dilute samples, if necessary. The stability experiments were performed during the validation concluded that the Diltiazem, desmethyl Diltiazem and desacetyl Diltiazem were stable at different condition like auto sampler (100 hours 40 minutes), bench top stability (16 hours 30 minutes), wet extract stability at room temperature (6 hours 30 minutes), wet extract stability at refrigerator temperature (46 hours 30 minutes), dry extract stability at -28 ± 5 °C (60 hours 10 minutes) and four freeze and thaw cycles at -28 ± 5 °C, blood stability at room temperature (06 hours 12 minutes) and blood stability at refrigerator temperature (05 hours 30 minutes) .

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