Quantitation of Diltiazem and Its Metabolite Desacetyl Diltiazem and N-Desmethyl Diltiazem in Human Plasma by Liquid Chromatography Mass Spectroscopy.

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ABSTRACT

A simple, sensitive, selective and rugged liquid chromatography coupled with mass spectrometry (LCMS/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; LCMS/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 after load. 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 electron spray 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, rapid and 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 electron spray 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 diltiazem 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]. To demonstrate the suitability of the method, validation was done as per US FDA guide lines[11].

MATERIALS AND METHOD

Diltiazem, Desmethyl Diltiazem, Desacetyl Diltiazem and Verapamil obtained from Vivan life science Mumbai India. Methanol, acetonitrile and methyl tertiary butyl ether were purchased from local distributor of Finar with LC Grade. Ammonium acetate purchased from the S D. Fine Chemical with AR Grade. Drug free human plasma was procured from Amclin Life Sciences, Pune.

Instrumentation:

Mass Spectrometry API 4000 triple quadrupole instrument (AB Sciex, Toronto, Canada) was used as Ultra Performance Liquid Chromatography from Shimadzu. Data processing was done using Analyst software 1.6.3 (SCIEX).

Instrument Method

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization were in the positive ion mode. Sources dependent parameters were as follows. Ion Spray Voltages 5500 and temperature was 400. The compound dependent parameters such as declustering potential (DP), Focusing potential (FP), Entrance potential (EP), Collision Energy (CE) and cell exit potential (CXP) were optimized during tuning as 20, 400, 10, 40 and 5 eV for Diltiazem, 20, 400, 7, 80 and 5 eV for Desacetyl Diltiazem, 20, 400, 9, 60 and 5 eV for N-Desmethyl Diltiazem and 20, 400, 10, 50 and 5 eV for Verapamil respectively. The collision activated dissociation (CAD) gas was set at 8 psi by using nitrogen gas. Dwell time was set at 200 msec for analytes and IS. The mass transition selected was 415.0-137.30 for Diltiazem, 373.00-109.00 for Desacetyl Diltiazem, 401.00-150.10 for N-Desmethyl Diltiazem and 455.20-260.30 for Verapamil.

Chromatographic conditions:

During the optimization of chromatography, selection of suitable mobile phase and the column is the initial aspects. The mobile phase containing ammonium acetate, acetic acid, Methanol and
Acetonitrile in varying combinations were tried. The low response, poor chromatography, low sensitivity was observed. Finally, the mobile phase was selected, 10 mM ammonium acetate: Acetonitrile (10:90) v/v was selected for appropriate sensitivity of analyte and internal standard with good chromatography. Different columns like Agilent 15cm, 4.6mm 5µ, Inertsil ODS with the same specification column were used but the chromatography was not found acceptable. Good chromatography was observed in Gemini C18, 4.6×50 mm, 5µ. This column was selected for further analysis; it gives an adequate response for three analyte and internal standard at a flow rate of 1.0 mL/min with 80% splitter with a run time of 2.50 mins. Retention time for Diltiazem, Desacetyl Diltiazem, N-Desmethyl Diltiazem and Verapamil were 0.82, 0.81, 0.95 and 1.04 min respectively.

Solution Preparation

Standard Solution:

Stock solutions of Diltiazem, N-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 (50:50% v/v) and the solutions were stored at 5 ± 3°C. Internal standard solution of verapamil, with concentration of 1.0 mg/mL was prepared in methanol. Internal standard working stock solution was prepared by diluting stock solution 1.0 mg/mL in acetonitrile: water (50:50% v/v) and the solution was stored at 5 ± 3°C. The concentration of Internal Standard was 3 µg/mL.

Sample Preparation:

A liquid-liquid extraction method was used for separation of drug from plasma. Tert butyl methyl ether used for extraction to separate drug from plasma, 200 µL of plasma transfer into the RIA vial, add 50 µL of Internal standard (verapamil 3µg/mL) then vortex approximately followed by addition of 3 mL of tert butyl methyl ether and vortex for 5 minutes. These tubes were centrifuge at 4500 rpm for 7 minutes at 4°C. Separate out 2 mL of supernatant organic layer and transferred in the prelabeled RIA vial. Evaporate to dryness these samples at 40°C under the stream of nitrogen gas. This dried residue was reconstituted in 200 µL mobile phase. These samples were directly transferred to the auto sampler vial for analysis and injected in optimized chromatographic condition.

Validation:

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 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 relative standard deviation (% RSD). It was evaluated by the % RSD at different concentration levels corresponding to lower limit of quantification (LLOQ), lower quality control (LQC), Medium quality control (MQC) 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, N-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, MQC 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.

The stability of analytes in plasma was investigated in order to characterize each operation. i.e. freeze and thaw stability, bench top stability, auto sampler stability, wet extract stability at room temperature, wet extract stability at refrigerated temperature and dry extract stability. 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.

RESULTS AND DISCUSSION

Method Development

To develop a simple LC-MS/MS method for quantitation of Diltiazem and its metabolites (N-Desmethyl Diltiazem, Desacetyl Diltiazem) and Verapamil, wide spectrum of organic solvents from
different physicochemical categories with different volume fractions and combinations were tested in terms of the analysis condition. Various mobile phases, in different proportions, buffered and non-buffered at various pH were attempted to provide concomitantly the best peak resolution and retention times. After considering all the data, the optimum method conditions described earlier were selected for method validation.

**Method Validation**

The LLOQ established in Method Validation was 7.28 ng/mL for Diltiazem, 0.49 ng/mL for Desacetyl Diltiazem and 1.82 ng/mL for N-Desmethyl Diltiazem. Figures of blank and LLOQ sample are given in Figure 1 and 3 for Diltiazem, 5 and 6 for Desacetyl Diltiazem and 8 and 9 for N-Desmethyl Diltiazem.

The Linear range was determined from eight concentration levels covering the range from 7.28 to 311.99 ng/mL for Diltiazem, 0.49 to 20.80 ng/mL for Desacetyl Diltiazem and 1.82 ng/mL to 78.00 ng/mL for N-Desmethyl Diltiazem.

Intra-day and Inter-day accuracy and precision were evaluated from replicate analyses (n=6) of quality control samples containing Diltiazem, Desacetyl Diltiazem and N-Desmethyl Diltiazem at four different concentrations of QC samples against calibration standards.

The percent recovery of analytes and IS from K3EDTA based human plasma was determined by comparing the mean peak area of six extracted and six unextracted samples at three different concentrations (LQC, MQC and HQC). Percent recovery found for Diltiazem, Desacetyl Diltiazem N-Desmethyl Diltiazem and Verapamil was 104.94, 99.45, 98.49 and 102.44 respectively (Table 06). Haemolysis and lipemic effect was also evaluated by calculating the % RSD and % nominal of back calculated concentration.

Ruggedness test was performed for different column, different equipment as well as different analyst. Precision and accuracy batches were processed for evaluation of ruggedness of the method at five different concentration levels.

Stability experiments were performed based on the comparison of stability samples against freshly prepared samples of the same concentration. Stability was evaluated by calculating percentage difference between the back calculated concentrations of stability samples and freshly prepared sample.

**Selectivity and Specificity:**

Selectivity was assessed to show that the quantitation of intended analyte was not affected by the presence of endogenous matrix components, metabolites, degradation products or co administered drugs. Blank and LLOQ level sample from six different lots along with one Haemolysed and one
hyperlipidemic lot were processed and extracted as per the extraction procedure. Interference was checked at the retention time of Analytes or internal standard. Quantitation of analytes was not affected by the presence of the biological matrix and there was no interference of the biological matrix in the quantitation of Diltiazem, Desacetyl diltiazem, N-Desmethyl diltiazem and Verapamil. Hence, the method is selective. Representative chromatograms of Diltiazem, N-Desmethyl Diltiazem and Desacetyl Diltiazem were represented in Figure 2.

**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 3.

**Sensitivity:**
For sensitivity experiment, six samples of blank biological matrix were spiked with spiking solution standard A (LLOQ) and extracted along with precision and accuracy batch as per extraction procedure. Signal to Noise ratio (S/N) was calculated for each sensitivity sample. The % RSD and % Nominal of back calculated concentration of analytes at LLOQ level was calculated. Sensitivity results were represented for Diltiazem, N-Desmethyl Diltiazem and Desacetyl Diltiazem in Table 1.

**Linearity:**
Linearity was evaluated using spiked plasma samples and calibration curves were constructed using eight non-zero standard points for Diltiazem, Desacetyl Diltiazem and N-Desmethyl diltiazem covering the range of 7.21 to 309.01 ng/mL, 0.49 to 21.10 ng/mL and 1.76 to 75.33 ng/mL respectively. The Calibration Curve was generated using linear regression y = ax + b with weighting (1/x²).

All the three calibration curves analyzed during the course of validation were found to be linear for the standards Diltiazem, N-Desmethyl Diltiazem and Desacetyl Diltiazem respectively. The correlation coefficient ($r^2$) was observed to be ≥ 0.99. The overall % mean accuracy for the calibration curve standards were found to be in between 93.52-107.62, 91.85-113.27 and 95.51-104.54 % for Diltiazem, Desacetyl diltiazem and N-Desmethyl diltiazem respectively. The overall precision was found to be 3.05-7.71, 2.43-8.34 and 2.54-6.90 % for Diltiazem, Desacetyl diltiazem and N-Desmethyl diltiazem respectively.

**Precision and Accuracy:**
For all precision and accuracy experiments % RSD and % accuracy of back calculated concentration of analytes was calculated. Intra-day and inter-day accuracy and precision were evaluated from replicate analyses (n=6) of quality control samples containing Diltiazem, Desacetyl diltiazem and N-Desmethyl diltiazem at different concentrations (LLOQ QC, LQC, LMQC, MQC and HQC).
Intra-day and inter-day accuracy and precision were also assessed from the analysis of the same QC samples on different days in replicate (n=6). QC samples were analyzed against calibration standards. Data of precision and accuracy is summarized in Table 02.

**Recovery:**
The % recovery of analytes and IS from K3EDTA based human plasma was determined by comparing the mean peak area of six extracted and six unextracted samples at three different concentrations (LQC, MQC and HQC). The data of recovery is summarized in Table 6.

**Haemolysis and Lipemic effect:**
Haemolysis and lipemic effect of analytes was evaluated by calculating the % RSD and % nominal of back calculated concentration. From results it was observed that quantitation of Diltiazem, Desacetyl diltiazem and N-desmethyl diltiazem was not affected by haemolysis and lipid content of samples. (Table 3)

**Dilution Integrity:**
The dilution integrity experiment was performed by diluting the sample by 2 and 10 times. Dilution integrity samples were having the concentration of 49.44 ng/mL - 247.21 ng/mL, 3.38 ng/mL -16.89 ng/mL and 12.05 ng/mL-60.27 ng/mL for Diltiazem, Desacetyl diltiazem and N-desmethyl diltiazem respectively. Six replicates of each diluted DI samples were analyzed for dilution integrity. % RSD and % Nominal was concluded that analyte can be accurately and precisely quantified after dilution up to 10 times. (Table 5)

**Ruggedness Test:**
Ruggedness test was performed for different column, different equipment as well as different analyst. Precision and accuracy batches were processed for evaluation of ruggedness of the method at five different concentration levels (LLOQ QC, LQC, LMQC, MQC and HQC). All ruggedness tests were meeting the acceptance criteria of accuracy and precision.

**Long Batch Performance:**
The long batch performance experiment was performed to evaluate any change over time within one run and to demonstrate accuracy and precision of QC samples with a size equivalent to a prospective analytical run. The experiment was performed with multiple sets of QC samples at five different concentrations (LLOQ QC, LQC, LMQC, MQC and HQC). The data generated in this Long Batch indicates acceptable and satisfactory instrument performance over the period of time for which the Long batch was run (consisting of 130 samples). This Long batch was a simulation of the proposed subject sample analysis batch. Also no trend within a run was observed over the period in which 130 samples of the Long Batch were analyzed. Hence analysis of plasma samples of the subjects from
the proposed diltiazem and its metabolites bio-study can be successfully done in analytical runs comprising of 130 samples.

**Stability**

Evaluation of the stability of samples was based on the comparison of stability samples against freshly prepared samples of the same concentration. Percentage difference between the back calculated concentrations obtained for the sample under investigation and freshly prepared sample was evaluated. Six aliquots, each of LQC and HQC concentrations were used for stability study. In bench top stability the low and high QC sample were thawed and left at room temperature for 24 hours. Comparison of the results for QC sample (low and high) with freshly prepared samples showed that there was no significant difference between response of freshly prepared samples and stability samples after 24 hours.

Freeze-thaw stability was determined after five freeze-thaw cycles for six replicate of low and high QC sample. The samples were stored at −20°C temperature. Then samples were thawed at room temperature and processed with freshly prepared samples as per extraction procedure. No significant difference between freeze thaw samples and freshly prepared samples was observed. Dry extract stability was evaluated by extracting samples till step of drying. Dry extract samples were stored at 2-8°C in refrigerator for 24 hours. After stability duration (24 hours), these samples are reconstituted and analysed along with fresh samples for stability evaluation. For wet extract stability, samples were extracted and stored at 2-8°C. After 24 hours, stability samples were processed along with fresh samples. % difference was calculated between stability samples and fresh samples. All stability results met acceptance criteria. Results of stability experiments are shown in Table 04.

**Table 1: Sensitivity of Diltiazem**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diltiazem</th>
<th>Desacetyl Diltiazem</th>
<th>N-Desmethyl Diltiazem</th>
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<tr>
<td>Mean Concentration</td>
<td>6.97</td>
<td>0.46</td>
<td>1.84</td>
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<tr>
<td>SD</td>
<td>0.3031</td>
<td>0.0531</td>
<td>0.0512</td>
</tr>
<tr>
<td>% RSD</td>
<td>4.35</td>
<td>11.54</td>
<td>2.78</td>
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<tr>
<td>% Mean Accuracy</td>
<td>95.74</td>
<td>93.88</td>
<td>104.55</td>
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**Table 2: Intraday and Inter-day precision and Accuracy**

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<th>Inter-day</th>
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<tr>
<td></td>
<td>Mean Concentration (ng/mL)</td>
<td>SD</td>
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<td>Diltiazem</td>
<td>LQC</td>
<td>22.15</td>
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<tr>
<td></td>
<td>LMQC</td>
<td>63.52</td>
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<td>MQC</td>
<td>152.44</td>
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Table 3: Matrix Effect and Haemolized Effect

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<th>Parameters</th>
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<th>Haemolized Effect</th>
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<tr>
<td>Diltiazem</td>
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<td></td>
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<tr>
<td>LQC</td>
<td>24.52</td>
<td>1.8593</td>
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<td>HQC</td>
<td>279.13</td>
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<td>Desacetyl Diltiazem</td>
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<tr>
<td>LQC</td>
<td>1.57</td>
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<td>HQC</td>
<td>17.13</td>
<td>0.7296</td>
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<tr>
<td>N-Desmethyl Diltiazem</td>
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<tr>
<td>LQC</td>
<td>6.06</td>
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<td>HQC</td>
<td>72.70</td>
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Table 4: Stability

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<tr>
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<th>N-Desmethyl Diltiazem</th>
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<tbody>
<tr>
<td>Dry Extract (24 Hrs.)</td>
<td>101.57</td>
<td>95.27</td>
<td>93.75</td>
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<tr>
<td>Freeze Thaw Cycle (4 Cycle)</td>
<td>97.50</td>
<td>100.00</td>
<td>91.25</td>
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<tr>
<td>Bench Top (24 Hrs)</td>
<td>106.10</td>
<td>97.30</td>
<td>89.88</td>
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<td>Autosampler Stability (24 Hrs)</td>
<td>111.14</td>
<td>93.41</td>
<td>91.33</td>
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<td>Long term stability (25 days)</td>
<td>93.42</td>
<td>93.55</td>
<td>92.80</td>
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Table 5: Dilution Integrity

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<th>SD</th>
<th>% RSD</th>
<th>% Mean Accuracy</th>
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<td>Diltiazem</td>
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<td></td>
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</tr>
<tr>
<td>1:2</td>
<td></td>
<td>52.87</td>
<td>1.1443</td>
<td>2.16</td>
<td>106.94</td>
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<tr>
<td>1:10</td>
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<td>244.23</td>
<td>26.2861</td>
<td>10.76</td>
<td>98.79</td>
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<tr>
<td>Desacetyl Diltiazem</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td></td>
<td>3.01</td>
<td>0.1281</td>
<td>4.26</td>
<td>89.05</td>
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<td>15.56</td>
<td>1.2113</td>
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<td>N-Desmethyl Diltiazem</td>
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<td></td>
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<tr>
<td>1:2</td>
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<td>12.64</td>
<td>0.3772</td>
<td>2.98</td>
<td>104.90</td>
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<tr>
<td>1:10</td>
<td></td>
<td>59.40</td>
<td>5.6482</td>
<td>9.51</td>
<td>98.56</td>
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Table 6: Recovery

<table>
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<tr>
<th>Parameters</th>
<th>Diltiazem</th>
<th>Desacetyl Diltiazem</th>
<th>N-Desmethyl Diltiazem</th>
<th>Internal standard</th>
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<tbody>
<tr>
<td>LQC</td>
<td>105.68</td>
<td>97.10</td>
<td>97.45</td>
<td>98.06</td>
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<tr>
<td>MQC</td>
<td>99.87</td>
<td>98.43</td>
<td>96.45</td>
<td>96.04</td>
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<tr>
<td>HQC</td>
<td>109.28</td>
<td>102.83</td>
<td>101.58</td>
<td>93.11</td>
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<tr>
<td>Mean Recovery</td>
<td>104.94</td>
<td>99.45</td>
<td>98.49</td>
<td>95.74</td>
</tr>
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</table>

Figure 1: Blank of Diltiazem

Figure 2: Internal Standard

Figure 3: LLOQ of Diltiazem
Figure 4: ULOQ of Diltiazem

Figure 5: Blank of Desacetyl Diltiazem

Figure 6: LLOQ of Desacetyl Diltiazem
Figure 7: ULOQ of Desacetyl Diltiazem

Figure 8: Blank of N-Desmethyl Diltiazem

Figure 9: LLOQ of N-Desmethyl Diltiazem
CONCLUSION

The experiments performed during the validation, concluded that the method is validated for the simultaneous quantitation of Diltiazem, N-desmethyl Diltiazem and desacetyl Diltiazem human plasma over the concentration range of 7.21 to 309.01 ng/mL, 0.49 to 21.10 ng/mL and 1.76 to 75.33 ng/mL respectively, using Verapamil, as an internal standard. The precision and mean accuracy are within the acceptable limits. Consistent recoveries were observed for LQC, LMQC, MQC and HQC. The method is specific enough in the presence of K2EDTA 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, N-Desmethyldiltiazem and Desacetyl Diltiazem were stable at different condition like auto sampler, bench top stability, dry extract stability and four freeze and thaw cycles at -28 ± 5 °C.

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