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STABILITY INDICATING ASSAY FOR DILTIAZEM AND ITS METABOLITES IN HUMAN PLASMA BY ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY FOR PHARMACOKINETIC APPLICATION

Rajeev Kumar Gupta [*1] and Anand Chaurasiya [2]

MJRP University [1], Ram Nagar Extension, Jaipur - 302019, Rajasthan, India.
Millennium College of Pharmacy [2], Bhopal - 462044, Madhya Pradesh, India.

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Correspondence to Author:
Rajeev Kumar Gupta

Research Scholar,
MJRP University, Ram Nagar Extension, Jaipur - 302019, Rajasthan, India.

E-mail: rkpipraiya@gmail.com

ABSTRACT: A simple and sensitive Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry method was developed to perform a stability study of diltiazem and its metabolites in human plasma using various buffer reagents at a different strength. The method was applied for the quantification of diltiazem and its two major metabolites N-desmethyl diltiazem and desacetyl diltiazem in human plasma. The analytes were separated using a binary solvent delivery mode on a reversed-phase column and analyzed by Mass Spectrometry in the multiple reaction monitoring mode using the respective $(M+H)^+$ ions, m/z 415.05/178.03 for diltiazem, m/z 401.09/150.04 for N-desmethyl diltiazem m/z 373.21/108.85 for desacetyl diltiazem, m/z 419.22/314.0 for diltiazem-D4 (internal standard). The linearity was 0.93 to 250.10 ng/mL for diltiazem, 0.24 to 64.00 ng/mL for N-desmethyl diltiazem and 0.15 to 40.69 ng/mL for desacetyl diltiazem in human plasma. The lower limit of quantification was 0.93 ng/mL, 0.24 ng/mL and 0.15 ng/mL for diltiazem, N-desmethyl diltiazem, and desacetyl diltiazem, respectively. The plasma samples buffered with 1% of 0.1 M NaF solution was able to limit the degradation of diltiazem to desacetyl diltiazem for longer storage periods at -70 °C.

INTRODUCTION: Diltiazem hydrochloride (2S, 3S)- 5-(2- (dimethylamino) ethyl)-2- (4-methoxy phenyl)-4-oxo-2, 3, 4, 5-tetrahydro benzo [b][1,4] thiazepine-3-yl acetate hydrochloride is a calcium channel blockers indicated not only in the treatment of classical and vasospastic angina pectoris but also in the treatment of supraventricular tachyarrhythmia and essential hypertension. Diltiazem (DTZ) is metabolized by N-demethylation, *O*-demethylation, and deacetylation [1].

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The metabolites produced through these metabolic pathways may influence the pharmacodynamic reaction or interacting potential in patients treated with DTZ. The metabolites desacetyl diltiazem and N-desmethyl diltiazem **Fig. 1** noticeably possess stronger ability to reduce the thrombocyte aggregation as compared the DTZ, and it is probable that the metabolites may well affect the response after administration of DTZ. DTZ and its major metabolite N-desmethyl diltiazem are readily decomposed to desacetyl diltiazem and desacetyl N-desmethyl diltiazem [2, 3, 4, 5].

The present gap in research is the instability of metabolite during the analytical methodology. Thus, the objective of the present research work is to perform the stability study of DTZ and desacetyl diltiazem by a simple and sensitive LC-MS/MS

method and study of optimization of buffer to prevent the conversion of diltiazem to desacetyl diltiazem. Although, diltiazem and its metabolites have been analyzed by HPLC [6-14] and LC-MS/MS [15-16], and other methods [17-24] these methods possess a higher range of quantification for the metabolite and insufficient stability data [25].

UPLC/MS is one of the best recent analytical techniques [26] for the characterization of the different organic molecules as reported in biologically active extracts [27], glibenclamide [28], bisphenols [29-30], resin-based dental composites [31] and extracts of *Boerhaavia diffusa* [32], *etc.*

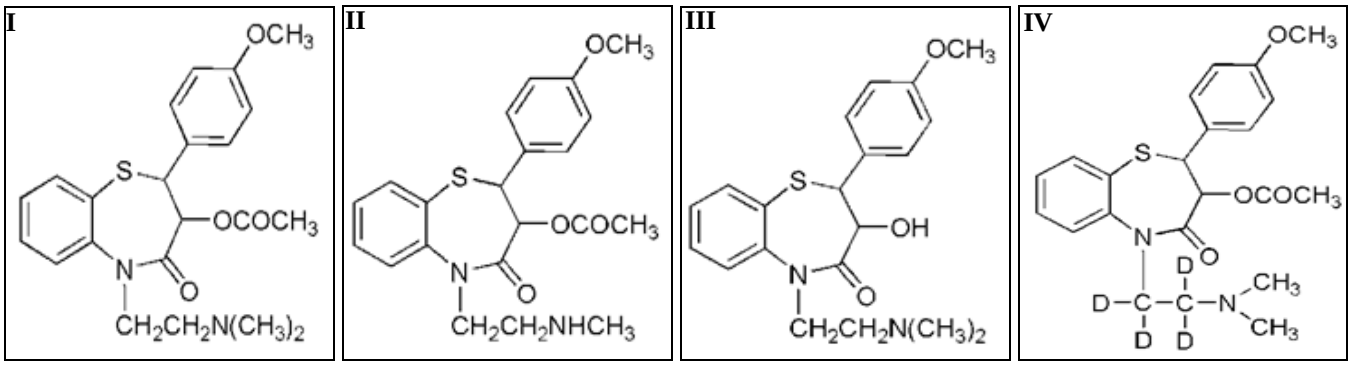

FIG. 1: CHEMICAL STRUCTURE OF DILTIAZEM (I), N-DESMETHYL DILTIAZEM (II), DESACETYL DILTIAZEM (III), DILTIAZEM D4 (IV)

EXPERIMENTAL:

Chemicals: Diltiazem, N-desmethyl diltiazem, desacetyl diltiazem, and diltiazem-D4 (internal standard) **Fig. 1** reference standards were obtained from a commercial laboratory. The drug-free plasma is containing dipotassium EDTA as an anticoagulant was collected from Sai Laxmi labs, Hyderabad. Methanol (HPLC Grade), formic acid (GR Grade), and hydrochloric acid [GR Grade] were purchased from Merck India. Ammonium formate (AR) was purchased from Sigma Aldrich and water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the study.

Chromatography: The UPLC system (Waters, milford, USA) was equipped with an acquity SM sample manager, acquity BSM binary solvent manager and thermostated column compartment. Chromatography was performed on a thermo hypersil gold column (5µm, 150 × 4.6 mm) at a temperature of 40 °C. The binary mobile phase comprised of ammonium formate buffer (pH 3.0; 5 mM)-methanol (10:90, v/v) and was pumped at a flow rate of 0.6 mL/min. Mass spectrometric detection was performed on Micro mass (WATERS, UK) using multiple reaction monitoring (MRM) with positive ionization mode **Table 1**.

Sample Preparation: Standard stock solution of diltiazem (1 mg/mL) was prepared in methanol-hydrochloric acid (0.1%) solution mixture (60:40, v/v); N-desmethyl diltiazem (1 mg/mL), desacetyl diltiazem (1 mg/ mL), and diltiazem-D4, were separately prepared in methanol. Spiking solutions for the calibration curve and quality control were prepared by appropriate dilution in methanol-water (70:30, v/v) solution. Spiking solution was prepared as a mixture of above three analytes. These spiking solutions (0.2 ml) were added to drug-free human plasma (9.8 mL) as bulk, to obtain 0.93 to 250.10 ng/mL for diltiazem, 0.24 to 64.00 ng/mL for N-desmethyl diltiazem and 0.15 to 40.69 ng/mL for deacetyl diltiazem, respectively in human plasma. This mixture was buffered with NaF solution (1%, 0.1 M) to prevent the degradation of diltiazem to its metabolite.

TABLE 1: TANDEM MASS SPECTROMETRY PARAMETERS FOR DTZ AND ITS METABOLITES

Compound Name	m/z	Dwell (sec)	Cone Voltage (V)	Collision Energy (eV)
Diltiazem	415.05/178.03	0.200	22	16
N-Desmethyl diltiazem (meta 1)	401.09/150.04	0.200	30	46
Desacetyl diltiazem (meta 2)	373.21/108.85	0.200	25	54
Diltiazem D4 (IS)	419.22/314.00	0.200	30	32

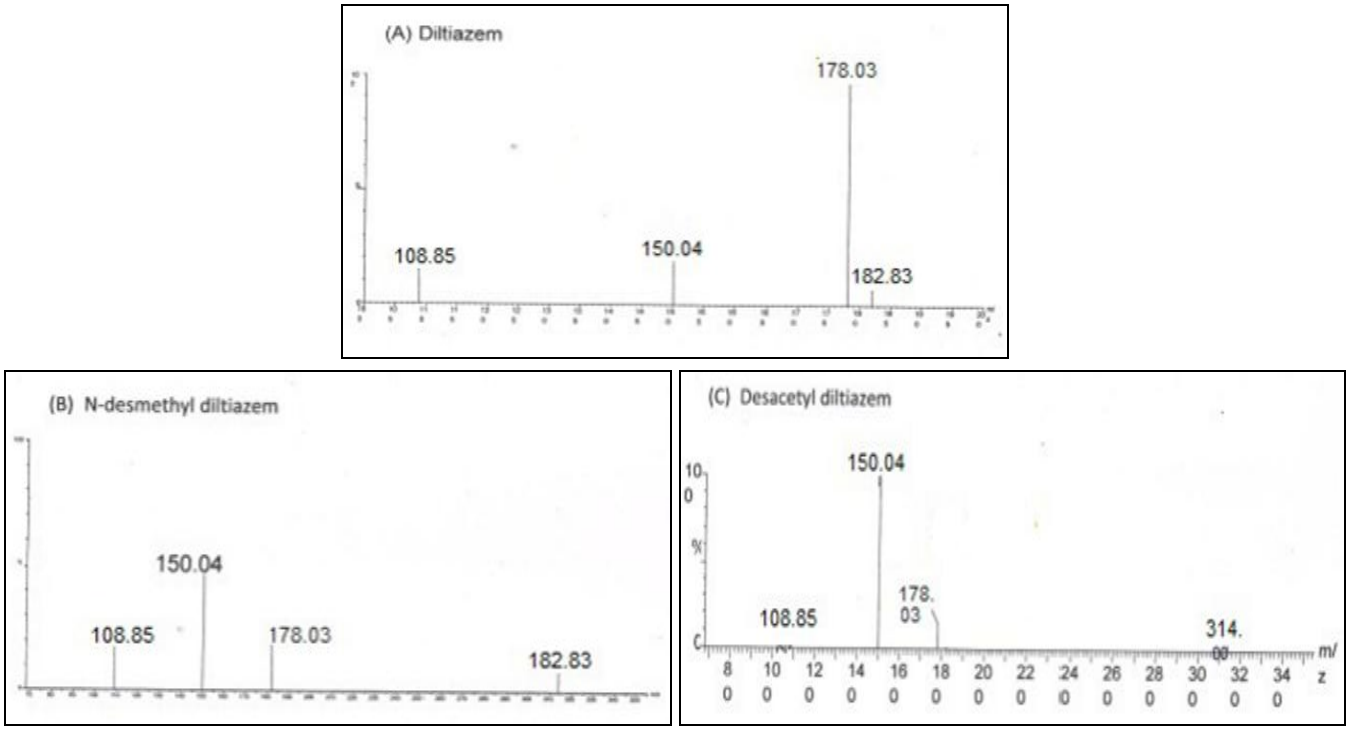

FIG. 2: MASS SPECTRA OF DILTIAZEM, N-DESMETHYL DILTIAZEM AND DESACETYL DILTIAZEM

Liquid-Liquid Extraction: The mixture of plasma sample (0.5 mL) and 50 µL of diltiazem D4 (IS) working solution (2000 ng/mL) was vortexed for 60 sec in a glass tube. After the addition of 2.5 ml, methyl *tert*-butyl ether to the above mixture was vortexed for 10 min and centrifuged (4000 rpm) for 5 min at 4 °C. The plasma layer was flash-freeze, and the organic layer was transferred to a pre-labeled tube. The sample was evaporated to dryness at 40 °C under a stream of nitrogen. The dried extract was reconstituted with 200 µL of the mobile phase. 10 µl of the reconstituted extract was injected into the chromatographic system & analyzed. Sample processing was performed in ice-cold water, maintaining temperature 2-8 °C throughout processing.

Method Validation: Working solutions (0.2 ml) were added to drug-free human plasma (9.8 ml) as a bulk, to obtain concentration levels of 0.93, 1.85, 12.76, 36.46, 121.55, 180.07, 225.09 and 250.10 ng/ml for diltiazem; 0.24, 0.47, 3.27, 9.33. 31.10, 46.08, 57.60 and 64.00 ng/ml for N-desmethyl diltiazem and 0.15, 030, 2.08, 5.93, 19.77, 29.30, 36.62 and 40.69 ng/ml desacetyl diltiazem as a single batch at each concentration. Quality control (QC) sample were also prepared at concentration of 0.94 (LLOQ), 2.78 (low), 117.55 (medium) and 200.08 ng/ml (high) for diltiazem; 0.25 (LLOQ), 0.71 (low), 30.08 (medium) and 51.20 ng/ml (high) for N-desmethyl diltiazem and 0.15 (LLOQ), 0.45 (low), 19.12 (medium) and 32.55 ng/ml (high) for desacetyl diltiazem. The spiked calibration and control bulk samples were stored in the freezer at below -65 °C until analyzed.

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero sample covering the total range 0.93-250.10 ng/ml for diltiazem; 0.24-64.00 ng/ml for N-desmethyl diltiazem and 0.15-40.69 ng/ml for desacetyl diltiazem including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratio by weighted ($1/X^2$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r^2) of 0.99 or better, and that each back-calculated standard concentration must be within ± 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at ± 20%.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LOQ QC, low, medium, and high concentration) each comprised of six replicates in a batch. Between-Batch precision and accuracy were determined by analyzing five different batches. The acceptance criteria for within-and between-batch

precision were ± 15% or better for all concentration, except at LOQ QC with acceptance of ± 20 %, and the accuracy was 100 ± 15% or better for all concentrations, except at LOQ QC with acceptance of 100 ± 20%. Recovery of analytes from the extraction procedure was determined by a comparison of the peak area of analytes in spiked plasma samples (six each of low, medium and high QCs) with the peak area of analytes in samples prepared by spiking extracted drug-free plasma samples with the same amounts of analytes at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak area of extracted QC samples (n = 6) to mean peak area of IS in samples prepared by spiking extracted drug-free plasma samples with the same amount of IS at the step immediately before chromatography.

The stability of the analytes and IS in human plasma under different temperature and timing condition, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term storage conditions (2-8 °C) to long-term storage conditions (2-8 °C) and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels 2.78 and 200.08 ng/mL for diltiazem, 0.71 and 51.20 ng/ml for N-desmethyl diltiazem and 0.45 and 32.55 ng/ml for desacetyl diltiazem, as low and high QC values with six replicates for each.

RESULTS AND DISCUSSION:

Method Development: Pharmacokinetic application requires highly selective assay method with high sample throughput capacity quantification of the drug in biological matrices. LC-MS/MS is one of the most common techniques with improved sensitivity and selectivity to achieve the desired LLOQ levels. The chromatographic conditions were optimized to achieve better resolution and symmetric peak shapes for the diltiazem and desacetyl diltiazem along with internal standard by using a mixture of ammonium formate (5 mM; pH 3.0)-methanol (10:90, v/v) as mobile phase. The diltiazem, N-desmethyl diltiazem, desacetyl diltiazem, and diltiazem D4 were eluted at 2.50, 2.48, 2.54, and 2.52 min, respectively at the flow rate of 0.6 ml/min in a run time of 4.25 min **Fig. 3**. Liquid-liquid extraction was used for the sample preparation, which was cost-effective.

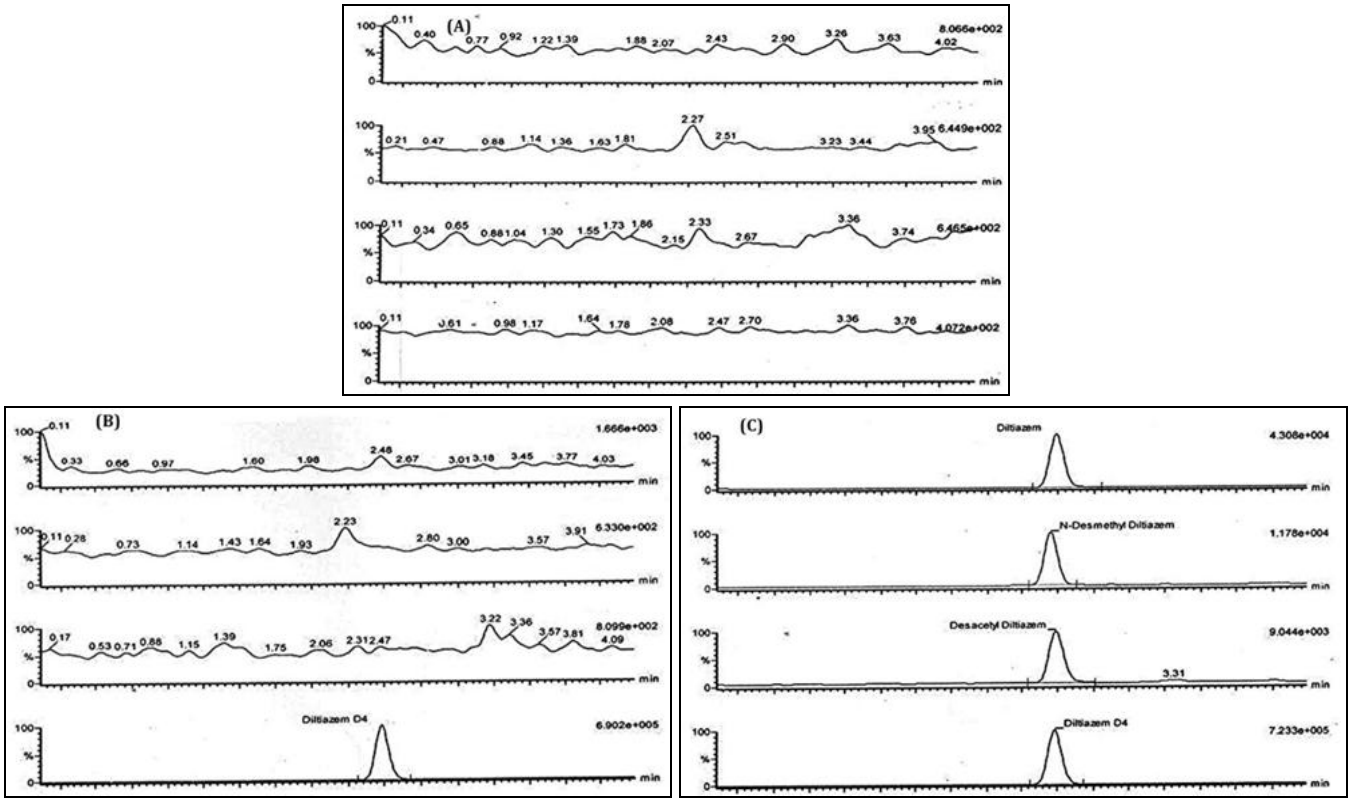

FIG. 3: CHROMATOGRAM OF BLANK PLASMA (A); PLASMA SPIKED WITH INTERNAL STANDARD (B); PLASMA SPIKED WITH DILTIAZEM, DESMETHYL DILTIAZEM, DESACETYL DILTIAZEM AND INTERNAL STANDARD (C)

Diltiazem is metabolized to desacetyl diltiazem in plasma, immediately, *i.e.,* it is unstable in normal conditions. To improve the stability of diltiazem, sodium fluoride (1-2 %), 0.1 M potassium fluoride (1-2%) and hydrochloric acid (0.1 N) were used upto 125 h at different temperature (+ 25°C, -20 °C and -70 °C). Sodium fluoride was optimized as sodium fluoride (0.1 M; 1%) at -70 °C stops the conversion of diltiazem into desacetyl diltiazem.

The aqueous solution of diltiazem converted into desacetyl diltiazem at room temperature due to hydrolysis. Different acid and basic solution (0.1N HCI, 1M NaOH) were used to prevent the hydrolysis of the drug and allowed to stand at (25 °C, -20 °C). 0.1 N HCl at -20 °C was optimized for preventing the hydrolysis. Hence, all aqueous solutions were also kept at -20 °C temperature. It was followed during sample separation of subject samples.

The average best recovery of diltiazem from spiked plasma samples at low, medium and high level is 82.9%, 75.4%, and 76.7% respectively, for N-desmethyl diltiazem at low, medium and high level is 76.6%, 69.4%, and 71.9% respectively and for desacetyl diltiazem at low, medium and high level is 72.9% 66.9% and 92.4% respectively and diltiazem-d4 is 80.5% of the concentration used in the method. Hence, the method is consistent, precise, reproducible, and robust in high throughput bioanalysis **Table 2**.

TABLE 2: RECOVERY OF LQC, MQC AND HQC SAMPLES

QC Level	Diltiazem (%)	N-desmethyl diltiazem (%)	Desacetyl diltiazem (%)
LQC	82.9	76.6	72.9
MQC	75.4	69.4	66.9
HQC	76.7	71.9	92.4

The retention behavior of IS was similar to that of the target analyte, and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. The importance of matrix effect in any LC-MS/MS method has been reported by Matuszewski and co-workers [26]. The data strongly emphasizes the need to use a blank matrix from (at least six) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this method were performed with matrices obtained from different individuals. Also, validation experiments were performed using hemolytic and strongly lipemic matrices. As all data falls within the guidelines, it was concluded that the degree of matrix effect was sufficiently low to produce acceptable data and the method can be considered as valid.

The eight-point calibration curve was linear over the concentration range 0.93-250.10 ng/ml for diltiazem, 0.24-64.00 ng/ml for N-desmethyl diltiazem and 0.15-40.69 ng/ml for desacetyl diltiazem. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/X^2$ weighing factor, giving a mean linear regression equation for the calibration curve.

Selectivity: The selectivity of the method was examined by analyzing (n=6) blank human plasma extract **Fig. 3A** and an extract spiked only with the IS. As shown in **Fig. 3B**, **3C** no significant direct interference in the blank plasma traces was observed from an endogenous substance in drug-free human plasma at the retention time of the diltiazem, N-desmethyl diltiazem and desacetyl diltiazem, similarly, **Fig. 3B** shows the absence of direct interference from the IS the MRM channel of the analyte. **Fig. 3C** depicts a representative ion chromatogram for the LLOQ (0.93 ng/ml) for diltiazem and (0.24 ng/ml) for N-desmethyl diltiazem and (0.15 ng/ml) for desacetyl diltiazem. Excellent sensitivity was observed for a 10-µl-injection volume; the LLOQ corresponds to on-column.

The between-batch coefficient of variance (CV) and within batch at all QC level for diltiazem, N desmethyl diltiazem and desacetyl diltiazem are presented in **Table 3**, **4** and **5** respectively. The CV and accuracy for the analytes met the acceptance criteria (<15%).

TABLE 3: PRECISION AND ACCURACY OF METHOD IN DETERMINING DILTIAZEM IN HUMAN PLASMA

Concentration Added (ng/ml)	Within-batch (n=5)			Between-batch (n=5)		
	Concentration Found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)	Concentration found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)
0.94	0.92 ± 0.037	4.0	97.87	0.92 ± 0.040	4.4	97.87
2.78	2.87 ± 0.083	2.9	103.24	2.86 ± 0.092	3.2	102.88
117.55	117.14 ± 1.844	1.6	99.65	115.60 ± 3.369	2.9	98.34
200.08	201.80 ± 5.932	2.9	100.86	200.70 ± 10.084	5.0	100.31

TABLE 4: PRECISION AND ACCURACY OF METHOD IN DETERMINING N-DESMETHYL DILTIAZEM IN HUMAN PLASMA

Concentration Added (ng/ml)	Within-batch (n=5)			Between-batch (n=5)		
	Concentration Found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)	Concentration found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)
0.25	0.22 ± 0.017	7.9	88.00	0.22 ± 0.026	12.1	88.00
0.71	0.68 ± 0.024	3.5	95.77	0.68 ± 0.030	4.4	95.77
30.08	29.72 ± 0.771	2.6	98.80	29.54 ± 1.470	5.0	98.20
51.20	50.71 ± 1.033	2.0	99.04	51.43 ± 1.581	3.1	100.45

TABLE 5: PRECISION AND ACCURACY OF METHOD IN DETERMINING DESACETYL DILTIAZEM IN HUMAN PLASMA

Concentration Added (ng/ml)	Within-batch (n=5)			Between-batch (n=5)		
	Concentration Found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)	Concentration found (Mean ± SD) (ng/ml)	Precision (%)	Accuracy (%)
0.15	0.17 ± 0.013	7.8	113.33	0.17 ± 0.016	9.6	113.33
0.45	0.50 ± 0.044	9.0	111.11	0.48 ± 0.027	5.7	106.67
19.12	19.98 ± 0.558	2.8	104.50	19.85 ± 0.914	4.6	103.82
32.55	32.57 ± 0.793	2.4	100.06	32.36 ± 1.008	3.1	99.42

Stability Studies: For short-term stability determination stored plasma aliquots were thawed and kept in an ice-cold water bath (2-8 °C) for a period exceeding that expected to be encountered during routine sample preparation (around 10 h). The sample was extracted and analyzed as described above, and these results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the auto-sampler for 140 h, analytes in plasma over five freezes/thaw cycles, analytes in plasma over dry extract, and analytes in plasma on the bench for 5 h in the cold water bath was also assessed **Table 6**. The results indicate that solutions of diltiazem, N-desmethyl diltiazem, desacetyl diltiazem and the IS can remain in the autosampler for at least 140 h without showing significant loss in quantified values indicating that sample should be analyzed within this period time. The analytes are stable in human plasma for five freezes/thaw cycles when stored at below -70 °C and thawed in a water bath maintained at a temperature between 2-8 °C; for 120 h as a dry extract; and were also stable in human plasma.

TABLE 6: STABILITY OF DILTIAZEM AND ITS METABOLITES IN HUMAN PLASMA

Condition	Diltiazem concentration	% Change	N-Desmethyl diltiazem concentration	% Change	Desacetyl diltiazem concentration	% Change
Autosampler stability for 140 h	2.78	-2.2	0.71	1.4	0.45	6.7
	200.08	-2.6	51.20	-3.3	32.55	2.7
Five freeze and thaw cycle	2.78	-2.5	0.71	-2.8	0.45	-4.4
	200.08	-1.6	51.20	-3.1	32.55	0.5
Dry extract stability for 120 h	2.78	1.4	0.71	1.4	0.45	2.2
	200.08	-3.1	51.20	-5.6	32.55	-7.4
Bench top stability for 5 h	2.78	-1.1	0.71	2.8	0.45	-2.2
	200.08	-1.2	51.20	-4.5	32.55	-3.7

These findings indicate that storage of the analytes in plasma samples at below -70 °C is adequate, and no stability-related problem would be expected during routine analyses for pharmacokinetic, bioavailability, or bioequivalence studies. The stability of the stock solutions was tested and established at room temperature for 10 h and under refrigeration (2 to 8 °C) for 14 days. The results

revealed optimum stability for the prepared stock solutions throughout the periods intended for their daily use. Extensive sets of experiments up to 125 h were performed to select the best buffering solution for preventing the degradation of diltiazem to desacetyl diltiazem. It was clearly evident that diltiazem converted to desacetyl diltiazem at room temperature but we buffered plasma with 0.1% HCl (100 µl & 200 µl), 1% & 2% 0.1 M KF, 1% & 2% 0.1 M NaF at various conditions of stability like room temperature, –20 °C & -70 °C for up to 125 h, to optimize the degradation. As the peak area of diltiazem was much higher than the peak area of desacetyl diltiazem, for better comparison in a bar graph, the Y-axis was taken as multiple of 10 **Fig. 4-7**.

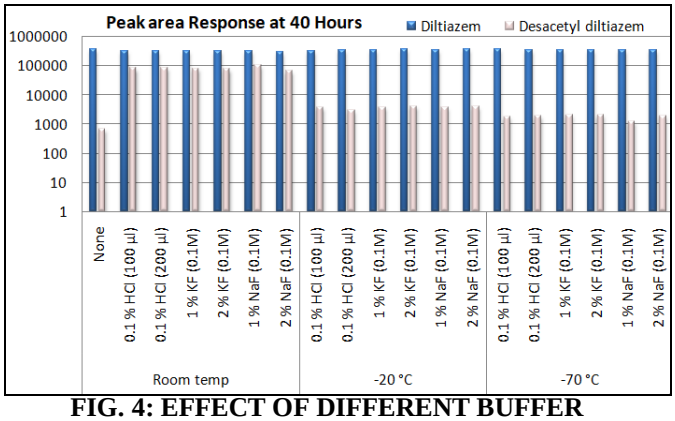

FIG. 4: EFFECT OF DIFFERENT BUFFER SOLUTIONS AND TEMPERATURE ON THE CONVERSION OF DILTIAZEM TO DESACETYL DILTIAZEM AT 40 h

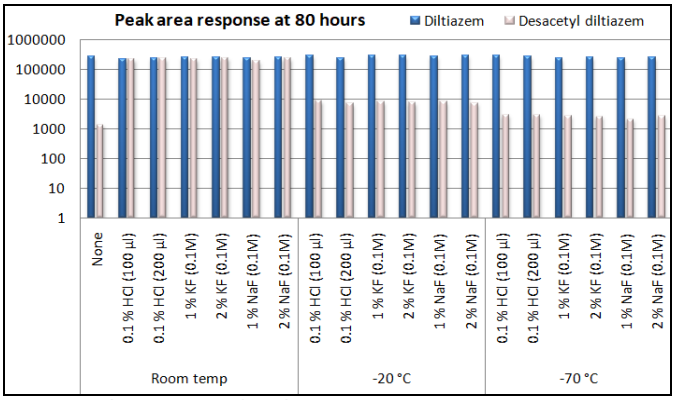

FIG. 5: EFFECT OF DIFFERENT BUFFER SOLUTIONS AND TEMPERATURE ON THE CONVERSION OF DILTIAZEM TO DESACETYL DILTIAZEM AT 80 h

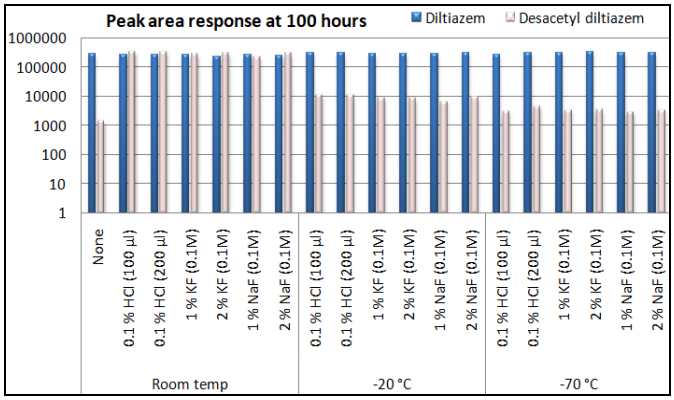

FIG. 6: EFFECT OF DIFFERENT BUFFER SOLUTIONS AND TEMPERATURE ON THE CONVERSION OF DILTIAZEM TO DESACETYL DILTIAZEM AT 100 h

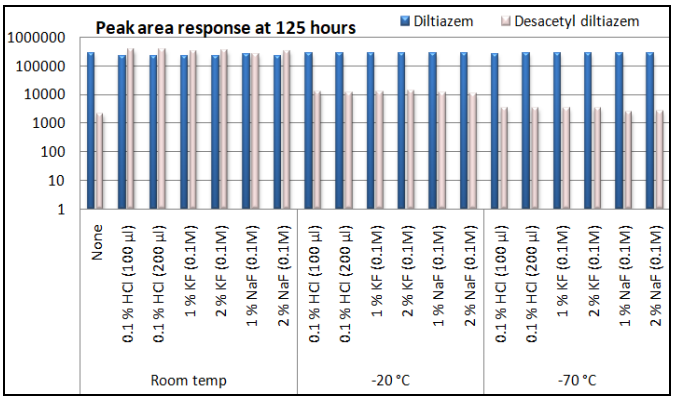

FIG. 7: EFFECT OF DIFFERENT BUFFER SOLUTIONS AND TEMPERATURE ON THE CONVERSION OF DILTIAZEM TO DESACETYL DILTIAZEM AT 125 h

The "none" bar present in room temperature section was immediate sample without any addition of buffer, which was used for the comparison at the respective time of analysis. At room temperature, the action of buffer was negligible on inhibition of the conversion of diltiazem to desacetyl diltiazem, while at lower temperatures (-20 °C and -70 °C) the buffer action was observed. At different time intervals (40, 80. 100 and 125 h), the maximum inhibition of conversion was observed at -70 °C.

It was concluded that spiking of 1% solution of 0.1 M sodium fluoride buffer was the best inhibitor for the conversion of diltiazem to desacetyl diltiazem at -70 °C temperature among all studied buffers.

CONCLUSION: The metabolic conversion of diltiazem into desacetyl diltiazem and its inhibition by different buffer solutions were studied in human plasma by LC/MS/MS in positive ESI mode using multiple reaction monitoring. The method was acceptable at adequate sensitivity for the quantification of diltiazem, N-desmethyl diltiazem and desacetyl diltiazem in human plasma for its pharmacokinetic application. The sensitivity of the method for diltiazem was achieved with an LLOQ

of 0.93 ng/ml, for N-desmethyl diltiazem 0.24 ng/ml and desacetyl diltiazem 0.15 ng/ml with a coefficient of variance of 4.4%, 12.1%, and 9.6% respectively.

The liquid-liquid extraction was used for sample preparation for high-throughput bioanalysis of diltiazem, N-desmethyl diltiazem, and desacetyl diltiazem. The validated method was applied for quantification of diltiazem, N-desmethyl diltiazem, and desacetyl diltiazem range of 0.93-250.10 ng/ml range 0.24-64.00 ng/ml and range 0.15-40.69 ng/ml, respectively. The stability of diltiazem in plasma solutions could be maintained over up to 125 h by buffering with 1% of 0.1M NaF solution, thereby making the method very viable for routine analysis and bioequivalence studies.

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CONFLICTS OF INTEREST: None of the conflict of interest.

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