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HPLC method development for the simultaneous determination and validation of Diltiazem hydrochloride and its major metabolite desacetyl Diltiazem hydrochloride

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ABSTRACT

A stability-indicating liquid chromatographic method has been developed and validated for the determination of Diltiazem Hydrochloride and its major metabolite desacetyl Diltiazem Hydrochloride. The concentration range was 25% to 250% and the flow rate was 1.0 mL/min. The mobile phase was 650:350 v:v acetate buffer and acetonitrile, respectively. The method was proved to be linear for the determination of Diltiazem Hydrochloride and the metabolite in the range of 25% to 250% of the specified limit i.e. 0.5%; the correlation coefficient met the acceptance criteria (≥ 0.998). The smaller point in the calibration curve was optimal for low concentration samples. The limit of detection and quantification were found to be 0.0633 and 0.450 µg/mL, respectively for desacetyl Diltiazem hydrochloride and 0.0408 µg/mL and 0.2668 µg/mL, respectively for Diltiazem hydrochloride. The degradation reaction of desacetyl Diltiazem hydrochloride by stress conditions was found to be faster and more intensive than Diltiazem hydrochloride.

Keywords: Diltiazem Hydrochloride, Desacetyl Diltiazem hydrochloride, degradation, validation, stability indicating, LOD, LOQ.

INTRODUCTION

Diltiazem Hydrochloride is a member of calcium channel blockers drugs [1,2]. It works by relaxing the muscles of heart and blood vessels [3]. Diltiazem is used to treat hypertension, angina, and certain heart rhythm disorders [4]. Diltiazem hydrochloride (Fig. 1, A) is used to maintain the blood flow to the heart and reduce the frequency and severity of angina attacks [5]. Studies indicate that Diltiazem is metabolized into its major metabolites such as desacetyl Diltiazem (Fig. 1, B) in plasma. Desacetyl Diltiazem weakly inhibits nucleoside triphosphate diphosphohydrolases, which is believed to follow a mechanism that reduces the concentration of calcium produced by the enzymes catalytic site [6]. Studies using gas chromatography proved that Desacetyl Diltiazem can be separated from its parent molecule, Diltiazem, using a nitrogen detector, which used for the determination of each molecule in plasma [7].

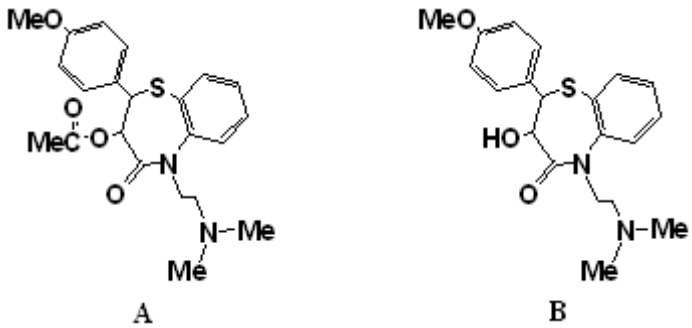


Figure 1: The structure of A) Diltiazem Hydrochloride and B) Desacetyl Diltiazem Hydrochloride

A liquid chromatographic method was developed for stability-indicating of Diltiazem Hydrochloride together with its six related substances (Diltiazem sulphoxide, Imp-A, Imp-B, Imp-D, Imp-E, and Imp-F) in a laboratory mixture as well as in tablet formulation developed in-house [8]. Several experiments were tested to choose a suitable stationary phase to analyze Diltiazem Hydrochloride. Among the tested stationary phases were the RP-8 and monolithic RP-18. Both showed a good selectivity, but the monolithic RP-18 column was much more sensitive and faster [9].

A sensitive and specific reversed-phase high-performance liquid chromatographic method was developed for the determination of diltiazem and three of its main metabolites in human plasma. A solid-phase extraction method (C18) was described for isolating diltiazem and its metabolites [10]. FT-Raman spectroscopy was used to quantitatively analyze diltiazem hydrochloride in commercially available tablets [11]. An HPLC assay was developed for the quantification of diltiazem in small-volume blood specimens and applied to pharmacokinetic studies in rats [12]. Number of binding sites and binding constant between diltiazem hydrochloride and human serum albumin were determined by ultrasonic microdialysis coupled with online capillary electrophoresis electrochemiluminescence [13].

Diltiazem hydrochloride was also determined in pharmaceutical formulations by spectrophotometric methods using bromothymol blue, bromophenol blue and bromocresol green [14]. A similar work has been performed by Abu-Nameh to study the stability indicating method of diltiazem hydrochloride [15], but the concentration range was (70%-130%) which is much narrower than those in the present study (25%-250%). In the present work, the development and validation was applied on Diltiazem Hydrochloride and extended to measure the degree of appearance of Desacetyl Diltiazem hydrochloride (the major metabolite) as degradation product (Figure 1B). The concentration range was expanded over a range of 25%-250%. The flow rate was 1.0 mL/min. The smaller point in the calibration curve is optimal for low concentration samples.

MATERIALS AND METHODS

2. Experimental Part:

2.1 Mobile phase preparation:

Sodium acetate buffer (0.1 M, 8.2 g in 1 liter of distilled water) was prepared. Camphorsulfonic acid (0.5 g) was added with stirring. 650 mL of the sonicated solution was mixed with 350 mL of acetonitrile. The pH of resulting mixture was adjusted to 6.3 by 0.1 M HCl then filtered through a 0.45 µm Whatman filter paper.

2.2 HPLC system

A spectraphysics HPLC system type SP8700 with an ultraviolet detector was used. The wavelength was set at 240 nm. The separation was performed by using a Waters associated microbonapack C18 column, 5µm (4.6 x250mm) at a flow rate of 1.0 mL/min.

2.3 Diltiazem hydrochloride Stock solution:

Accurately weigh an amount of 50.0 ±0.1 mg of Diltiazem hydrochloride powder and transfer it into a 50 mL volumetric flask. Add a rough amount of acetonitrile, and sonicate for 15 minutes until a clear solution is obtained then dilute to volume with acetonitrile.

2.4 Diltiazem hydrochloride calibration standards:

Using a micropipette, pipette accurately 15, 30, 60, 90, 120 and 150 µL of the stock solution into separate six volumetric flasks (10 mL). Fill the volumetric flasks to the mark by the mobile phase. This corresponds with 1.50, 3.0, 6, 9, 12 and 15 µg/mL respectively and 25%-250% concentration range for Diltiazem hydrochloride.

2.5 Desacetyl Diltiazem hydrochloride stock solution:

Accurately weigh an amount of 20.0 ±0.1 mg of Diltiazem hydrochloride powder and transfer it into a 200 mL volumetric flask. Add a rough amount of acetonitrile, and sonicate for 15 minutes until a clear solution is obtained then dilute to volume with acetonitrile.

2.6 Desacetyl Diltiazem hydrochloride calibration standards:

Using a micropipette, pipette accurately 150, 300, 600, 900, 1200 and 1500 µL of the stock solution into separate six volumetric flasks (10 mL each). Fill the volumetric flasks to the mark by the mobile phase. This corresponds with 1.50, 3.0, 6, 9, 12 and 15 µg/mL respectively and 25%-250% concentration range for desacetyl Diltiazem hydrochloride.

2.7 Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride samples:
Ten tablets of the purchased material were weighed and finely powdered. Tablet powder equivalent to 200 mg Diltiazem hydrochloride was accurately weighed and transferred into a 100 mL volumetric flask. Acetonitrile (50 mL) was added and the flask was sonicated for 15 min. An amount equivalent to 200 mg of desacetyl Diltiazem hydrochloride was then spiked in the solution. The flask was shaken, and the volume was made up to the mark with the mobile phase. The above solution was then filtered through 0.45 µm Whatman filter paper to get rid of exepients.

2.8 General procedure:
Separately into the chromatographic system, inject equal volumes of 20 µL of standard preparation, assay preparation, test solution, 0.5% solution, for Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride standards. Treatment of the resulted chromatograms includes area calculation, retention times, resolution and theoretical plates.

2.9 Acidic degradation
Use the analytical balance to accurately weigh powder equivalent to 20 mg of Diltiazem hydrochloride (standard and sample) and desacetyl Diltiazem hydrochloride (standard and sample) into four separate 10 mL volumetric flasks. To each solution, 5 mL of 1N HCl was added and the resulting solution was kept at 60^0C for three hours in water bath. Allow the solution to attend ambient temperature, neutralized with 1N NaOH solution to pH 7 and the volume was made up to the mark with the mobile phase. Further dilution to this solution was performed to achieve a concentration of 15 µg/mL of Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride standard.

2.10 Alkali degradation
Use the analytical balance to accurately weigh powder equivalent to 20 mg of Diltiazem hydrochloride (standard and sample) and desacetyl Diltiazem hydrochloride (standard and sample) into four separate 10 mL volumetric flasks. To each solution, 5 mL of 1N NaOH was added and the resulting solution was kept at 60^0C for three hours in water bath. Allow the solution to attend ambient temperature, neutralized with 1N HCl solution to pH 7 and the volume was made up to the mark with the mobile phase. Further dilution to this solution was performed to achieve a concentration of 15 µg/mL of Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride standard.

2.11 Oxidative degradation
Use the analytical balance to accurately weigh powder equivalent to 20 mg of Diltiazem hydrochloride (standard and sample) and desacetyl Diltiazem hydrochloride (standard and sample) into four different 10 mL volumetric flasks. To each flask, 5 mL of 30% hydrogen peroxide was added and the resulting mixture was kept at 60^0C for one hour in water bath. Allow the solution to attend ambient temperature and the volume was made up to the mark with the mobile phase. Further dilution to this solution was performed to achieve a concentration of 15 µg/mL of Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride standard.

2.12 Thermal degradation
Use the analytical balance to accurately weigh powder equivalent to 20 mg of Diltiazem hydrochloride (standard and sample) and desacetyl Diltiazem hydrochloride (standard and sample) into four petri dishes. The solid materials were kept at 90 ^{0}C for 24 hrs. Then a solution from each powder was prepared to achieve 15 µg/mL solution concentration of Diltiazem hydrochloride and desacetyl Diltiazem hydrochloride.

2.13 Hydrolysis degradation
20 mg of Diltiazem hydrochloride (standard and sample) and desacetyl Diltiazem hydrochloride (standard and sample) were accurately weighed and placed in four separate 10 mL volumetric flasks. To each solution, 5 mL of water was added and the resulting solution was kept at 60^0C for 24 hours in water bath. Allow the solution to attend ambient temperature. Further dilution to this solution was performed to achieve a concentration of 15 µg/mL of Diltiazem hydrochloride and Desacetyl Diltiazem hydrochloride standard solutions.

RESULTS AND DISCUSSION

3.1 Optimization of the chromatographic conditions
To develop a method, different column stationary phases like C18, C8 and CN are tested. Different mobile phases containing HPLC grade solvents such as acetonitrile and methanol are tested. Also, different buffer solutions like phosphate, acetate are used.

Our objective of the chromatographic method development was to separate Diltiazem hydrochloride peak from other degradation products especially the major metabolite desacetyl Diltiazem hydrochloride. The chromatographic

separation was achieved using a Waters associated microbonapack C18 column, 5µm (4.6 x250mm) at a flow rate of 1.0 ml/min.
After changing the composition of the mobile phase to obtain good system suitability parameters, a mixture of acetate buffer (0.1 M) and acetonitrile with a ratio of (650:350, v:v) with pH 6.3 was optimum. The system suitability tests showed that the retention times were 15.7 and 26.4 min for desacetyl Diltiazem hydrochloride and Diltiazem hydrochloride respectively. The theoretical plate counts were 3675 and 6733 for desacetyl Diltiazem hydrochloride and Diltiazem hydrochloride respectively.

3.2 Linearity

Graphs were plotted between the theoretical concentrations and the corrected peak areas of standard solutions. Slope, y-intercept, and correlation coefficient (R) were calculated. Results are shown in table 1 for Diltiazem hydrochloride standard and table 2 for desacetyl Diltiazem hydrochloride.

The obtained equation for the standard curve of Diltiazem hydrochloride standard was Y= 21411.75X – 6982.34 with a correlation coefficient R of 0.9994. The calibration plot is shown in figure 2. The results are found to be within the acceptance criteria. Therefore, the method is linear for Diltiazem hydrochloride between 25% and 250% concentration range.

Table 1: Standard curve for Diltiazem hydrochloride

Percentage from Nominal	Conc. From Nominal (µg/mL)	Actual Conc. (µg/mL)	Average Area	R.S. D	Corrected AUC	Average corrected AUC
		1.4946	27158.67	1.23	27256.76	
25	1.50	1.4931	27961.67	1.17	28090.76	27771.54
		1.4945	27863.67	5.23	27967.10	
		2.9892	57078.67	0.89	57284.82	
50	3.00	2.9862	57549.67	7.20	57815.36	58023.54
		2.9889	58752.33	8.90	58970.43	
		5.9784	121598.00	1.44	122037.18	
100	6.00	5.9724	120590.33	2.05	121147.07	120525.15
		5.9778	117953.33	0.98	118391.19	
		8.9676	182262.00	1.80	182920.28	
150	9.00	8.9586	180109.67	1.19	180941.19	181749.32
		8.9667	180715.67	1.37	181386.51	
		11.9568	244824.00	0.74	245708.24	
200	12.00	11.9449	244123.67	1.34	245250.72	245850.43
		11.9556	245680.33	1.95	246592.33	
		14.9460	308967.33	2.13	310083.24	
250	15.00	14.9311	310516.67	1.48	311950.24	319832.49
		14.9445	337464.00	1.84	337464.00	

Correlation Coefficier	Slope = 21
Intercept = -6982.34	SD of Inter

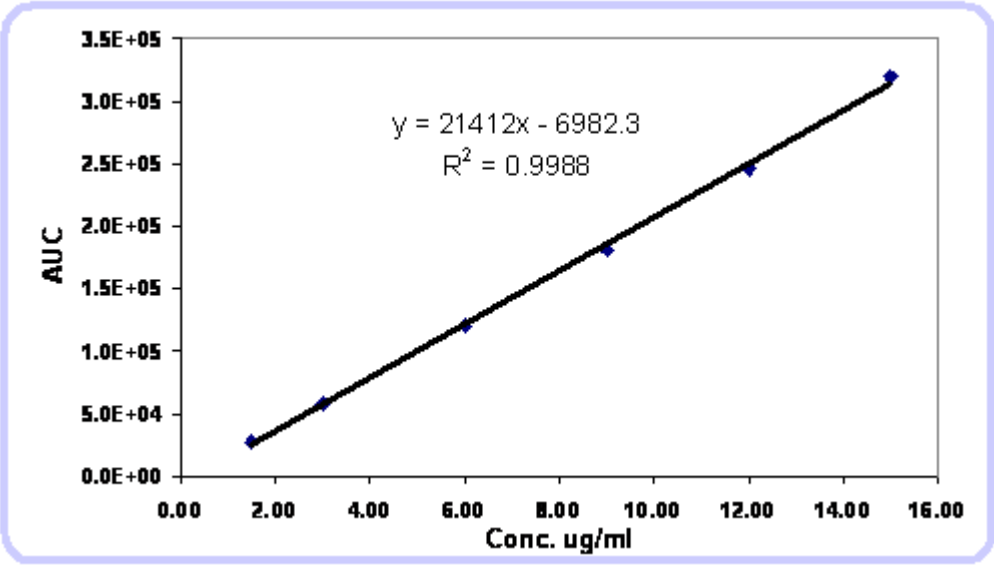


Figure 2: Standard curve (concentration vs area under the curve) for Diltiazem hydrochloride in the range 1.50-15.0 µg/mL

The obtained equation for the standard curve of desacetyl Diltiazem hydrochloride standard was: Y= 24078.80 – 3710.47 with a correlation coefficient R of 0.9998. The calibration plot is shown in Figure 3.

The results are found to be within the acceptance criteria. Therefore, the method is linear for desacetyl Diltiazem hydrochloride between 25% and 250% concentration range.

Table 2: Standard curve for desacetyl Diltiazem hydrochloride

Percentage From Nominal	Conc. From Nominal (µg/mL)	Actual Conc. (µg/mL)	Average Area	R.S.D	Corrected AUC
25	1.50	1.62	36075.67	3.23	33403.40
50	3.00	3.24	73388.00	0.38	67951.85
100	6.00	6.48	150692.3	1.75	139529.94
150	9.00	9.72	232986.0	0.48	215727.78
200	12.00	12.96	303793.00	0.87	281289.81
250	15.00	16.20	388258.33	1.84	359498.46

Correlation Coefficie	Slope = 24(
Intercept = -3710.47	SD of Inter

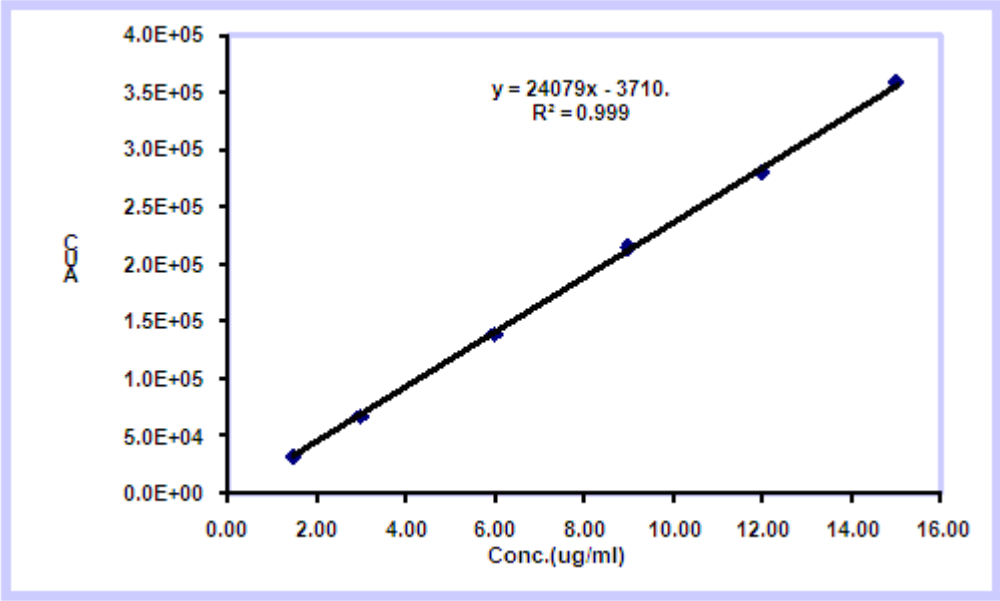


Figure 3: Standard curve (concentration vs area under the curve) for desacetyl Diltiazem hydrochloride in the range 1.50-15.0 µg/mL.

3.3 Stability indicating study

The stability of the drug and its major metabolite was carried out according to the ICH guideline. The forced degradation study was performed at acidic, basic, oxidative stress, hydrolysis and the stability against direct heat. The results are illustrated in table 3. Selected chromatograms are shown in figure 4.

Table 3: Stress testing and stability investigation studies for Diltiazem hydrochloride and desacetyl diltiazem hydrochloride

Test	Recovery% for Diltiazem hydrochloride	Recovery% for Desacetyl Diltiazem hydrochloride
heat	84.67%	69.92%
hydrolysis	99.27%	89.15%
acidic	86.81%	61.13%
Basic stress	45.94%	35.97%
Oxidative stress	91.14%	51.33%

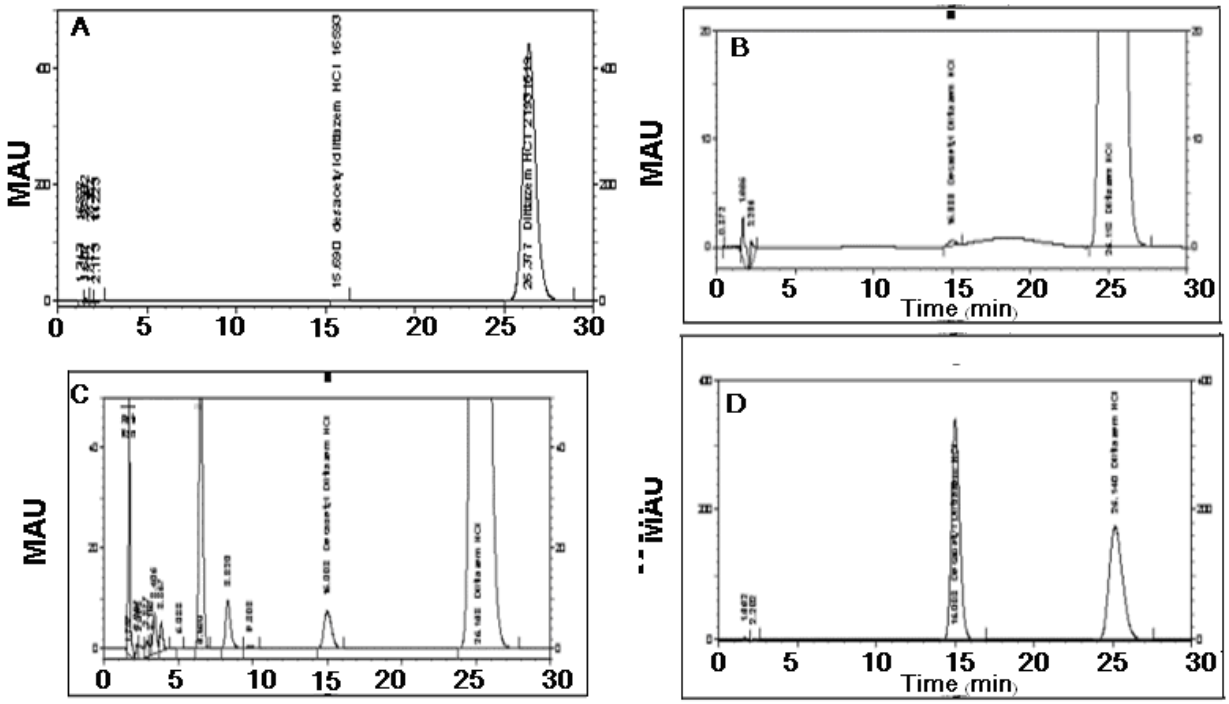
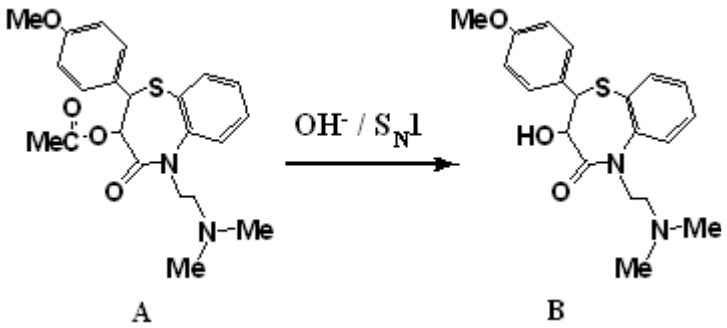


Figure 4: Stability studies Chromatograms for Diltiazem hydrochloride A) standard B) hydrolysis stress, C) oxidative stress, D) basic stress

It is found that the trend of increasing degradation reaction of Diltiazem hydrochloride by stress conditions was in the following order: hydrolysis, oxidation, acidic, heat and basic. This can be clearly observed by the area decrease of Diltiazem hydrochloride and the area increase of Desacetyl Diltiazem hydrochloride, see scheme 1 for basic degradation. The degradation process of desacetyl Diltiazem hydrochloride by stress conditions is faster and more intensive than Diltiazem hydrochloride [16], See table 3.



Scheme1: Mechanism of conversion of Diltiazem hydrochloride (A) to Desacetyl Diltiazem hydrochloride (B).

The overall degradation of desacetyl Diltiazem Hydrochloride was a result of two competitive processes, epimerization and decomposition of the benzothiazipin ring. These two processes depend on the pH of the solution, concentration of analyte and the type of solvent [16].

3.4 Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of detection (LOD) and limit of quantification (LOQ) are two essential characteristics in method validation. LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably observed and measured by an analytical procedure [17-19] . In this work, low concentration standards of desacetyl Diltiazem hydrochloride (0.02%, 0.03%, 0.07% and 0.14%) were prepared. A straight line (Figure 5) was plotted between concentration and average MAU. The following equation was resulted Y= 1.8986X - 0.056.

Given that the Absorbance of LOD estimated from the peak is equal to 3 x mAU of the noise and the Absorbance of LOQ estimated from the peak is equal to 10 x mAU of the noise, the Limit of detection for desacetyl Diltiazem hydrochloride was calculated and found to be 0.0408 µg/mL and the limit of quantitation for desacetyl Diltiazem hydrochloride was calculated and found to be 0.2668 µg/mL. For Diltiazem hydrochloride, the limit of detection and quantification were found to be 0.0633 and 0.450 µg/mL, respectively.

Table 4: Spiked standard solutions of desacetyl Diltiazem hydrochloride prepared to calculate LOD and LOQ

%	Desacetyl Diltiazem. HCl Conc. µg/ml	inj 1 (mAU)	inj 2(mAU)	Average (mAU)
0.14	1.62000	0.8831	0.8706	0.8769
0.07	0.81000	0.4697	0.4658	0.4678
0.03	0.40500	0.2485	0.2450	0.2468
0.02	0.20250	0.1260	0.1270	0.1265

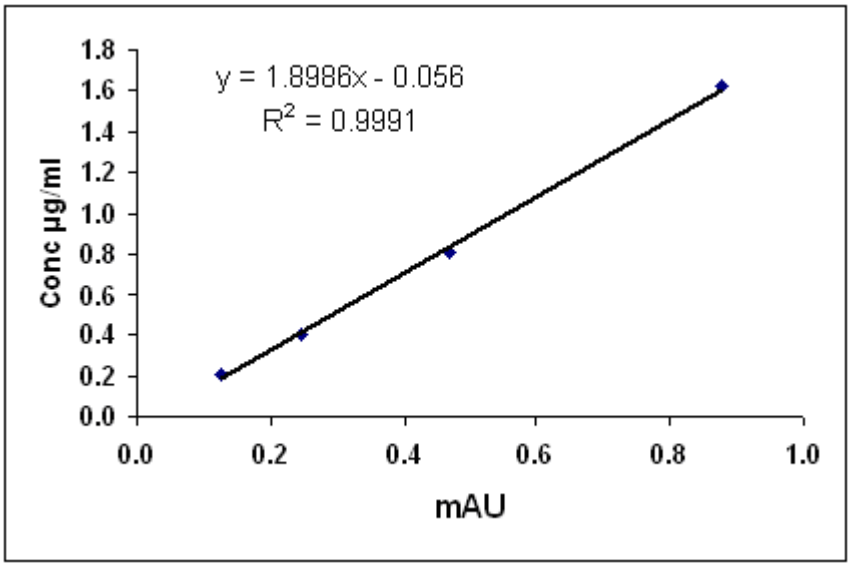


Figure 5: Desacetyl Diltiazem hydrochloride concentrations (µg/ml) versus mAU. A trial performed to calculate LOD and LOQ

CONCLUSION

An HPLC method was developed for the study of stress degradation of Diltiazem hydrochloride (the drug) and Desacetyl Diltiazem hydrochloride (the major metabolite) in pharmaceutical preparations. The method was precise, accurate and linear in the range of 25%-250% for both the drug and its major metabolite. The method was fully validated showing a satisfactory data for all method validation parameter tests. The developed method was found to meet the stability indicating conditions and can be conveniently used by quality control department to determine the assay of pharmaceutical preparations of Diltiazem hydrochloride (the drug) and Desacetyl Diltiazem hydrochloride (the major metabolite).

Acknowledgments
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