Asian Journal of Chemistry Vol. 21, No. 7 (2009), 5048-5054

High-Performance Liquid Chromatography Method for Determination of Diltiazem in Human Plasma

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A simple rapid, selective sensitive and novel high pressure liquid chromatographic method was developed and validated for the assay of diltiazem. Sample preparation was performed by liquid-liquid extraction in *t*-butyl methyl ether. The organic solvent was evaporated and the residue dissolved in a mobile phase. Aliquots of 20 µL were injected automatically into the chromatograph. The separation of diltiazem was achieved on a reversed phase (C18) column with a mobile phase consisting of 10 mM ammonium acetate:acetonitrile (55:45 v/v). Quantitation of diltiazem was performed by UV detection at 239 nm. The linearity range was 20 to 640 ng/mL. Inter-day and intra-day accuracy and precision was found 90.44-109.34, 3.74-3.80 and 99.89-103.71, 7.00-10.15, respectively. Long term stability for 30 days of diltiazem in human plasma was proved as per guidelines.

Key Words: High performance liquid chromatography, Diltiazem.

INTRODUCTION

Diltiazem is a benzothiazepine calcium channel blocker with peripheral and coronary vasodilator properties. It lowers the blood pressure and has some effect on cardiac conduction. It is administered orally in the treatment of angina pectoris and hypertension and may be given intravenously in the treatment of atrial fibrillation or flutter and paroxysmal supraventricular tachycardia[1].

Several investigation have been performed on the determination of diltiazem in pharmaceutical preparations by high performance liquid chromatography[2,3]. High performance liquid chromatography methods for determination of diltiazem in human plasma or serum have also been reported. Investigators[4,5] have proposed automated high performance liquid chromatography analysis of diltiazem in human plasma using a column-switching technique for online clean-up. These methods have a very low limit of detection and their only disadvantage is the high reagent consumption and the need for an HPLC system with two pump.

Hubert *et al.*[6] proposed an automated method for the determination of diltiazem in human plasma using liquid-solid extraction on disposable cartridge coupled to HPLC. Some investigators have performed the separation of diltiazem from plasma

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with solid-phase extraction on C_{18} cartridges[7,8]. These methods overcome problems caused by endogenous compounds, but extraction on this type of cartridge gave relatively low recoveries (75-80 %).

Ascalone *et al.*[8] and Lena Kristoffersen[9] reported the methods for determination of diltiazem and its main metabolites in human plasma by automated solid-phase extraction and high-performance liquid chromatography. The methods was developed on the basics of solid phase extraction, which is more costly than liquid-liquid extraction. Ke Li *et al.*[10] reported HPLC determination of diltiazem in human plasma and its application to pharmacokinetics in humans. In this method run time and linearity range was not reported.

The purpose of this work is to develop and validate a method for determination of diltiazem in human plasma. In this paper, a new method for determination of diltiazem in plasma samples is proposed. A simple, economical, sensitive and easy to operate with high through put HPLC method using liquid-liquid extraction with ethyl acetate for the determination of diltiazem in human plasma. Validation studies for the diltiazem were performed with respect to recovery, selectivity, linearity, precision and accuracy. The procedure has been used for the analysis of plasma samples of patients taking diltiazem.

EXPERIMENTAL

Diltiazem provided by Golden Cross Pharma, Daman. They also supply the COA of that drug. All reagents were of analytical grade. Ammonium acetate (Rankem), *tert*-butyl methyl ether (Rankem), hydrochloric acid (Merck) and acetonitrile (HPLC, Rankem). Milli-Q water, millipore water was used for the preparation of buffer and other aqueous solutions. Extraction method is liquid-liquid extraction.

Chromatographic separation of the diltiazem was performed on a Merck, Purosphere C_{18} column (150 mm × 4.6 mm i.d.). The HPLC equipment comprised of a solvent delivery system (Agilent 1100 series) and UV detector. The auto ampler (Agilent) was used to sample injection and the column was kept in oven (Agilent), UV detection was performed at 239 nm. Chemstation software was used for data analysis. A lab India pH meter was used to measure the pH of the aqueous mobile phase. The mobile phase was delivered at an isocratic rate of 1.5 mL/min with a pump pressure of *ca.* 150 bar. Total run time for plasma samples was 7 min.

Chromatographic conditions: The separation of diltiazem was achieved on a reversed phase (C_{18}) column with a mobile phase consisting of 10 mM ammonium acetate: acetonitrile in the ratio 70:30 v/v. Quantitation of diltiazem was performed by UV detection at 239 nm, at CTO 40 ºC and flow 1.5 mL/min.

Preparation of stock solution: Stock solution of diltiazem was prepared in methanol (1000 ppm), weigh accurately 25 mg of diltiazem transfer to volumetric flask, add 10 mL of methanol sonic add it, adjust the volume with methanol. Solutions were stored at 2-8 ºC was used to spike plasma samples. Standards and quality control samples were made by addition of the determined quantity of stock solution

to drug free plasma and stored at -20 ºC in aliquots. Plasma quality control sample concentrations were 60, 300 and 600 ng/mL.

Preparation of calibration curve standards and quality control samples: Assay performance was determined in accordance with FDA guidance for bioanalytical method validation for human studies[11]. Standard curve were constructed using seven standards non-zero concentrations in plasma and run in duplicate daily for 3 consecutive days. Calibration curve standards of diltiazem were prepared by spiking blank plasma at concentration of 20.00, 40.00, 60.00, 80.00, 160.00, 320.00 and 640.00 ng/mL. The quality control samples were prepared in blank plasma at concentration 60.00, 300.00 and 600.00 ng/mL. (LQC, MQC and HQC, respectively). Calibration curves were generated using weighted least squares regression analysis and obtained over the respective standard concentrations in plasma. All standards and quality control samples were stored at -20 ºC until analysis.

Preparation of plasma samples: Take 0.5 mL of plasma in ria vial, add 100 µL of 0.1 N HCl, vortex the vials on vortexer, then add 3 mL of *t*-butyl methyl ether, vortex vials for 3 min on vortexer, then centrifuge the vial in cold centrifuge for 10 min at 4500 rpm on 4 ºC. Separate 2 mL of upper layer of organic solvent in vial, evaporate to dryness at 50 ºC under a steady stream of nitrogen. Following reconstitution of the residue in 100 µL of mobile phase, the mixture was transferred to a micro insert and 60 µL was then injected on to HPLC.

Precision and accuracy: Standards and replicate quality control samples of plasma at each concentration were analyzed on 3 consecutive days, after which Inter-and Intra-day means, % Nominal and coefficients of variation (% CV) were calculated by standard method.

Method validation: Method validation were carried out according to the currently accepted USFDA (food and drug administration) bioanalytical method validation guidelines.

Selectivity: Blank plasma samples of the healthy human used for testing specificity of the method were obtained from 6 different sources. Each blank sample was tested for the visible interference. Six blank samples and six lower limits of concentration samples were analyzed for specificity.

Linearity: Calibration curves were generated by using the analyte peak area. The sample concentrations were calculated using weighted (1/x) least squares regression. Acceptance criteria were not more than 20 % deviation at LLOQ and not more than 15 % deviation at above LLOQ levels. The LOD values were calculated by using a signal-to-noise ratio of 3.2, while LOQ value was calculated by using a signal-to-noise ratio of 10.

Precision and accuracy: The Intra and Inter-day precision and accuracy was determined by analyzing six replicates of spiked plasma samples of diltiazem at each quality control (60.00, 300.00 and 600.00 ng/mL) levels. Inter-day precision and accuracy was observed in 3 consecutive batches. The accuracy was expressed by (mean concentration)/(spiked concentration) × 100 % and the precision by relative

standard deviation (% RSD). The concentration of each sample was calculated using standard curve prepared and analyzed on the same day.

Recovery: Recovery of diltiazem was performed at three concentration levels. Recovery was evaluated by comparing detector response for analyte obtained from extracted plasma samples with the true concentration of the analyte (unextracted)

Stability: The stability of diltiazem in the reconstituted solution was assessed by placing quality control samples in autosampler conditions for 36 h. The freeze-thaw stability of diltiazem was also assessed by analyzing quality control samples undergoing three freeze (-20 ºC)-thaw (room temperature) cycles. Short term and long term stock solution stability (for 6.0 h and 12 d), short term and long term (for 6 d and 30 d) stability of drug in matrix was evaluated. Short term and long term stock solution stability was also evaluated.

Anticoagulant effect: Anticoagulant effect was performed by comparing high and low quality control samples prepared in EDTA, anticoagulant based human plasma and compared against calibration curve prepared in CPDA anticoagulant based plasma.

Biological application: To demonstrate the applicability of the procedure for measurement of diltiazem levels in pharmacokinetics studies on healthy male volunteers. We are performing to study the new coating formulation.

RESULTS AND DISCUSSION

Method development: To develop a sensitive, selective and simple assay method for the extraction and quantification of diltiazem during method development different options were evaluated to optimize detection and chromatography parameters. First we have to find out the wavelength using the references. Then select the mobile phase varying the composition and pH, optimize it. Further optimization in chromatography conditions increased the response of analyte. A mobile phase containing 10 mM ammonium acetate buffer in combination with acetonitrile resulted in improved response. Use of purosphere (C_{18} 150 × 4.6 mm × 5 µm) column resulted in HPLC run time of 7 min.

Selectivity: Selectivity of the method was demonstrated by the absence of endogenous interfering peaks at the retention times of drug in six different lots of extracted blank plasma. Representative chromatographs of extracted blank plasma, extracted plasma samples containing 20 ng/mL of diltiazem are given in Figs. 1 and 2.

Linearity and LLOQ: The peak area of calibration standards was proportional to the concentration of analyte in each assay over the nominal concentration range of 20.00 to 640.00 ng/mL for diltiazem. The calibration curves appeared linear and were well described by least squares lines. A weighting factor of 1/x concentration was chosen to achieve homogeneity of variance. The correlation coefficients were = 0.99 (n = 4) for diltiazem. The mean (± SD) slopes of the calibration

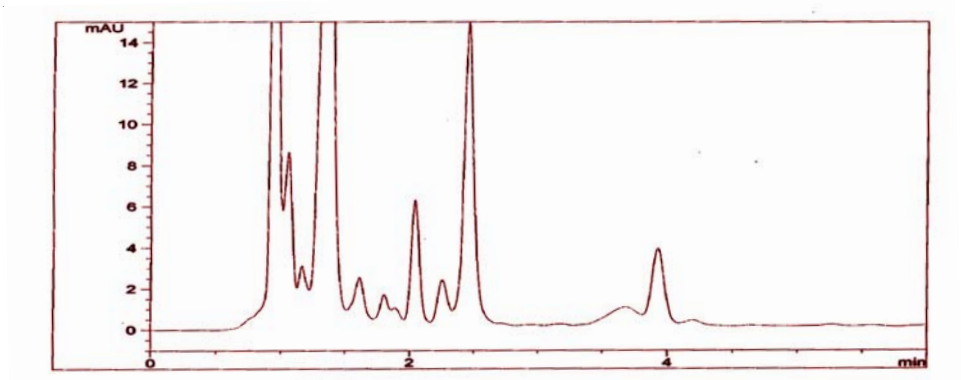

Fig. 1. Chromatograph of blank sample there is absence of endogeneous material of drug at room temperature

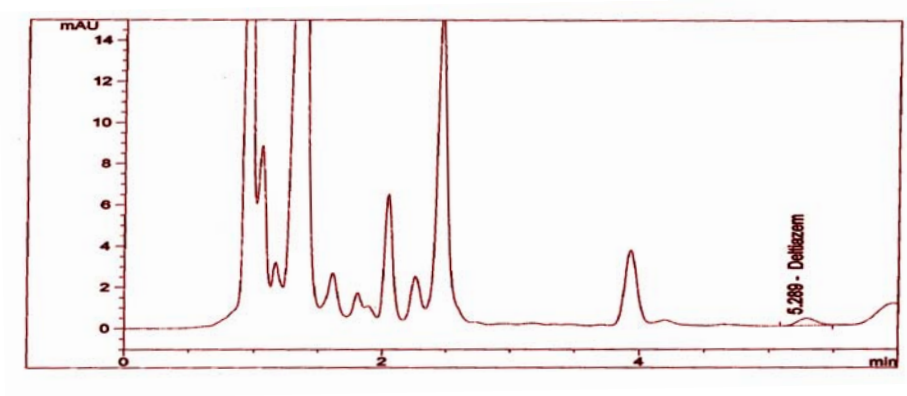

Fig. 2. Chromatograph of LLOQ sample

curves (n = 4) for diltiazem were 0.1830 (± 0.0166). Results of four representative standard curve for HPLC determination of diltiazem are given in Table-1. The lower imit of quantification for diltiazem proved to be 20.00 ng/mL.

TABLE-1
RESULTS OF FOUR CALIBRATION CURVES FOR DETERMINATION OF DILTIAZEM IN HUMAN PLASMA

Assay	Concentration (ng/mL)						
	20	40	60	80	160	320	640
1	18.030	41.870	59.750	91.200	153.940	310.270	644.950
2	17.510	36.130	64.700	85.250	153.410	324.270	638.740
3	17.630	42.370	63.010	97.090	138.870	316.480	644.550
4	20.540	39.670	66.980	84.770	151.780	310.910	645.360
Average	18.428	40.009	63.609	89.579	149.500	315.481	643.398
SD	1.427	2.841	3.043	5.800	7.144	6.488	3.125
% RSD	7.740	7.100	4.780	6.470	4.780	2.060	0.490
% Nominal	92.140	100.020	106.010	111.970	93.440	98.590	100.530

Precision and accuracy: Intra-batch and inter-batch precision and accuracy of the method for diltiazem are presented in Tables 2 and 3. The precision deviation values for intra-batch and inter-batch are all within 15 % of the relative standard deviation (RSD) at each quality control level. The accuracy deviation values for intra-batch and inter-batch are all within the (100 ± 15) % of the actual values at each quality control level.

TABLE-2
INTRA BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF DILTIAZEM IN HUMAN PLASMA

Intra-batch precision and accuracy n = 6			
Concentration (ng/mL)	Concentration Mean ± SD (ng/mL)	Accuracy (%)	Precision (%)
60.00	59.93 ± 5.76	99.89	9.61
300.00	302.38 ± 30.69	100.79	10.15
600.00	622.24 ± 43,58	103.71	7.00

TABLE-3
INTER BATCH PRECISION AND ACCURACY OF THE METHOD FOR DETERMINATION OF DILTIAZEM IN HUMAN PLASMA

Intra-batch precision and accuracy n = 6			
Concentration (ng/mL)	Concentration Mean ± SD (ng/mL)	Accuracy (%)	Precision (%)
60.00	54.27 ± 2.06	90.44	3.80
300.00	329.35 ± 12.46	109.78	3.78
600.00	656.05 ± 24.55	109.34	3.74

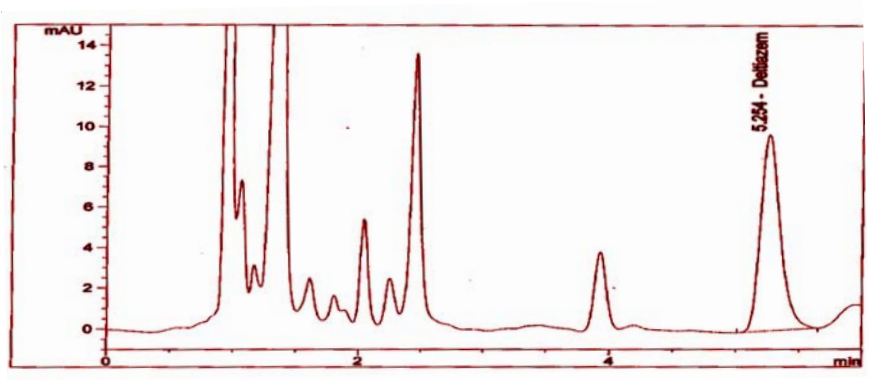

Fig. 3. Chromatograph at HLOQ level

Recovery: Results of extraction efficiency measured for diltiazem was consistent, precise and reproducible. The mean related extraction recovery of diltiazem at each quality control level (60.00, 300.00 and 600.00 ng/mL) was 68.05, 66.40 and 64.04 %, respectively.

Stability: Results of autosampler stability, freeze thaw stability, long term stability are given in Table-4. Stability data gives the idea about reliable stability of deltiazem in tested conditions. Diltiazem was found stable during short term and long term stock solution stability. During short term stock solution stability % mean stability of diltiazem was found 99.54 %. Long term stock solution stability was performed for 6 days and % mean stability of diltiazem was found 100.05 %.

TABLE-4
STABILITY OF DILTIAZEM IN HUMAN PLASMA UNDER TESTED CONDITIONS

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	60 ng/mL	600 ng/mL
Autosampler stability (36 h at 15 ºC)	98.06±3.17	109.51±57.96
Freeze thaw stability (3 cycles at -20 ºC)	98.69±4.60	103.22±64.05
Bench top stability (6 h at room temperature)	96.92±4.71	100.40±57.96
Short term stability in matrix (6 days at -20 ºC)	99.63±3.83	99.94±19.50
Long term stability (30 days at -20 ºC)	104.22±4.75	101.43±34.76

Anticoagulant effect: As method validation was performed in CPDA base human plasma anticoagulant effect was performed for EDTA base human plasma. Results of anticoagulant effect are summarized in Table-5.

TABLE-5
RESULTS OF ANTICOAGULANT EFFECT

Concentration (ng/mL)	% Accuracy (Mean ± SD)	
	60 ng/mL	600 ng/mL
Anticoagulant effect	106.00±12.32	103.41±32.90

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(*Received*: 13 March 2008; *Accepted*: 20 April 2009) AJC-7434