Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 6, Issue 9, 2014

Original Article

SIMPLE HPLC VALIDATED METHOD FOR THE DETERMINATION OF DILTIAZEM HYDROCHLORIDE IN HUMAN PLASMA

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Received: 26 Jun 2014 Revised and Accepted: 18 Aug 2014

ABSTRACT

Objectives: To develop and validate a high-performance liquid chromatographic method for determination of diltiazem hydrochloride (DLZ) in human plasma.

Methods: Mixture of n-hexane and 2-propanol (96:4, ratio) was added to plasma at sample preparation time followed by centrifuging the samples. The obtained upper organic layer was transferred and evaporated to dryness. The residue was reconstituted with a mobile phase and the supernatant was then injected onto the column. The mobile phase used was consisted of 0.2 M ammonium dihydrogen phosphate, acetonitrile, isopropyl alcohol and triethylamine (55:43:1.7:0.3, v/v) with pH adjusted to 4.5 using 85% phosphoric acid. The flow rate was 0.7 ml/min. UV detector set at 240 nm and samples were quantified using peak area.

Results: A well-resolved DLZ peak and free of interference from endogenous compounds in plasma with a retention time of 6.03 min was achieved. Recovery of DLZ was satisfactory (≥ 91.3%) over the concentration range tested 0.25 - 20 µg/ml. LOD of this assay was 0.125 µg/ml and LOQ was 0.25 µg/ml and, at this concentration, intra- and inter-day CV were 6.8 and 9.2 %, respectively. DLZ was found to be stable in plasma after storage at -80ºC, over 90 days.

Conclusion: The HPLC method described in this article was simple, sensitive, selective, reproducible, linear, precise, accurate, stability indicating and requires only a small sample volume, lending it suitable for the determination of DLZ concentration in routine measurements for pharmacokinetic/bioavailability studies.

Keywords: Diltiazem hydrochloride, HPLC, Assay validation, Stability indicating method.

INTRODUCTION

Diltiazem hydrochloride (DLZ) is a benzothiazepine calcium-channel antagonist with proven antianginal and antihypertensive efficacies [1, 2]. It is marketed for many years as a leading product for the treatment of cardiac disorders [3]. It can be given orally and intravenously for the treatment of angina pectoris, hypertension, arterial fibrillation and paroxysmal supra ventricular tachycardia [3]. DLZ like most calcium channel antagonists, it is a short acting compound and relies on a variety of slow release delivery systems to increase its duration of action and thereby decrease the dosing frequency [4]. The mechanism of action of DLZ is through decreasing the myocardial contractility and peripheral vascular resistance of smooth muscle.

This is due to inhibiting calcium influx which resulted in reducing the coronary vasospasm. A vasodilation in arteriolesthereby can occur [5]. In this way, DLZ causes a modest lowering of heart rate and subsequently lowers systolic as well as diastolic blood pressures [6]. Recently, a large prospective study demonstrated that DLZ was as effective as β-blocker and thiazide diuretics in preventing cardiovascular events in hypertensive patients [7]. Thus, DLZ appears to be a first line alternative among antihypertensive drugs from evidence based point of view. Drug content, concentration, loading, kinetic release and pharmacokinetics issues in relation to DLZ can be addressed using in vitro, in vivo and other characteristic studies. The determination of analytes in biological and non-biological fluids using a reliable, sensitive and validated analytical method is required. A reversed phase high performance liquid chromatography (HPLC) with ultraviolet (UV) is most commonly applied among various analytical reported methods. Validation of the analytical method prior the drug analysis in various matrices and media must be conducted to determine the following parameters: specificity, sensitivity, linearity, limit of quantification (LOQ) and limit of detection (LOD), accuracy, precision (reproducibility of inter-day and intra-day variation), recovery (extraction efficacy) and drug stability in the sample (stability indicating method (SIM)).

A number of analytical methods however have been developed for the determination of DLZ in biological samples, including using thin-layer chromatography (TLC)[8], gas chromatography (GC)[9 - 11] and HPLC [12 – 20]. Although, they were generally using a reversed phase systems with UV detection but the procedure of sample preparation were differed, not sensitive and no comment on long-term stability indicating method were made. Thus in this article, a sensitive, selective, reproducible and stability indicating HPLC-UV assay of DLZ concentration in human plasma was developed and validated for accuracy, precision, recovery, linearity and long-term stability.

MATERIALS AND METHODS

Materials

DLZ was obtained from Sigma Aldrich (St. Louis, USA). HPLC grade acetonitrile and n-hexane were purchased from Merck (Darmstadt, Germany). 2–propanol and isopropyl alcohol were purchased from J. T. Baker (New Jersey, USA). Ammonium dihydrogen phosphate, phosphoric acid and triethylamine were obtained from BDH Chemicals Ltd (Poole. UK). All solvents and reagents used were of HPLC grade and used with further manipulations.

Methods

HPLC system for plasma samples

A complete Shimadzu's HPLC system (Shimadzu Corporation, Japan) was used consisting of a LC-10A vp Intelligent HPLC pump and a SPD-10A vp detector set at an operation wavelength of 240 nm and a Rheodyne 7725i injector fitted with a 50 µl sample loop (Rheodyne, USA). This HPLC system was typically used to analyze all the DLZ spiked samples. All obtained data were collected and processed by Shimadzu Class VP version 7.4 software. A Zorbax SB-C18 (Palo Alto, CA, USA) reversed phase column, (4.6 mm x 250 mm, 5 µm) fitted with refillable guard column (30 - 40 µm) packed with Perisorb RP-18 (Upchurch Scientific, Oak Harbour, WA, USA) was used with a

mobile phase (degassed and filtered just before use) consisted of 0.2 M ammonium dihydrogen phosphate, acetonitrile, isopropyl alcohol and triethylamine (55:43:1.7:0.3, v/v) with pH adjusted to pH 4.5 using 85% phosphoric acid. The flow rate was 0.7 ml/min and samples were quantified using peak area.

Sample preparation

Prior to analysis, DLZ was extracted from the plasma samples according to the following procedure, 100 µl aliquot of plasma was measured accurately into an eppendorf micro-centrifuge tube and extracted by adding 400 µl extraction solvent of n-hexane and 2-propanol (96:4 ratio) The mixture was vortex-mixed for 50 s (stuart scientific, UK). The sample was then centrifuged (Eppendrof, Hamburg, Germany) at 12 000 g for 5 min. The upper organic layer was transferred into a reactivial (Pierce Reacti-vial, USA) and evaporated to dryness at 40°C under a gentle stream of nitrogen gas. The residue was reconstituted with 100 µl of mobile phase and 60 µl aliquot of the supernatant was then injected onto the column.

Assay validation

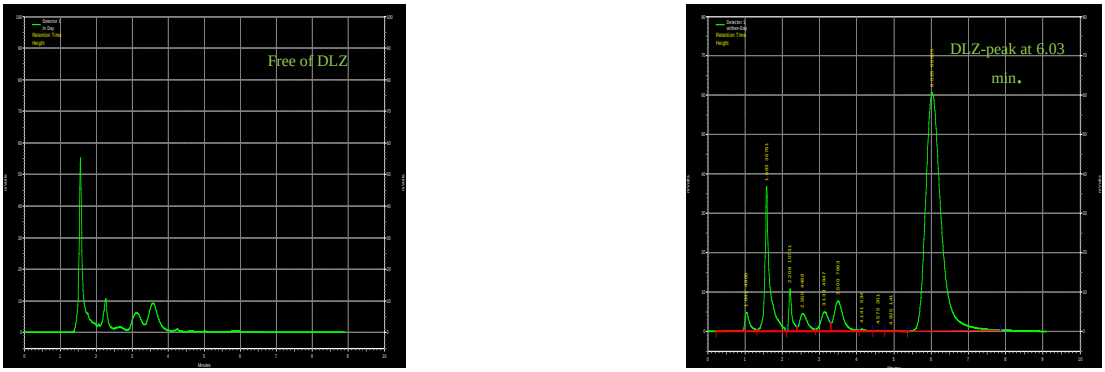
A DLZ stock solution (1mg/ml) was prepared and stored at -80ºC until required. Six batches of DLZ standard curves were prepared in water and by spiking human serum or plasma with DLZ at final concentrations of 0.25, 0.5, 1, 2, 5, 10 and 20 µg/ml. The calibration standards were kept frozen (-80ºC) in the freezer until being used for the assay validation. Quantitataion was based on peak area of DLZ. The LOQ is defined as the concentration with signal-to-noise ratios of 10:1 whereas, LOD 3:1. Linearity was tested by minitab 15 software using linear–square regression and lack-of-fit analysis.

To determine intra-day accuracy and precision, six replicates of each concentration were determined in a single sample of each concentration were determined in a single day. For inter-day accuracy and precision evaluations, analyses were carried out on a single sample of each concentration daily for six consecutive days. The standard curves for DLZ were constructed by plotting area against concentration.

DLZ concentrations were calculated using HPLC Shimadzu Class VP version 7.4 software. The absolute recovery (n=6) of the method was estimated by comprising peak areas of drug samples with drug solutions at corresponding concentrations. The stability of DLZ was tested in serum, at various concentrations. Short term stability (STS) was tested over 2days while long term stability (LTS) was tested over 90 days.

RESULTS

Chromatograms of plasma spiked with 10 µg/ml DLZ and plasma free of DLZ shows the DLZ peak with retention time of approximately 6.03 min was obtained and was well resolved and free of interference from endogenous compounds in plasma(Figure 1A,Figure 1B). The total run time for each sample was 10 min. At least six batches of human plasma were tested. Thus, blank human plasma was used in the preparation of the calibration curves. DLZ was found to be stable in human plasma for at least a day at room temperature with no noticeable changes in DLZ-plasma concentrations. The calibration curve of DLZ in plasma (Figure 2) was linear over the concentration range 0.25 - 20 µg/ml (r^2 0.9996 ± 0.0008) with a mean intercept of 0.0696 ± 0.02(Table 1). Similarly, the calibration curve of DLZ in water(Figure 2) was linear over the concentration range 0.25 - 20 µg/ml (r^2 0.9999 ± 0.0004) with a mean intercept of -0.1104 ± 0.05 (Table 1). The absolute recovery, intra- and inter-day accuracy and precision values over the DLZ were over concentration range 0.25 -20 µg/ml (Table 2). Intra-day and inter-day accuracies were 91.5 - 108.4 % and 91.3 - 104.8 %, respectively, with precision (CV) ≤ 9.3 %. The LOD was 0.125 µg/ml whereas LOQ as the lowest concentration used in the construction of calibration curves was 0.25 µg/ml. At this concentration, intra- and inter-day C. V. were 6.8 and 9.2 %, respectively. This value is comparable to that reported by Parissi-Poulou et al.,[22], even though a smaller supernatant volume was injected in the study reported here. Furthermore, the present method was testing the DLZ stability in plasma before extraction. STS and LTS tests, of DLZ in human plasma after storage at -80ºC for 2, 30 and 90 days (Table 3).



(A) Blank plasma **(B) DLZ spiked plasma (10 µg/mL)**

Fig. 1: Chromatograms of DLZ obtained from (A) plasma free of DLZ and (B) plasma spiked with10 µg/ml DLZ.

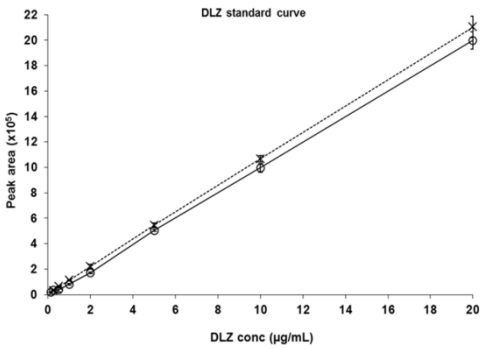


Fig. 2: Mean calibration curves for the determination of DLZ in water (x) and in human plasma (o) by HPLC using UV detector. (data are means ± S. D., n = 6).

Table 1: Comparison between r^2, slops and intercepts of DLZ standards curve (data are means ± S. D., n=6).

Standard Curve	Regression r^2	Slope (mean ± SE)	Intercept (mean ± SE)
plasma spiked DLZ	0.9996 ± 0.0008	1.0029 ± 0.2	+ 0.0696 ± 0.02
water spiked DLZ	0.9999 ± 0.0004	1.0489 ± 0.3	- 0.1104 ± 0.05

Table 2: Extraction recovery, Intra-day and Inter-day accuracy and precision of HPLC validation for the DLZ in human plasma (n=6).

Concentrations of DLZ (µg/ml)	Recovery Mean (%)	Recovery C. V. (%)	Intra-day Accuracy (%)	Intra-day precision (C. V. %)	Inter-day Accuracy (%)	Inter-day precision (C. V. %)
0.25	109.4	4.4	91.8	6.8	104.8	9.2
0.5	108.2	8.2	108.4	3.3	99.8	8.9
1	98.1	8.4	94.9	8.2	102.4	9.0
2	91.3	9.9	91.5	5.0	91.3	4.4
5	101.4	6.6	102.8	3.1	103.1	9.3
10	100.2	4.8	100.3	4.3	99.0	4.7
20	102.5	0.4	101.5	0.4	100.3	1.7

Table 3: DLZ stability in human plasma after 2, 30 and 90 days at -80ºC (data are means ± S. D., n=6).

[DLZ] (µg/mL)	[DLZ] at 2 days (µg/mL)	[DLZ] at 30 days (µg/mL)	[DLZ] at 90 days (µg/mL)
0.50	0.49 ± 0.10	0.49 ± 0.30	0.48 ± 0.50
1.0	0.9 ± 0.40	0.9 ± 0.50	0.9 ± 0.70
10.0	9.9 ± 0.50	9.8 ± 0.96	9.8 ± 1.30

DISCUSSION

This paper presents analytical methods for the determination of DLZ in human plasma. Samples from any further in vivo and pharmacokinetic studies can be analysed for DLZ using this current HPLC method. A number of analytical methods have been reported for determination of DLZ in plasma [8 - 21]. However, the HPLC method developed here was based on the method reported by Parissi - Poulou et al.,[22], but with some modifications to the mobile phase, extraction solvent, pH and HPLC flow rate.

The mobile phase was modified to contain 55% of 0.2 M potassium dihydrogen phosphate, 43% of acetonitrile,1.7% of isopropyl alcohol and 0.3% triethylamineas compared to that of Parissi - Poulou et al.,[22] which consists only 0.5 M potassium dihydrogen phosphate and acetonitrile at a ratio of 30:70. The addition of 1.7% isopropyl alcohol and increase the buffer pH value from 2.5 to 4.5 played an important role in producing sharp and well-resolved drug peaks free from interference from endogenous compounds in the plasma.

The acetonitrile content in the mobile phase was also found to be critical in separating DLZ from endogenous compounds and the best resolution was achieved at an acetonitrile concentration of 43% (v/v). When the acetonitrile content was below 35% (v/v), the elution time of DLZ was increased and the peak was not well separated from an endogenous peak being eluted later. Two peaks gradually overlapped as the acetonitrile content was further reduced to 30% (v/v).

Since DLZ is a weakly basic amine, the interaction between its amino group and the stationary phase could lead to peak tailing and broadening[23]. The use of short-chain tertiary amine modifiers such as triethylamine is very effective in improving peak symmetry and reducing retention of solutes with amino functional groups[24]. Therefore, triethylamine in 0.3% was added into the mobile phase to improve the resolution as well as peak symmetry of DLZ.

It is evident that a linear correlation is existed between the peak area ratio of plasma spiked DLZ and water spiked DLZ. This result also demonstrates that no considerable interference is existed. The extraction recovery of DLZ was determined by comparing the peak area obtained by direct injection of standard aqueous solutions to those obtained after the plasma extraction procedure. An extracting solvent mixture of n-hexane and 2-propanol (96:4 v/v) was found to give satisfactory recoveries for DLZ and provide cleaner chromatograms as compared to using n-hexane, diethyl ether, methyl tertiary butyl ether, ethyl acetate or chloroform alone as the extracting solvent.

DLZ was found to be stable in plasma after storage at -80ºC for 2, 30 and 90 days. No considerable differences were observed. The result demonstrates that the assay is selective, stable and sufficiently sensitive for the determination of DLZ concentrations in any future pharmacokinetic/bioavailability studies.

CONFLICT OF INTERESTS

Declared None

CONCLUSION

In conclusion, the HPLC method described here is simple, sensitive, selective, reproducible, linear in the specified range, precise, accurate, stability indicating and requires only a small sample volume, lending it suitable for the determination of DLZ concentration in routine measurements for pharmacokinetics / bioavailability studies.

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