Simple and effective HPLC method development and its validation for clindipine in human drug free plasma

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Abstract: Simple and effective high performance liquid chromatographic (HPLC) method was developed for estimation of Clindipine in drug free human drug free blank plasma. The internal standard used as Nifidipine (IS). The current method was used protein precipitating extraction of Clindipine from blank plasma. Separation was achieved on reversed-phase c_{18} column (25cm × 4.6mm, 5µ) and the detection was monitored by UV detector at 260 nm. The optimized mobile phase was used acetonitrile: 5mM potassium dihydrogen orthophosphate (pH 4.5), in the ratio of 60:40% v/v at a flow rate of 1.0 ml/min. This linearity was achieved in this method range of 10.0-125.0 ng/ml with regression coefficient range is 0.99. The present method is suitable in terms of precise, accurate and specific during the study. The simplicity of the method allows for application in laboratories that lack sophisticated analytical instruments such as LC-MS/MS or GC–MS/MS that are complicated, costly and time consuming rather than a simple HPLC–UV method. The present method was successfully applied for pharmacokinetic studies.

Keywords: Simple method; HPLC; validation; clindipine; human plasma; pharmacokinetic study.

INTRODUCTION

Clindipine [2-methoxyethyl-(E)-3-phenyl-2-propen-1-yl (_)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate], a dihydropyridine calcium channel antagonist and antihypertensive agent, has been reported to have a long-lasting anti-hypertensive effect and unique inhibitory actions on sympathetic neurotransmission; (Ikeda *et al*., 1992; Zhang *et al*., 2007; Hosono *et al*., 1995). Only limited number of analytical and bioanalytical methods have been reported for the quantification of Clindipine in various matrices. HPLC (shen *et al*., 2002)[4], HPTLC (Karmalkar *et al*., 2008) HPLC with fluorescence detection (Tan *et al*., 2005), LC-MS (Hatada *et al*., 1992; Lee *et al*., 2008). To the best of our knowledge no reports were found for the validation of Clindipine in drug free human plasma. The objective of this study was to develop and validate an assay for the estimation of Clindipine using HPLC.

MATERIALS AND METHODS

Materials and reagents

Acetonitrile HPLC grade was procured from E.Merck (India) Ltd, Mumbai. Potassium dihydrogen ortho-phosphate AR grade were procured from Qualigens fine chemicals, Mumbai. Water HPLC grade was obtained from a Milli-QRO water purification system. Reference standards of Clindipine and Nifidipine were procured from Sashan pharmaceuticals, Pondicherry, India. orthophosphoric acid (HPLC grade) were purchased from Merck (Mumbai, Maharashtra, India). Milli-Q water purification system supplied by Millipore (Bangalore, Karnataka, India) was used for the preparation of the aqueous mobile phase.

Equipment

HPLC chromatographic separation was achieved on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan). Princeton SPHER C_{18} (25cm x 4.6 mm i.d., 5µ) was used for the present analysis.

Preparation of the calibration standards and quality control (QC) samples

The stock solutions of Clindipine and Nifidipine were prepared using water and acetonitrile mixture 1:1 at a concentration of 1.0 mg/mL each. Clindipine working solution was used to prepare the spiking stock solutions for construction of six-point calibration curve (10.0-125.0 ng/mL) and QC samples at three different levels (25.0, 50.0, 100.0 ng/mL). All the stock solutions were refrigerated (2-8°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 25.0µL of respective spiking stock solutions. These were stored at -70°C until analysis.

Sample preparation for analysis

Calibration standards, validation QC samples and healthy volunteer plasma samples were prepared by adding 0.5ml plasma to 2.0ml centrifuge tube and added 0.5ml (10 µg/ml) of internal standard and 0.5ml of precipitating agent (10% v/v perchloric acid) vortexed for 2 min. The

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resulting solution was centrifuged at 4000 rpm for 7 min. The supernatant layer was separated and estimated by HPLC.

Chromatographic conditions

Standardization of clindipine by RP-HPLC method was carried out using the optimized chromatographic conditions. The mobile phase used was acetonitrile: 05mM potassium dihydrogen orthophosphate (ph 4.5). Potassium dihydrogen ortho phosphate used was 5 mM solution in water with pH being adjusted to 4.5 with orthophosphoric acid solution. The injection volume was 20.0 µL. The UV-visible detector was set at 260 nm.

Validation

The method was validated (FDA 2001) for selectivity, sensitivity, recovery, linearity, precision, accuracy and stability.

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms obtained from the samples containing Clindipine and the internal standard with those obtained from blank samples.

Sensitivity

Sensitivity was achieved in terms of LLOQ (Lower Limit of Quantification) where the response of LLOQ was at least five times greater than the response of interference in blank matrix at the retention time or mass transitions of the analyte.

Linearity

Different concentrations of standard solutions were prepared from 10.0ng/mL to 125.0ng/mL of Clindipine containing 5.0µL of internal standard (25.0µg/mL Nifidipine). These solutions were analysed and the peak areas and response factors were calculated. The calibration curve was plotted using response factor vs concentration of the standard solutions.

Precision and accuracy

The precision of the method was determined by intraday precision and interday precision. The intra-assay precision and accuracy was calculated for five replicates at each Lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) levels, each on the same analytical run, and inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs.

Stability studies

Various stability study was carried out. Room temperature stock solution stability, refrigerated stock solution stability, freeze thaw stability, short term stability and long term stability were determined. Room temperature stock solution stability was carried out at 0, 3 and 8 hours by injecting four replicates of prepared stock dilutions of Clindipine equivalent to middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. Comparison of the mean area response of Clindipine and internal standard at 3 and 8 hours was carried out against the zero hour value. Refrigerated stock solution stability was determined at 7, 14 and 27 days by injecting four replicates of prepared stock dilutions of the analyte equivalent to the middle quality control sample concentration and the stock dilution of internal standard equivalent to the working concentration. The stability studies of plasma samples spiked with Clindipine were subjected to three freeze - thaw cycles, short term stability at room temperature for 3 h and long term stability at 70^{o}C over four weeks. In addition, stability of standard solutions was performed at room temperature over 6 h and after freezing for four weeks. The stability of triplicate spiked human plasma samples following three freeze thaw cycles was analysed. The mean concentrations of the stability samples were compared to the theoretical concentrations. The stability of triplicate short term samples spiked with Clindipine was investigated at room temperature for 1.00 to 3.00 h before extraction. The plasma samples for long term stability were stored in the freezer at -70^{o}C until the time of analysis.

RESULTS

Selectivity

No interfering endogenous compound peak was observed at the retention time of drug and internal standard. Under chromatographic conditions, the retention times of Clindipine and Nifidipine were 7.82 min and 11.0 min respectively. Representative chromatograms of Lower Limit of Quantitation (LLOQ) and one study sample containing Clindipine is shown in (fig. 1) respectively.

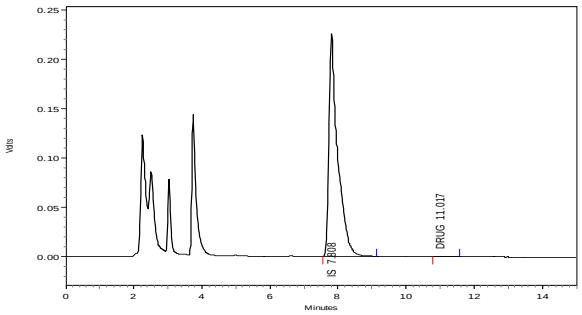

Fig. 1: Typical chromatogram of Isradipine sample

Sensitivity (Lower limit of quantitation)

The sensitivity of the experiment was carried out at LLOQ level.

Linearity

The calibration curves correlation coefficient was > 0.999. Calibration curve data of Clindipine result presented in table 1 and fig. 2.

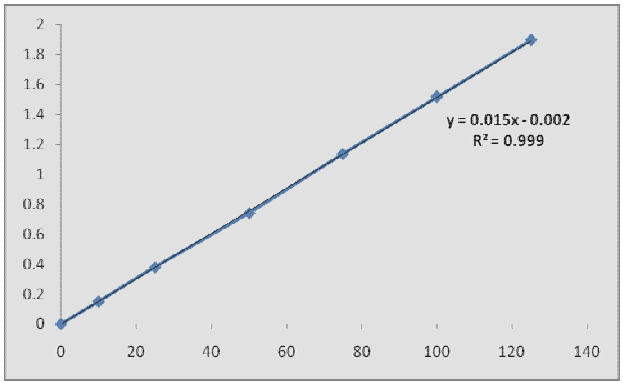

Fig. 2: Calibration Curve of Isradipine

Precision and Accuracy

Intra-day and inter-day accuracy and precision of the method were determined by Analysis of the control rat plasma spiked with Clindipine at LLOQ, LQC, MQC and HQC. All QCs concentration was calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage relative standard deviation; the results are given in table 2.

Table 1: Inter-run accuracy and precision of plasma calibration standards for Clindipine

Standard concentration(ng/mL)	Average calculated Concentration (ng/mL)	SD
10	9.86	0.75
25	24.79	1.02
50	49.88	0.47
75	73.97	1.42
100	99.94	0.39
125	124.91	0.57

SD=Standard deviation

Table 2: Intraday and Interday accuracy and precision of Clindipine in plasma

Standard concentration (ng/mL)	Average calculated concentration (ng/mL)	SD
Inter-day (n=3)		
10	9.69	0.57
50	48.57	1.02
100	99.86	0.861
Inter-day (n=3)		
10	9.56	0.58
50	49.25	0.91
100	98.67	1.34

SD=Standard deviation

Stability

Stock solution analysis was performed at 100.0 ng/mL. proper storage after for 15 days at 2-8°C and at room temperature for 6h more than 98% of Clindipine remained unchanged, based on peak areas in comparison with freshly prepared solution of Clindipine. This suggests that the Clindipine in standard solution is stable for at least 15 days when stored at 2-8°C and for 6h at room temperature. Bench top stability of Clindipine in plasma was investigated at LQC and HQC levels. This revealed that the Clindipine in plasma was stable for at least 6 h at room temperature. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Clindipine at LQC and HQC level did not affect the stability of Clindipine Long term stability of the Clindipine in plasma at -70°C was also performed after 30 days of storage at LQC, HQC levels. The results of the stability studies are shown in table 3. The average long term stability was 96.82%. The above results indicated that the Clindipine was stable in the studied conditions.

Table 3: Stability Study of Clindipine

Standard concentra-tion (ng/mL)	Average calculated concentration (ng/mL)	SD
Bench top (n=5)		
50	48.68	1.54
100	149.25	0.91
Freeze thaw stability (n=5)		
50	49.14	0.85
100	149.52	0.96
Long term stability (n=5)		
50	48.33	1.05
100	148.93	1.27

SD=Standard deviation

CONCLUSION

A simple and sensitive method for the determination of Clindipine in plasma by HPLC was developed and validated. Adequate specificity, precision and accuracy of the proposed method were demonstrated over the concentration range of 10.0-125.0 ng/mL. The method was accurate, reproducible, specific and applicable to the evaluation of pharmacokinetic profiles of Clindipine and suitable for the pharmacokinetic study of Clindipine.

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