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LC determination of the anti-ischemic and anti-hypertensive agent CDRI-93/478 in rat serum☆

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Abstract

CDRI-93/478 is a potent anti-ischemic and anti-hypertensive agent. This compound is in advanced stage of pre-clinical trials. A high-performance liquid chromatographic (HPLC) method was developed for the analysis of CDRI-93/478 in rat serum, a species used for safety evaluation. The HPLC analysis, applicable to 1 ml volumes of serum, involved double extraction of serum samples with diethyl ether at alkaline pH followed by separation on a spheri-5 cyano column and the use of fluorescence detector at excitation wavelength 250 nm and emission wavelength 372 nm. The method was sensitive with a limit of quantitation of 10 ng ml^{-1} in rat serum and the recovery was more than 84%. The linearity was satisfactory as indicated by correlation of >0.99, in addition to the visual examination of the calibration curves. The precision and accuracy were acceptable as indicated by relative standard deviation (R.S.D.) ranging from 1.73 to 9.51%, bias values ranging from -7.31 to 8.68%. Moreover, CDRI-93/478 was stable in rat serum after being subjected to three freeze–thaw cycles. In-process stability evaluation showed the stability of the compound in processed samples lasted up to 168 h. The assay was found to be sensitive, specific, accurate, precise, and reliable for use in pharmacokinetic or toxicokinetic studies.

Keywords: Reversed phase chromatography; CDRI-93/478; Anti-ischemic agent; Anti-hypertensive agent; Serum

1. Introduction

The advances in pathophysiology of hypertension have been the driving forces for the development of new anti-hypertensive drugs. Compound CDRI-93/478, 1-[4-(4-flurophenyl) piperazine-1-yl]-3-(2-oxopyrrolidin-1-yl) propane hydrochloride (Fig. 1) was synthesised in house. It is an arylpiperazine derivative and has shown its usefulness in the treatment of hypertension and in preventing post-ischemic reperfusion injury (ischemia) [1]. The candidate drug acts by blocking the peripheral α_1-adrenergic receptors. This compound is currently in advanced stage of preclinical stage of development.

There is no published assay method for the determination of CDRI-93/478 in serum. This paper describes on the development and validation of sensitive, precise and accurate high-performance liquid chromatographic (HPLC) method for quantitative determination of CDRI-93/478,

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which can be further used for the pharmacokinetic, toxicokinetic/metabolic studies of this candidate drug in rats.

2. Experimental

2.1. Materials

Compound CDRI-93/478 was synthesised in house (purity > 99%) and was used in the present study. HPLC grade acetonitrile was purchased from Ranbaxy Laboratories, SAS Nagar, India. Analytical grade glacial acetic acid was procured from Qualigens Fine Chemicals, Bombay, India. Ammonium acetate was obtained from HIPersolv, UK. Diethyl ether was further purified by distillation before use. Triple distilled water from all Quartz glass apparatus was used in the preparation of the buffer.

Blood was collected from healthy male Sprague–Dawley rats and was centrifuged to separate serum so as to generate a drug-free serum pool.

2.2. Instrumentation

The HPLC system consisted of a solvent delivery system with a controller (Model PU 4003; Pye Unicam, Cambridge, UK) equipped with a 7125 injector (Rheodyne, Berkeley, USA) fitted with a fixed 100 μl loop and a RF-530 fluorescence detector (Shimadzu, Kyoto, Japan). The samples were injected with a 250 μl syringe. The eluants were monitored at excitation wavelength 250 nm and emission wavelength 372 nm and chromatograms were integrated using C-R5A Chromatopac integrator (Shimadzu). Separation was achieved on a spheri-5 cyano column (5 μm, 220 × 4.6 mm, i.d.), coupled with a guard column packed with the same material (5 μm, 30 × 4.6 mm, i.d.; Pierce, Rockford, IL). The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml min^{-1} before analysis commenced. A vortex-mixer (Cecon, India), ultrasonic bath (Bransonic, Shelto, CY), a Model SVC-220H Speed vac concentrator (Savant, NY) and a Model C-30 centrifuge (Remi, India) were used for sample preparations.

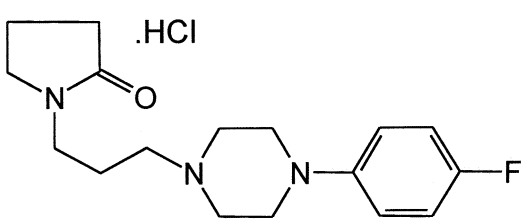

Fig. 1. Chemical structure of CDRI-93/478.

2.3. Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile and 50 mM ammonium acetate buffer (pH 3.5; 35:65, v/v). The mobile phase was degassed for 15 min before use and was pumped at a flow rate of 1 ml min^{-1}. The chromatography was performed at ambient temperature.

2.4. Stock and standard solutions

A stock solution of CDRI-93/478 was prepared in acetonitrile at 100 μg ml^{-1} and was used to prepare working standards containing 0.4, 0.8, 2, 4, 8 and 20 μg ml^{-1} CDRI-93/478 in acetonitrile. Each working standard (1.25 ml) was diluted to 10 ml with the mobile phase to prepare analytical standards (0.05, 0.1, 0.25, 0.5, 1 and 2.5 μg ml^{-1}).

Serum calibration standards containing 10, 20, 50, 100, 200 and 500 ng ml^{-1} of the compound were prepared individually by diluting 25 μl of working standards to 1 ml with serum. This method was used for the preparation of appropriate standards in serum in replicate.

2.5. Extraction procedure

To 1 ml serum (blank, spiked or test) in 10 ml test tube, 100 μl of 1 M potassium hydroxide solution was added and vortex-mixed for 15 s. To this, 2 × 3 ml diethyl ether was added, vortex-mixed for 1 min and centrifuged at 1000 × g for 10 min. The supernatant was transferred to a 10 ml conical tube by snap freezing the aqueous layer in liquid nitrogen and evaporated to dryness in Savant speed vac concentrator. The residue was

reconstituted in 0.2 ml of mobile phase, centrifuged and the resulting solution was injected onto the HPLC system. The external standard method was used for quantitation.

2.6. Calibration curve: model selection and verification

Quadruplicate of each calibration standard was assayed. The spiked concentration and their respective peak heights were subjected to least-squares regression (with and without intercepts) and a weighted least-squares regression ($1/x$ or $1/x^2$) using WinNonlin [2]. After examining the residuals and the percent deviation, a proper model was chosen.

2.7. Recovery

For calculation of recovery of the compound, spiked control samples were prepared at low (10 ng ml^{-1}), medium (50 ng ml^{-1}), and high (500 ng ml^{-1}) concentrations. The samples were processed as mentioned above and the concentration of the compound was determined from the regression of the analytical standard calibration curve. Recovery was calculated by comparing the observed concentrations with the spiked concentrations.

2.8. Accuracy and precision

To determine accuracy and precision, replicates of the spiked control samples at low, medium, and high concentrations were prepared. One set of the samples consisting of low, medium and high concentrations of the compound was assayed on the day of preparation. The concentration of the compound in the samples were determined from the calibration curve constructed from the standards prepared by spiking the serum CDRI-93/478 to their true or normal value. The assay was repeated with another set of samples spiked at the three concentrations on three more occasions. Intra- and inter-batch accuracy was determined by calculating the percent bias from the theoretical concentration. Precision, in terms of relative standard deviation (R.S.D.) was obtained by subjecting the data to one way analysis of variance (ANOVA).

2.9. Stability of CDRI-93/478 in processed samples

The stability of the compound in the processed (residue of the serum extract) samples on storage at -20 °C was studied at low, medium and high concentrations. Replicate samples of serum were processed as outlined above and one set (comprising triplicates of each concentration level) was reconstituted in the mobile phase and analysed on the day of preparation (day 1). The remaining three sets of processed samples were stored at -20 °C. These samples were assayed over a period of 4 days and their concentrations were determined with the respective calibration curve. The results of day 1 were taken as standard (100%) and the subsequent results were compared with the standard and are expressed as percent deviation.

2.10. Effect of freeze–thaw cycles on the stability of CDRI-93/478

Drug-free serum was spiked at low, medium and high concentrations. One set (comprising triplicates of each concentration level) was assayed on the day of preparation as described above with serum calibration standards, without being subjected to the freeze–thaw cycle and the results were referred as standard (100%). The remaining three sets of samples were stored frozen at -20 °C and were assayed after one, two and three freeze–thaw cycles and their concentrations were determined with the respective calibration curve. Thawing was achieved by keeping the tubes containing the sample at room temperature for 30 min. Data was analysed as described for the stability of CDRI-93/478 in processed samples.

2.11. Degradation of CDRI-93/478

The compound was subjected to acid and base hydrolysis to identify the degradation product or the putative metabolite(s) of the compound.

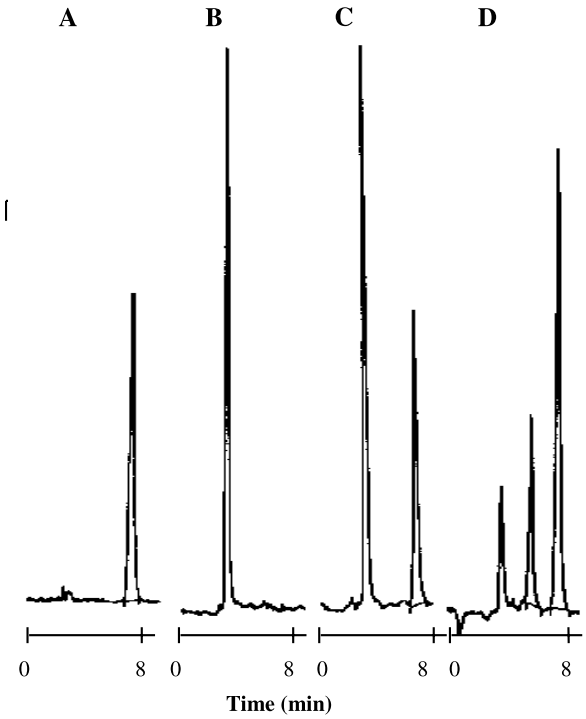

Fig. 2. Chromatograms of (A) standard containing 500 ng ml^{-1} CDRI-93/478; (B) drug-free rat serum; (C) serum containing 100 ng ml^{-1} CDRI-93/478; (D) rat sample taken 4 h after 8 mg kg^{-1} intravenous dose of CDRI-93/478.

2.12. Acid hydrolysis

To study the degradation of the compound in acidic condition, the compound (5 mg) was dissolved in a methanolic solution of hydrochloric acid (2 ml, 1 M) and heated at 60 °C for 2 h. After cooling, the pH of the reaction mixture was adjusted to neutral with a methanolic solution of potassium hydroxide (5% w/v). An aliquot of the neutral reaction mixture was diluted with the mobile phase and injected on to the HPLC.

2.13. Base hydrolysis

The compound (5 mg) was refluxed with a methanolic solution of potassium hydroxide (5% w/v) for 5 h. The pH of the reaction mixture was adjusted to neutral with a methanolic solution of hydrochloric acid (1 M). An aliquot of the neutral reaction mixture was diluted with the mobile phase and subjected to HPLC analysis.

3. Results and discussion

Initially, CDRI-93/478 when subjected to spectroscopic analyses showed both ultra violet (UV) absorbance (λ_{max}, 243 nm; ε, 7973) and fluorescence (excitation λ 250 nm and emission λ 372 nm). However, the fluorescence of the compound was more sensitive than UV absorbance, therefore, the analysis were carried out by using fluorescence detector. In the beginning, the use of an RP-18 column (100×4.6 mm, 5 μm) with mobile phase [65% acetonitrile in 20 mM ammonium acetate buffer (pH 4.0)] eluted the compound at t_R 11 min with broad peak response. With the same conditions, use of a spheri-5 cyano column (100×4.6 mm, 5 μm) eluted the compound earlier (t_R 6 min) with sharp and enhanced peak response. By increasing the molar strength of the buffer from 20 to 50 mM and decreasing the pH from 4.0 to 3.5 and acetonitrile percentage from 65 to 35% and the use of a longer spheri-5 cyano column (220×4.6 mm, 5 μm) eluted the compound at optimum t_R (7.2 ± 0.3 min). The HPLC analysis showed that the lowest limit of quantitation (LLOQ) was 10 ng ml^{-1} of serum, which provided adequate sensitivity to conduct pharmacokinetic study in animals. Typical chromatograms of an analytical standard containing 500 ng ml^{-1} CDRI-93/478 (A); an extract of the drug-free rat serum (B); serum containing 100 ng ml^{-1} CDRI-93/478 (C); and rat serum sample taken 4 h after 8 mg kg^{-1} intravenous dose are depicted in Fig. 2. Moreover, the extraction procedure and the chromatographic conditions yielded a clean chromatogram for the compound. The endogenous impurities did not interfere with the elution of the compound, indicating that the method was selective.

Following both acid and base hydrolysis, the compound exhibited no additional chromatographic peaks indicating that it was stable upon acid and base hydrolysis. Moreover, the compound did not show any degradation with light, oxygen and long-term storage up to 6 months. Therefore, the assay method was validated only for the parent compound.

A validation protocol was prepared which contained the complete details of the compound,

Table 1
Recovery of CDRI-93/478 in rat serum

Concentration (ng ml^{-1})		CV (%)	Recovery (%)
Theoretical	Observed (mean ± S.D.)		
10	8.46 ± 0.14	1.6	84.6
50	52.12 ± 2.76	5.29	104.2
500	439.02 ± 4.66	1.06	87.8

chemicals and instrumentation, stocks and standards, serum requirements, spiking and storage requirements for the HPLC method and the plan of validation and was followed. All criteria commonly employed during the validation of the HPLC methods were assessed. The HPLC assay method was validated in terms of reproducibility, linearity, recovery, accuracy and precision and stability studies of the compound in processed (residue of the serum extract) samples stored at −20 °C and during freeze–thaw cycles.

HPLC system reproducibility was checked with pentaplet injections of each analytical standard in a single run. The variations in the peak heights of each standard was maximum (1.5%) at 0.05 μg ml^{-1} indicating that the system yields reproducible data. Moreover, the peak heights were linear with the concentrations.

Linearity was evaluated over a concentration range of 10–500 ng ml^{-1}. To verify the linearity of the concentration of compound and the chromatographic responses (peak heights), a calibration curve was created by plotting the peak heights of the compound against their corresponding concentrations in spiked serum. A 24-point calibration curve is necessary to provide enough information to discriminate between models [3]. In some cases, manipulations of the calibration data by applying weights or by transformation becomes necessary. In the present study, linear least square regression analysis (without intercept) showed highly reproducible relationship between the concentration of the analyte and the chromatographic response with correlation coefficient of >0.99, indicating that the response was linear over the concentration range studied. The residuals did not improve much with intercept and by weighted linear regression ($1/x$ and $1/x^2$). Thus, linear regression model (without intercept) was chosen for routine analysis of data.

The recoveries of the compound from the spiked serum samples were calculated at low, medium and high concentrations and ranged from 84.6 to 104.2% with a coefficient of variation (CV) of less than 7% (Table 1). The accuracy and precision of the method are summarised in Tables 2 and 3. Intra-day accuracy and precision were assessed at CDRI-93/478 concentrations of 10, 50 and 500 ng ml^{-1} by comparing the concentrations of the spiked control samples estimated from the calibration curve to their true values

Table 2
Intra-day accuracy and precision for CDRI-93/478 in rat serum

Nominal concentration (ng ml^{-1})	Observed concentration (mean; ng ml^{-1})	Accuracy (% bias)	Precision (% R.S.D.)
10	9.27	−7.31	9.51
50	54.34	8.68	4.02
500	466.68	−6.66	1.73

Table 3
Inter-day accuracy and precision for CDRI-93/478 in rat serum

Nominal concentration (ng ml^{-1})	Observed concentration (mean; ng ml^{-1})	Accuracy (% bias)	Precision (% R.S.D.)
10	9.46	−5.36	17.60
50	54.32	8.64	10.87
500	472.33	−5.53	7.99

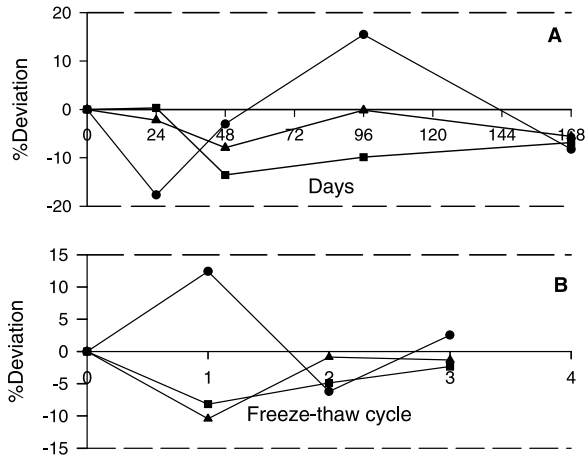

Fig. 3. Stability of CDRI-93/478 (A) in processed (residue of the serum extract) samples on storage at −20 °C; and (B) during freeze–thaw cycle, at low (●, 10 ng ml^{-1}), medium (■, 50 ng ml^{-1}) and high (▲, 500 ng ml^{-1}) of CDRI-93/478.

(Table 2). Three samples were analysed at each concentration on the same day. To determine the variance in precision (%R.S.D.), the observed concentrations at each level were subjected to ANOVA and the R.S.D. was calculated using the mean square value. An acceptance limit of 20% was employed for the low concentrations and 15% was applied for medium and high samples [4]. The results showed satisfactory intra-day precision and accuracy as indicated by R.S.D. $\leq$ 9.51% and bias of $\leq$ 8.68% (Table 2). Inter-day accuracy and precision were evaluated at the same concentrations as above and the samples were analysed on 4 separate days. The results demonstrated satisfactory inter-day precision and accuracy as shown by R.S.D. and bias values of $\leq$ 17.60 and $\leq$ 8.64%, respectively (Table 3).

In-process (residue of the serum extract) stability of CDRI-93/478 was evaluated at low, medium and high concentration up to 168 h after sample processing (Fig. 3A). The results of the first day analysis were considered as reference and the subsequent results and the subsequent results were compared with the first day result by ANOVA. The changes from the nominal concentrations were within the acceptable limits for the 10, 50 and 500 ng ml^{-1} concentrations, respectively, demonstrating that CDRI-93/478 was stable under the condition evaluated. The stability of CDRI-93/478 in serum was performed through three freeze–thaw-cycles at the above mentioned concentration and were assessed by analysing the samples at periodic intervals. After three cycles, the percent deviation from the concentration observed on day 1 were 2.53, −2.34 and −1.30% for the low, medium and high concentrations, respectively, demonstrating that the compound was stable in serum through three freeze–thaw cycles (Fig. 3B).

4. Conclusions

An HPLC assay was developed and validated for the quantitation determination of CDRI-93/478 in rat serum. The extraction procedure is easy. The moderate analysis time, together with rapid evaporation of extraction solvent, allowed rapid analysis. The method was shown to be accurate and reliable over a concentration range of 10–500 ng ml^{-1} with an acceptable R.S.D. and bias. The method is now in routine use in our laboratory.

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