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Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 1009–1015

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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LC determination of the anti-ulcer agent CDRI-85/92 in rat serum☆

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Received 25 July 2001; received in revised form 8 August 2001; accepted 28 August 2001

Abstract

CDRI-85/92 is a new potent anti-ulcer compound developed by Central Drug Research Institute, Lucknow (India). This compound is in advanced stage of preclinical trials. A High-performance liquid chromatographic (HPLC) method was developed and validated for the analysis of CDRI-85/92 using rat serum. The HPLC analysis, applicable to 0.5-ml volumes of serum, involved protein precipitation of serum samples with acetonitrile (1:3 v/v) followed by centrifugation and separation on a C-18 column and the use of UV detector at the wavelength 250 nm. The method was sensitive with a lowest limit of quantitation (LLOQ) of 1.25 ng ml^{-1} in rat serum and the recovery was more than 96%. 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 4.15 to 8.21%, bias values ranging from 2.96 to 11.18%. In-process stability evaluation showed the stability of the compound in processed samples lasted up to 168 h. The method was applied for analysing CDRI-85/92 in rat serum after administration of single oral or iv bolus dose of 20 mg kg^{-1}. The robustness/ruggedness of the HPLC procedure was tested using different HPLC instrumentation and column of different make. The assay was found to be sensitive (limit of quantification was 1.25 ng ml^{-1}), specific (retention time for CDRI-85/92 is 7.5 min), accurate (% bias is $<12\%$), precise (% R.S.D. is $<10\%$), robust (no significant change in peak profile in two HPLC Instruments) and reliable for use in pharmacokinetic or toxicokinetic studies.

Keywords: Reversed phase chromatography; CDRI-85/92; Anti-ulcer agent; Serum

1. Introduction

The proton pump inhibitors (PPIs), which are specific for H^+, K-ATPase, the proton pump responsible for the terminal step in gastric acid secretion, are considered to be the most effective medical treatment for the management of patients with acid peptic disease [1]. Comparative studies have demonstrated that PPIs provide acid suppression, pain relief and ulcer healing superior to that achieved with histamine type 2 receptor antagonists. Compound CDRI-85/92, 5-styryl-4, 5-*cis*-1, 3-oxazl-2-one-4-carboxylic acid was synthesised in house. It is an oxazole derivative,

☆ CDRI Communication Number: 6194.

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0731-7085/02/$ - see front matter
PII: S0731-7085(01)00600-8

and has shown its usefulness in the treatment of ulcers. This compound is currently in advanced stage of preclinical stage of development.

There is no published assay method for the determination of CDRI-85/92 in serum (Fig. 1). This paper describes the development and validation of simple, sensitive, precise and accurate HPLC method for quantitative determination of CDRI-85/92, which can be further used for the pharmacokinetic, toxicokinetic/metabolic study of this candidate drug in rats.

2. Experimental

2.1. Materials

Compound CDRI-85/92 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, England. 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 (Kontron HPLC System, Unicam, Cambridge, UK) equipped with a 7125 injector (Rheodyne, Berkely, USA) fitted with a fixed 20 μl loop and a Kontron UV Spectrophotometer detector (Uvikon 730SLC set at 250 nm). The samples were injected with a 100-μl syringe. The eluants were monitored at wavelength 250 nm and chromatograms were integrated using CR1B Chromatopac PM/Intg/084 integrator (Shimadzu, Kyoto, Japan). Separation was achieved on a C-18 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.), (E-merck, Darmstadt FR Germany No. 619429). The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml min^{-1} before analysis commenced. A vortex-mixer (Thermolyne, India), ultrasonic bath (Bransonic, Shelto, CY), a Model SVC-220H Speed vac concentrator (Savant, Nyor Heto/Maxi Dry Plus Germany) and a Model C-30 centrifuge (Remi, India) were used for sample preparations.

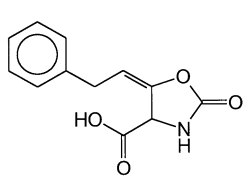

Fig. 1. Chemical structure of CDRI-85/92.

2.3. Chromatographic conditions

The mobile phase was prepared by mixing Methanol, acetonitrile and 10 mM ammonium acetate buffer (pH 4.0) (29:1:70 v/v) and mixed well. The mobile phase was degassed for 15 min in the sonicator 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 (SS) of CDRI-85/92 was prepared in methanol at 1000 μg ml^{-1} and was used to prepare working standards (WS) containing 4 μg ml^{-1} CDRI-85/92 in methanol in three different 10 ml volumetric flasks. 1.0 ml of each WS was serially diluted appropriately with the mobile phase to prepare analytical standards (12.5, 25, 50, 100, 200, 400 and 800 ng ml^{-1}).

Serum calibration standards containing 1.25, 2.5, 5.0, 10.0, 20.0 and 40 ng ml^{-1} of the compound were prepared by serially diluting WSs to 0.5 ml with serum. This method was used for the preparation of appropriate standards in serum in triplicate. All the three sets were mixed so that random selection of the complete range of concentrations could be obtained as and when needed.

2.5. Precipitation procedure

To 0.5 ml serum (blank, spiked or test) in 10 ml test tube, 1.5 ml of acetonitrile was added and vortex mixed for 15 s. The tubes were kept in fridge for 30 min for complete precipitation, vortex-mixed for 1 min and cold centrifuged at $1000 \times g$ for 10 min. Same volume of the supernatant (1.5 ml) was transferred to a 10 ml conical tube by Pipettes without disturbing the lower precipitate pellet and evaporated to dryness in Savant speed vac concentrator. The residue was reconstituted in 0.1 ml of mobile phase; cold centrifuged at $1000 \times g$ for 10 min 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 % deviation, a proper model was chosen.

2.7. Recovery

For calculation of recovery of the compound, spiked control samples were prepared at low (1.25 ng ml^{-1}), medium (5.0 ng ml^{-1}), and high (20 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.

$$\%\ \text{Recovery} = \frac{\text{Observed concentration}}{\text{Nominal concentration}} \times 100$$

2.8. Lowest limit of detection (LOD) and quantitation (LOQ)

The detection limit of the HPLC assay (LOD) of compound 85/92 after each sample clean up method was estimated as the drug quantity in serum, which corresponds to three times the baseline noise. The lowest LOQ was defined as the concentration quantity of the sample, which was quantified with less than 20% deviation in precision.

2.9. 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-85/92 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 % bias from the theoretical concentration. Precision, in terms of relative standard deviation (R.S.D.) was obtained by subjecting the data to one-way ANOVA.

$$\%\ \text{R.S.D.} = \frac{\text{S.D.}}{\text{Mean of observed concentration}} \times 100$$

$$\%\ \text{Bias} = \frac{(\text{Observed concentration} - \text{Nominal concentration})}{\text{Nominal concentration}} \times 100$$

2.10. Stability of CDRI-85/92 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 (compris-

ing 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.

2.11. Application of assay in rats

Application of the new HPLC method was conducted by measuring the concentration of CDRI-85/92 in male *Sprague–Dawley* rats. The rats (250 ± 25 g) were procured from the Laboratory Animal Division of the Institute and acclimatised for 3 days to study with 12 h alternate dark and light cycles. Each rat received either single oral or bolus iv via caudal vein. (20 mg kg^{-1}). Two blood samples were collected from each rat by cardiac puncture and finally from vena cava (terminal) so as to get a minimum of five samples at each time point (5,10, 15 and 30 min post dose). Serum required for analyses was separated and stored at −20 °C till use.

2.12. Robustness of the procedure

The robustness/ruggedness of the procedure was adjudged by using different instrumentation of the HPLC system consisting of a solvent delivery system with a System controller SCL-10Avp (Shimadzu HPLC System, Japan) equipped with an autoinjector SIL-10ADvp, set at the volume of 50 μl per injection and a UV detector (SPD-10Avp set at 250 nm). The eluants were monitored at wavelength 250 nm and chromatograms were integrated using System controller SCL-10Avp. Separation was achieved on a C-18 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.), (Perkin–Elmer). The HPLC system was equilibrated for approximately 30 min at a flow rate of 1 ml min^{-1} before analysis commenced. A vortex-mixer (Thermolyne, India), ultrasonic bath (Bransonic, Shelto, CY), a Model SVC-220H Speed vac concentrator (Savant, Nyor Heto/Maxi Dry Plus Germany) and a Model C-30 centrifuge (Remi, India) were used for sample preparations.

3. Results and discussion

CDRI-85/92, when subjected to spectroscopic analyses showed UV absorbance (λ_{max}, 250 nm; ε, 18250 M^{-1} cm^{-1}). In the beginning, the use of a C-18 column (100 × 4.6 mm, 5 μm) with mobile phase [50% acetonitrile in 10 mM ammonium acetate buffer (pH 4.0)] eluted the compound with the mobile phase front. By altering the pH of the buffer 3.5–6.8 and percentage of acetonitrile from 50 to 10, there was no appreciable alteration in the standard peak height also the retention time was 3–4 min which can be disguised with the impurity peaks of the biomatrices. However, change of mobile phase to methanol:10 mM ammonium acetate buffer pH 4.0::30:70 gave significantly good peak further addition of 1% acetonitrile as modifier in the mobile phase buffer eluted the compound at optimum t_R (7.5 ± 0.3 min). The HPLC analysis showed that the lowest limit of quantitation (LLOQ) was 1.25 ng ml^{-1} of serum, which provided adequate sensitivity to conduct pharmacokinetic study in animals. Typical chromatograms of an analytical standard containing 50 ng ml^{-1} CDRI-85/92 (A); an extract of the drug-free rat serum (B); serum containing 10 ng ml^{-1} CDRI-85/92 (C); and rat serum sample taken 1 h after 20 mg kg^{-1} intravenous dose are depicted in Fig. 2. Moreover, the precipitation 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. 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, chemicals and instrumentation, stocks and stan-

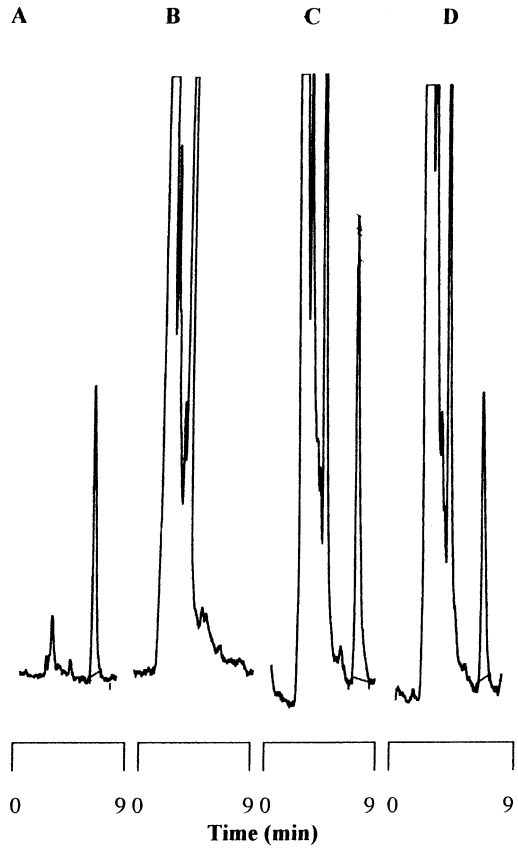

Fig. 2. Chromatograms of (A) standard containing 50 ng ml^{-1} CDRI-85/92; (B) drug free rat serum; (C) serum containing 100 ng ml^{-1} CDRI-85/92; (D) rat serum taken 1 h after 20 mg kg^{-1} intravenous dose of CDRI-85/92.

Table 1
Recovery of CDRI compound 85/92 in rat serum

Concentration (ng ml^{-1})		CV (%)	Recovery (%)
Theoretical	Observed (mean ± S.D.)		
1.25	1.23 ± 0.06	4.53	98.40
5.0	4.81 ± 0.14	2.89	96.20
20.0	19.58 ± 0.49	2.50	97.88

dards, 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. 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. Inclusion of all the above-mentioned parameters are in accordance to the GLP guidelines, which are routinely been followed in other laboratories for assay validation [3].

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 were maximum (1.5%) at 50 ng 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 12.5–800 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 [4]. In some cases, manipulations of the calibration data by applying weights or by transformation become 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 studies. 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 96.20 to 98.40% with a CV of less than 5% (Table 1). The accuracy and precision of the method are summarised in Table 2 and Table 3. Intra-day accuracy and precision of CDRI compound 85/92 were assessed at concentrations of 1.25, 5.0 and 20.0 ng ml^{-1} by comparing the concentrations of the spiked control samples estimated from the calibration curve to their true values (Table 3).

Table 2
Intra-day accuracy and precision for CDRI-85/92 in rat serum

Nominal concentration (ng ml^{-1})	Observed concentration (mean) (ng ml^{-1})	Accuracy (% bias)	Precision (% R.S.D.)
1.25	1.17	−6.61	5.58
5.0	4.42	−11.65	2.80
20.0	18.13	−9.34	3.24

Table 3
Inter-day accuracy and precision for CDRI compound 85/92 in rat serum

Nominal concentration (ng ml^{-1})	Observed concentration (mean) (ng ml^{-1})	Accuracy (% bias)	Precision (% R.S.D.)
1.25	1.22	−2.51	10.06
5.0	4.44	−11.65	3.28
20.0	18.16	−9.20	3.50

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 one-way 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 sample [5]. The results showed satisfactory intra-day precision and accuracy as indicated by R.S.D. $\leq 5.58\%$ and bias of $\leq 6.61\%$ (Table 2). Inter-day accuracy and precision were evaluated at the same concentrations as above and the samples were analysed on four separate days. The results demonstrated satisfactory inter-day precision and accuracy as shown by R.S.D. and bias values of ≤ 10.06 and $\leq 2.51\%$, respectively (Table 3).

In-process (residue of the serum extract) stability of CDRI-85/92 was evaluated at low, medium and high concentration up to 168 h after sample processing (Fig. 3). The results of the first day analysis were considered as reference 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 1.25, 5.0 and 20.0 ng ml^{-1} concentrations, respectively, demonstrating that CDRI compound 85/92 was stable under the condition evaluated.

Serum samples of rats receiving single 20 mg kg^{-1} iv or oral dose were analysed for CDRI-85/92 by the above mentioned proposed method. The concentration–time profile is given in the Table 4. The chromatograms did not exhibit any interfer-

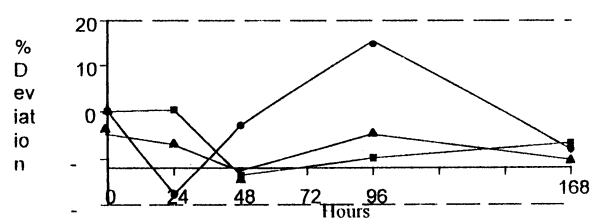

Fig. 3. Stability of CDRI-85/92 in processed (residue of the serum extract) samples on storage at −20 °C, samples at low (●, 1.25 ng ml^{-1}), medium (■, 5.0 ng ml^{-1}) and high (▲, 20 ng ml^{-1}) of CDRI-85/92.

Table 4
Serum levels of CDRI-85/92 in rats receiving single bolus dose of 20 mg kg^{-1} by oral or iv routes

Time (min)	Concentration (ng ml^{-1})	
	Oral	iv
5	31	112
10	29	94
15	45	46
20	11	33

ence from endogenous components of serum (Fig. 2D), also the blank serum was devoid of any interfering peak in the compound region (Fig. 2B).

To check the robustness/ruggedness of the HPLC procedure for the quantitation of CDRI-85/92, another HPLC instrumentation (Shimadzu) was used; the column make was also changed.

There was no visible difference in the peak profile of CDRI-85/92 in both the HPLC instruments.

4. Conclusions

An HPLC assay was developed and validated for the quantitative determination of CDRI compound 85/92 in rat serum, taking into account all the possible parameters of HPLC assay validation. 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 1.25–20.0 ng ml^{-1} with an acceptable R.S.D. and bias. The method is now in routine use in our laboratory.

Acknowledgements

The authors gratefully acknowledge the encouragement of Dr C.M. Gupta, Director, Central Drug Research Institute, Lucknow.

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