ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 533–538

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Liquid chromatographic determination of a non-steroidal oral contraceptive CDRI-85/287 in rat serum[1]

J. Lal, R.C. Gupta *

Pharmacokinetics & Metabolism Division, Central Drug Research Institute, Lucknow-226001, India

Received 1 October 1997; accepted 10 October 1997

Abstract

A precise and sensitive high performance liquid chromatographic (HPLC) assay method was developed and validated for the quantitation of 2-[4-(2-piperidinoethoxy) phenyl]-3-phenyl-(2H)-1-benzo(b)pyran (compound CDRI-85/287) in rat serum. This method, applicable to 0.5 ml volumes of serum, was validated according to GLP guidelines. It involved double extraction of serum samples with a mixture of hexane and iso-propanol (98:2 v/v) at alkaline pH and the use of UV detection at 332 nm. Linearity, precision and accuracy were acceptable (5–200 ng ml^{-1}). The absolute recovery was more than 75% and the lower limit of quantitation was 5 ng ml^{-1}. Freeze–thaw stability studies up to four cycles showed no apparent differences in the calculated spiked concentrations. However, in-process stability evaluation showed the stability of the processed samples lasted up to 85 h.

Keywords: Reversed phase chromatography; CDRI-85/287; Non-steroidal contraceptive serum

1. Introduction

Compound CDRI-85/287, 2-[4-(2-piperidinoethoxy) phenyl]-3-phenyl-(2H)-1-benzo(b)pyran (Fig. 1), is a new non-steroidal estrogen antagonist with potent anti-implantation activity [1,2]. It is virtually devoid of estrogen agonistic activity and does not induce vaginal cornification even at doses as high as 2.5 mg kg^{-1} [3–5]. This compound is currently in an advanced stage of preclinical trials. Pharmacokinetic data on the fate of the compound in experimental animals, viz rats and beagle dogs, are necessary to understand and predict the drug action. To evaluate this requires a sufficiently sensitive and reliable assay for the compound.

There is no published assay method for the determination of CDRI-85/287. This paper describes a rapid, precise and sensitive HPLC procedure for the determination of the compound in rat serum for its pharmacokinetic study.

2. Experimental

2.1. Materials

Compound CDRI-85/287 was synthesized in house (purity > 99%) and was used in the present study. HPLC grade acetonitrile, iso-propanol, and

* Corresponding author.

[1] CDRI communication no. 5625.

0731-7085/98/$19.00
PII S0731-7085(97)00231-8

n-hexane were purchased from S.D. (Boisar, India). *N*,*N*-dimethyloctylamine was prepared from octylamine in this laboratory. Analytical grade glacial acetic acid and sodium acetate were procured from Qualigens (Bombay, India) and were used without further purification. Triple distilled water from all quartz apparatus was prepared and used as solvent. Extraction solvent was prepared by mixing hexane and iso-propanol (98:2 v/v).

Blood was collected from healthy male albino 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 Shimadzu LC-10AD solvent delivery system equipped with a 7725i Rheodyne injector fitted with a fixed 50 μl loop, a SPD-10AD multiple wavelength UV-VIS detector and a CBM-10A Shimadzu LC workstation (Shimadzu, Kyoto, Japan). The samples were injected with a 100 μl syringe. The eluants were monitored at absorption wavelength 332 nm and chromatograms were integrated using model CLASS-LC10 software on a PC (model Compaq Prolinea 466). Separation was achieved on a reversed phase C18 column (5 μm, 100 mm × 4.6 mm i.d.), coupled with a guard column packed with the same material (5 μm, 30 mm × 4.6 mm i.d.) (Pierce, Rockford, IL). The HPLC system was equilibrated for approximatley 30 min at a flow rate of 1.2 ml min^{-1} before analysis commenced. A vortex mixture (Cecon, India), ultrasonic bath (Bransonic, Shelto, CY), a model C-30 centrifuge (Remi, India) and a model SVC-200H speed vac concentrator (Savant, NY) were used for sample preparation.

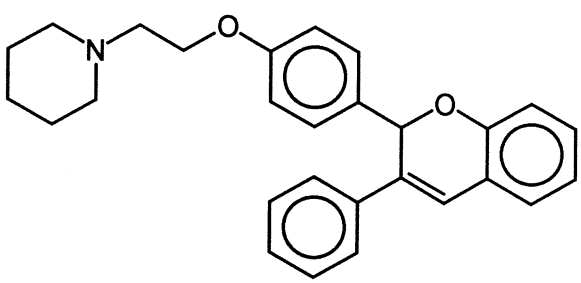

Fig. 1. Structure of compound CDRI-85/287.

2.3. Chromatographic conditions

The mobile phase was prepared by mixing acetonitrile and 10 mM sodium acetate buffer (pH 3) (50:50 v/v), to which *N*,*N*-dimethyl octylamine (1 ml l^{-1} v/v) was added and mixed well. The mobile phase was pumped at a flow rate of 1.2 ml min^{-1}. The chromatography was performed at ambient temperature.

2.4. Standard solutions

A 200 μg ml^{-1} stock solution of CDRI-85/287 was prepared by dissolving 5 mg of the compound in 25 ml acetonitrile and used to prepare working standards containing 50, 100, 200, 500, 1000 and 2000 ng ml^{-1} CDRI-85/287 in acetonitrile. Analytical standards (25, 50, 100, 250, 500 and 1000 ng ml^{-1}) were prepared by diluting each 2 ml of the corresponding working standards with 2 ml of the mobile phase.

Serum calibration standards containing 5, 10, 20, 50, 100 and 200 ng ml^{-1} of the compound were prepared by pipetting 50 μl of the working standards followed by drying and adding sufficient serum to make 500 μl. This method was used for the preparation of appropriate serum standards in replicates.

2.5. Extraction procedure

To 0.5 ml serum (blank, spiked or test) in 10 ml test tubes, 50 μl of 1 M potassium hydroxide solution was added and vortex-mixed for 15 s. To this, 2 × 3 ml hexane containing 2% isopropanol was added, vortexed 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. The residue was reconstituted in 0.1 ml of the mobile phase, centrifuged and the resulting solution was injected into the HPLC system. The external standard method was used for quantitation.

2.6. Calibration curve: model selection and verification

This study was carried out by spiking drug-free

rat serum with increasing amounts (5, 10, 20, 50, 100 and 200 ng ml^{-1}) of CDRI-85/287. These samples were then assayed according to the above procedure. Each standard was assayed in quadruplicate. The calibration curve from four replicate calibration standards was prepared at six concentrations. PCNONLIN software [6] was used to fit the data. A linear regression model and weighted least-squares regression ($1/x$ and $1/x^2$) were undertaken to check the suitability of the model.

2.7. Stability of processed samples

Drug-free serum was spiked at low (10 ng ml^{-1}), medium (50 ng ml^{-1}) and high (200 ng ml^{-1}) concentrations of the calibration standards and extracted as outlined above. The residues were reconstituted in the mobile phase. The reconstituted samples of the respective concentrations were combined and divided into 15 tubes. One set of the reconstituted samples at low, medium and high concentrations was assayed in quadruplicate immediately after distribution. The remaining 3 sets of samples were stored at −20°C. The remaining samples were assayed in replicates ($n = 3$ or 4) after 48, 72 and 192 h of storage and their concentrations were determined with the respective serum 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.

2.8. Stability during freeze–thaw cycles

To evaluate the impact of freeze–thaw cycles, spiked controls ($n = 4 \times 4$ assays $= 16$) in the matrix were prepared at low, medium and high concentrations. One set of spiked samples at the 3 concentrations was assayed in quadruplicate as outlined above with serum calibration standards, without being subjected to the freeze–thaw cycle and the results were referred as standard (100%). The remaining 3 sets of samples were frozen at −20°C for future studies. On the second occasion, the 3 sets of samples were taken out and allowed to stand at room temperature for 30 min. One set of samples, consisting of low, medium and high concentrations of CDRI-85/287, were assayed in quadruplicate with a freshly spiked and processed serum calibration standard and the remaining 2 sets of samples were stored frozen at −20°C. This freeze–thaw assay cycle was repeated two more times. The results of these three assays were compared with the standard and expressed as percent deviation.

2.9. Recovery

Drug-free serum was spiked with the compound at 3 concentrations (10, 50 and 200 ng ml^{-1}) of the calibration standards. The samples were chromatographed as described above and their concentrations were estimated from the regression of the analytical standard solution calibration curve. The recovery was calculated from:

$$\%\ \text{Recovery} = (\text{Mean measured value}/\text{Theoretical value}) \times 100. \quad (1)$$

2.10. Accuracy and precision

To assess accuracy and precision, replicates ($n = 16$) of the spiked samples were prepared at low, medium and high concentrations. The samples, in quadruplicate, were assayed by the proposed method with serum calibration standards. The concentrations of the compound in these spiked samples were estimated from the regression of the calibration curve. The between-day variation was assessed over a period of 15 days.

2.11. Degradation of CDRI-85/287

Acid and base hydrolysis of the compound was carried out to identify the degradation product or putative metabolite(s) of the compound.

2.12. Acid hydrolysis

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. 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 into the HPLC.

2.13. Base hydrolysis

The compound (5 mg) was refluxed with a methanolic solution of potassium hydroxide (2 ml, 5% w/v) for 24 h. The pH of the reaction mixture was adjusted to neutral with a methanolic solution of hydrochloric acid (1 M). An aliquot of this neutral reaction mixture was diluted with the mobile phase and injected into the HPLC.

3. Results and discussion

The method described here involves double extraction of serum samples with a mixture of hexane and iso-propanol (98:2 v/v), at an alkaline pH and with the use of UV detection at 332 nm.

The ultraviolet absorption spectrum of the compound in the mobile phase showed three bands (λ_{max}): 245 nm ($\varepsilon = 228 \times 10^2$), 297.8 nm ($\varepsilon = 140 \times 10^2$) and 332 nm ($\varepsilon = 155 \times 10^2$). A λ_{max} of 332 nm was selected because at this value the serum endogenous impurities interfere less than when at the more sensitive λ_{max} of 246 nm.

Representative chromatograms of a standard containing 250 ng ml^{-1} CDRI-85/287 (A); an extract of compound-free rat serum (B); serum containing 50 ng ml^{-1} CDRI-85/287 (C); rat serum taken at 4 h (D); and 24 h (E) after a 5 mg kg^{-1} oral dose of CDRI-85/287 are depicted in Fig. 2. Double extraction was essential to enrich the recovery of the compound. The residue, after evaporation of the organic layer, was reconstituted in 0.1 ml of the mobile phase and due to this 5-fold concentration of the sample, the lower limit of quantitation (LLOQ) could be achieved at 5 ng ml^{-1}. Moreover, the extraction procedure and chromatographic conditions yielded a clean chromatogram for the compound (Fig. 2). The endogenous impurities eluting before and after the elution of CDRI-85/287 did not interfere.

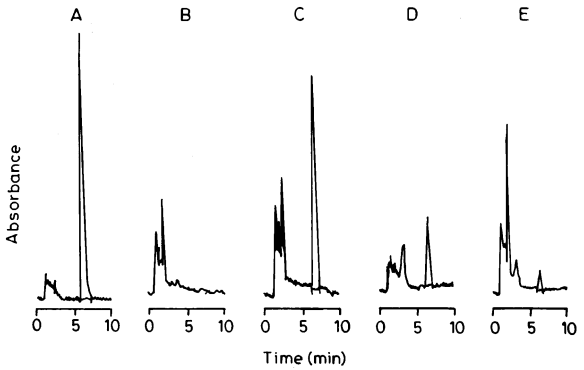

Fig. 2. Chromatograms of: (A) standard containing 250 ng ml^{-1} of CDRI-85/287; (B) drug-free serum; (C) serum containing 50 ng ml^{-1} of CDRI-85/287; (D) rat serum sample taken 4 h; and (E) 24 h postdose of CDRI-85/287.

All criteria commonly employed during the validation of HPLC methods were assessed. These criteria were linearity, accuracy, precision, sensitivity, freeze–thaw stability and in-process stability of the compound in the matrix.

To verify the linearity of the peak height responses, a calibration curve was created by plotting peak heights of the compound against their corresponding concentrations in spiked serum. Linear least-square regression analysis of the calibration curve showed excellent agreement between the peak heights of CDRI-85/287 versus their corresponding concentrations in spiked serum over the range 5–200 ng ml^{-1}. The residuals did not improve much with weighted least-squares regression ($1/x$ and $1/x^2$). The best-fit line was determined by least-squares regression analysis and found to have $Y = 17.48\ x \pm 24.92$ ($r = 0.999$); where Y is the peak height, x is the concentration in ng ml^{-1} and r is the correlation coefficient.

The recoveries (calculated as percent recovery) of the compound from spiked serum samples was calculated from

$$\%\ \text{Recovery} = (\text{Mean measured value/Theoretical value}) \times 100$$

at low (10 ng ml^{-1}), medium (50 ng ml^{-1}) and high (200 ng ml^{-1}) concentration levels with the

Table 1
Absolute recovery of CDRI-85/287 from spiked serum

Concentration (ng ml^{-1})		Recovery (%)	CV (%)
Theoretical	Observed		
10	7.65 ± 0.62	76.48	8.14
50	42.35 ± 3.57	84.70	8.43
200	161.21 ± 12.41	80.61	7.70

standard curve constructed from the analytical standards of the compound prepared in mobile phase (Table 1). The recovery ranged from 76.47–84.70% with the coefficients of variation less than 8.5%.

The accuracy and precision of this method are summarized in Table 2. Accuracy was assessed by comparing the concentrations of the spiked control samples, estimated from the calibration curve, to their true value. An acceptance limit of 15%, despite the concentration level in the calibration range [7], was applied for validation. Percent bias (accuracy) was calculated by

$$\% \text{ Bias} = (\text{Mean measured value} - \text{Theoretical value}) \times 100/\text{Theoretical value}. \quad (2)$$

The percent biases of the calculated values were well within the acceptance limit of 15%.

Precision was estimated in terms of coefficient of variation (CV) [8–10] of intra- and inter-assay at low, medium and high concentration levels. Acceptable intra- and inter-assay precision was obtained with CV values of 1.74–7.64% at the 3 concentrations levels of the compound (Table 2).

Table 2
Accuracy and precision of the assay

	Spiked control (ng ml^{-1})		
	Low	Medium	High
Theoretical (ng ml^{-1})	10	50	200
Mean (ng ml^{-1})	10.60	54.69	210.93
% Bias	6.00	9.38	5.47
$CV_{\text{within assay}}$	5.66	5.50	4.52
$CV_{\text{between assay}}$	7.64	4.13	1.74

The freeze–thaw assay stability study and in-process stability of CDRI-85/287 at low, medium and high concentration levels in the matrix were assessed [8–10]. The results of the first day analysis were considered as the reference and the subsequent results of the freeze–thaw study were compared with the first day result by the analysis of variance (ANOVA) using the STATIS-II program [11]. The results, up to four freeze–thaw assay cycles, showed no apparent differences in the calculated spiked concentrations (Fig. 3B) and indicated that the compound was stable under these conditions.

The ruggedness of this method was tested by varying the concentrations of CDRI-85/287 and changing the time for analysis after reconstitution. It was found that varying the concentrations of the compound did not change the analytical results. If the reconstituted samples were allowed to stand at -20°C, the variations in the concentrations were within the limit of 85 h (Fig. 3A). However, significant differences in the calculated concentrations were observed after 198 h of reconstitution. The errors are inherent to the analysis and are within the acceptable limit.

The compound was stable upon both acid and base hydrolysis and no additional chromatographic peaks were observed after analyzing the diluted reaction mixture. Therefore, the assay method was validated only for the parent compound.

3.1. Application of the method

The assay method described here was applied to a preclinical pharmacokinetic study of the compound CDRI-85/287 in female rats after a single 5 mg kg^{-1} oral dose. The method was sensitive enough to follow the compound up to 24 h post dose.

4. Conclusions

A method has been validated for the determination of compound CDRI-85/287. The extraction procedure was easy and required a simple pH

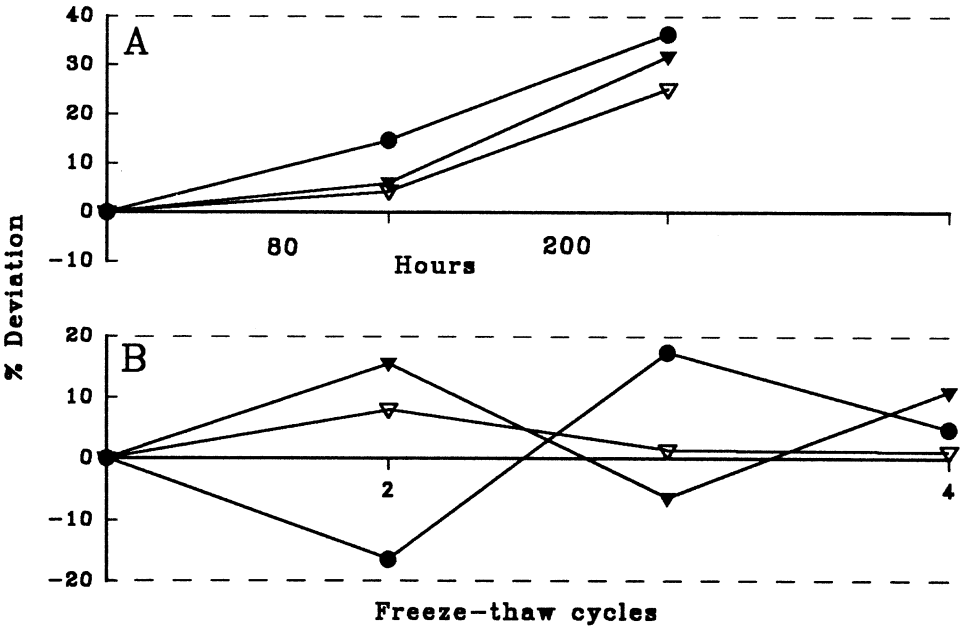

Fig. 3. Stability of compound: (A) in processed samples; and (B) during freeze–thaw cycle, at low (●, 10 ng ml^{-1}), medium (▼, 50 ng ml^{-1}) and high (▽, 200 ng ml^{-1}), of CDRI-85/287.

adjustment before extraction of the compound. The moderate analysis time (12 min), together with rapid evaporation of extraction solvent, allowed rapid analysis. The HPLC method provided reproducible estimates of the compound in rat serum with sufficient sensitivity to allow pharmacokinetic and bioavailability study. The method is now in routine use in our laboratory.

Acknowledgements

The authors gratefully acknowledge the encouragement of V.P. Kamboj, Director, Central Drug Research Institute, Lucknow.

References

[1] R.S. Kapil, S. Durani, J.D. Dhar, B.S. Setty, Indian Patent No. 1031/DEL/89, 1989.
[2] S. Sreenivasulu, M.M. Singh, A. Dwiwedi, B.S. Setty, V.P. Kamboj, Contraception 45 (1992) 81–92.
[3] N. Durani, R. Jain, A. Saeed, S. Durani, D.K. Dikshit, R.S. Kapil, J. Med. Chem. 32 (1989) 1700–1707.
[4] J.D. Dhar, B.S. Setty, S. Durani, R.S. Kapil, Contraception 44 (1991) 461–471.
[5] J.D. Dhar, A. Dwiwedi, A. Srivastava, B.S. Setty, Contraception 49 (1994) 609–616.
[6] PCNONLIN (version 4), SCI Software, Lexington, KY.
[7] R. DeAngelis, J. Hubbel, L. Kanics et al., Burroughs Wellcome, Research Triangle Park, NC (NAIC/91/001), 1992, pp. 1–49.
[8] A.J. Pateman, in: H.H. Blume, K.K. Midha (Eds.), Bio' 94, Bio-International 2, Bioavailability, Bioequivalence and Pharmacokinetic Studies, Medpharm Scientific, Stuttgart, Germany, 1995, pp. 348–398.
[9] R.D. McDowall, C. Hartman, W. Penninckx, Y.V. Heyden, P. Vankeerberghen, D.L. Massart, in: H.H. Blume, K.K. Midha (Eds.), Bio' 94, Bio-International 2, Bioavailability, Bioequivalence and Pharmacokinetic Studies, Medpharm Scientific, Stuttgart, Germany, 1995, pp. 331–336.
[10] J.W. Hooper, in: H.H. Blume and K.K. Midha (Eds.), Bio' 94, Bio-International 2, Bioavailability, Bioequivalence and Pharmacokinetic Studies, Medpharm Scientific, Stuttgart, Germany, 1995, pp. 348–398.
[11] A.W. Kelman. STATIS II (version 2.1), Statistical Package, Stobhill General Hospital, Glasgow, UK.