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Original Article

SIMPLE AND RAPID HPLC METHOD DETERMINATION OF CSR1 AND CSR2, NEW HETEROCYCLIC THIAZOLIDINEDIONE DERIVATIVES, IN RAT PLASMA

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ABSTRACT

Objective: This study aimed at developing a simple and rapid high-performance liquid chromatography (HPLC) method for determination of two thiazolidinedione derivatives, which were developed as anti-proliferative moieties (CSR1 and CSR2) in rat plasma. In addition, their oral pharmacokinetics were studied.

Methods: Drugs were recovered from plasma using acetonitrile and analyzed on a Kromasil C8 column (250 mm × 4.6 mm; 4 µm). HPLC running conditions (0.01M phosphate buffer [pH = 3.0]; flow rate, 0.9 ml/min; at 210 nm; run time, 17 min) were optimized and further used for the determination of pharmacokinetic parameters.

Results: At the described chromatographic conditions, CSR1, CSR2 and internal standard (metformin) eluted at 10.44, 9.41, and 3.15 min, respectively. The calibration curves were linear over the range of 0.25–20 µg/ml, with a correlation coefficient>0.999. The quantification limit was 0.25 µg/ml. Within- and between-day precision values were less than 15%. The developed HPLC method was successfully used to study the pharmacokinetics of CSR1 and CSR2 in rats. The developed method was successfully used to study the pharmacokinetics of CSR1 and CSR2. Cmax, $AUC_{0\text{-}12}$, Tmax, $t_{1/2}$ for CSR1 were 12.2±1.9 µg/ml, 65.34±0.12 µg h/ml, 4.07±0.23 h, $t_{1/2}$= 4.54±0.12 h, respectively, and those for CSR2 were 10.6±2.2 µg/ml, 62.45±0.31 µg h/ml, 3.56±0.23 h, 3.86±0.09 h, respectively.

Conclusion: A specific, linear, and reproducible method was successfully developed and implemented to determine pharmacokinetic activity for two thiazolidinedione derivatives (CSR1 and CSR2), which have been shown to have significant anti-proliferative activity.

Keywords: Bioanalytical method, Pharmacokinetics, Validation, Thiazolidinedione, HPLC

INTRODUCTION

The thiazolidinedione (TZD) class of peroxisome proliferator activated receptor γ (PPARγ) ligands are predominantly known for their insulin sensitization activity and also exhibit anticancer activity. Although use of TZDs has been controversial owing to their marked side effects at diabetic doses, it is important to note that there is a 3-order magnitude discrepancy between the concentration required to mediate antitumor effects and that for PPARγ activation [1, 2]. The toxicities of TZDs have also been attributed to the full activation of the receptor that eventually activates the genes responsible for the effects [3]. As in previous studies on PPARγ agonists such as efatutazone (Daiichi Sankyo), telmisartan, netoglitazone, α-aryloxy-α-methyl hydroxy cinnamic acid, and α-benzyl propionic acid derivatives, which were found to be effective against various cancer cell lines [4-6], we also prioritized on PPARγ agonism and fewer side effects when searching for novel PPARγ agonists. Thus, to overcome the toxicity issues related to full PPARγ agonists, we also reported novel TZDs and their bioisosteres, the oxazolone derivatives [2, 3]. These novel TZD molecules were designed by altering the structure of the well-known antidiabetic drug, pioglitazone. The TZDs synthesized and reported by us exhibited anticancer activity independently of PPARγ.

Of the various synthesized derivatives, two TZD derivatives (CSR1 and CSR2) (fig. 1) had potential anti-proliferative activity on breast cancer (MCF-7), oral cancer (GURAV), and chronic myeloid leukemia cell lines (K-562), with GI_{50} values ranging from 0.23 to 65µM. The antiproliferative potential of both the molecules was higher on the K-562 cell lines, with GI_{50} values of 0.9 µM (CSR1) and 0.23µM (CSR2) [7]. Moreover, both molecules arrested the cell cycle in the G0/G1 phase at a concentration of 10 µM. Furthermore, both molecules reduced cyclin D1 and proliferative cell nuclear antigen (PCNA) levels in a dose-and time-dependent manner, as determined using SDS PAGE analysis (data not published). *In vivo* tumor regression analysis of these two molecules is underway.

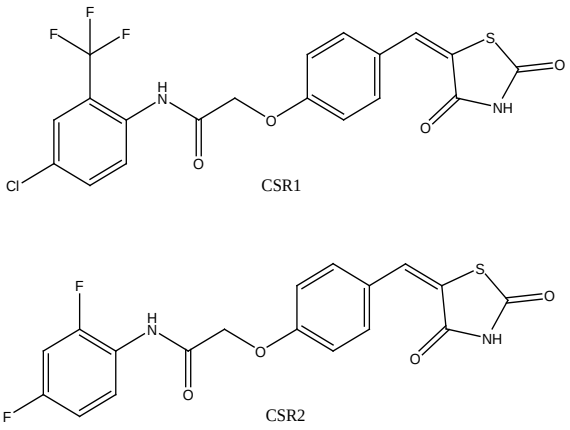


Fig. 1: Chemical structures of CSR1 and CSR2

Pre-clinical pharmacokinetic characteristics can help predict drug behavior. However, CSR1 and CSR2 pharmacokinetics have not been reported. Therefore, we developed a robust assay for pharmacokinetic study of CSR1 and CSR2.

In this study, we developed a high-performance liquid chromatography (HPLC) method using rat plasma; furthermore, representative plasma concentration versus time profiles of CSR1 and CSR2 are presented in this paper.

MATERIALS AND METHODS

Chemicals and reagents

CSR1 and CSR2 were synthesized as per reported scheme [8]. The synthesized compounds were purified by recrystallization with ethanol

and characterized by 1H NMR, ^{13}C NMR and mass spectrometry. Metformin (Supplier: Aarti Drugs Ltd., India; Batch No.: MET/14090518) was provided as a gift sample by Meyer Organics (Mumbai, India). HPLC grade acetonitrile and methanol were obtained from SD Fine Chemicals. Analytical grade diethyl ether and ethyl acetate were purchased from Thermo Fisher Scientific India Pvt. Ltd.

Animals and diet

Male Wistar rats weighing 150-170 g (National Institute of Nutrition, Hyderabad, India), were housed in a temperature-controlled (21±2 °C) room with 12 hour light-dark cycle. The experimental protocol was approved by Institutional Animal Ethical Committee as per the guidance of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (IAEC/PR/2014-2015/03), Ministry of Forest and Environment, Government of India, New Delhi, India. Prior to the experiment, rats were acclimatized for at least one week. During the acclimatization and study period, animals were maintained on normal chow diet.

Instrumentation

The HPLC system used was HPLC Shimadzu LC-2010C HT, series equipped with a 0.1 to 100 µl sample loop, and LC-100 UV detector and a PDA detector. The output signal was monitored and integrated using LC Solution® version 1.2 software.

Chromatographic conditions

Chromatographic separation was achieved using a Kromasil C8 column (250 mm × 4.6 mm; 4 µm particle size). The method involved use of 0.01M phosphate buffer (A) with a pH 3.0, adjusted with orthophosphoric acid and acetonitrile (B) as mobile phase. The mobile phase was prepared by initially passing through a 0.45 µm filter followed by ultrasonication for 15 min. The gradient program was (Time/%B)–0/2, 5/70, 10/98, 13/2, 17/2, with 0.9 ml/min flow rate 20 µl as sample volume. Detection was performed at 210 nm.

Standard solutions

CSR1, CSR2, metformin (IS) standard solution were prepared by dissolving 10 mg of drug in 10 ml methanol to give a final concentration of 1000 µg/ml. A serial dilution was carried out for CSR1 and CSR2 to give 0.25, 0.5, 1, 2, 5, 10 and 20 µg/ml standard solutions. Stock solution of IS was diluted to achieve a final concentration of 50 µg/ml.

Sample preparation

Ten microliter of standard solutions of CSR1, CSR2 and metformin (internal standard) were spiked to a 70 µl of plasma sample in a microcentrifuge tube and vortex mixed for 5 seconds. Later on, as determined from recovery studies, 900 µl of acetonitrile was added for liquid-liquid extraction. The microcentrifuge tubes were then vortex mixed for 40 seconds and centrifuged at 4000 rpm for 10 min. The supernatant was transferred to a microcentrifuge tube and was evaporated to dryness using nitrogen gas. The dried microcentrifuge tubes which contained the extracted drugs were reconstituted with 100 µl of mobile phase of which 20 µl was used for analysis. For pharmacokinetic studies, the same procedure was followed, and 20 µl of reconstituted supernatant was injected to HPLC system.

Method validation

Calibration curve and lower limit of quantification (LLOQ)

Calibration curve was obtained by spiking series of standard solutions of CSR1 and CSR2 (0.25, 0.5, 1, 2, 5, 10 and 20 µg/ml) along with IS in rat plasma. The lowest concentration of the analyte that gives at least 5 times the response as compared with a blank was considered as the Lower Limit of Quantification (LLOQ). The drugs were extracted as described in sample preparation method. The procedure was repeated three times for the said chromatographic method. The peak areas ratios of CSR1 and CSR2 to IS (Y axis) were plotted against the corresponding concentrations (X axis).

Recovery studies

To determine highest recovery of drug in a particular solvent after protein precipitation, four different solvents were used i.e. acetonitrile, diethyl ether, ethyl acetate and methanol. A typical set for each solvent consisted of a zero sample (plasma with IS) and plasma sample spiked with CSR1 and CSR2 at three different concentrations (0.5, 5 and 20 µg/ml) and metformin (IS) (50 µg/ml). Ten microliter of CSR1 and CSR2 standard solutions and 10 µl of IS solution were added to a 70 µl of plasma sample in a microcentrifuge tubes. Liquid-liquid extraction was performed according to the method described for sample preparation. Reconstituted 20 µl of sample from respective sets of solvent was injected into the HPLC system. The experiment was performed in triplicate. The peak areas ratios of CSR1 and CSR2 to IS were compared among various solvents used.

Accuracy and precision

Accuracy and precision of this method was established by within-day and between-day analysis. Three different concentrations (0.25, 2 and 20 µg/ml) of CSR1 and CSR2 were assayed on six consecutive days. The procedure was repeated three times for the said concentrations of CSR1 and CSR2.

Sample collection

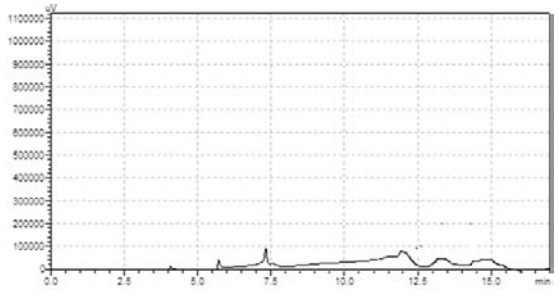
To test the applicability of bioanalytical method developed, the plasma concentration of CSR1 and CSR2 was measured in rat plasma after administration of a single oral dose of CSR1 (250 mg/kg) and CSR2 (250 mg/kg) in overnight fasted animals. The dose of CSR1 and CSR2 was selected based on maximum tolerated dose (MTD) study. The CSR1 and CSR2 were found to be safe up to 2500 mg/kg and one-tenth of MTD dose was used to study pharmacokinetic data. Suspensions of CSR1 and CSR2 were freshly prepared in 1% w/v sodium carboxymethyl cellulose (CMC-Na).

Blood samples were collected by retro-orbital puncture into microcentrifuge tubes containing heparin at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following drug administration. The study was carried out by sparse sampling design using three rats for each time point. Blood samples were centrifuged at 2000 g for 10 min, and plasma samples were separated and stored at–20 °C until analysis.

RESULTS AND DISCUSSION

Chromatography conditions

Various mobile phases and columns were tested to determine CSR1 and CSR2. The best result was obtained using a Kromasil C8 column and 0.01M phosphate buffer (pH 3.0) and acetonitrile as the mobile phase.



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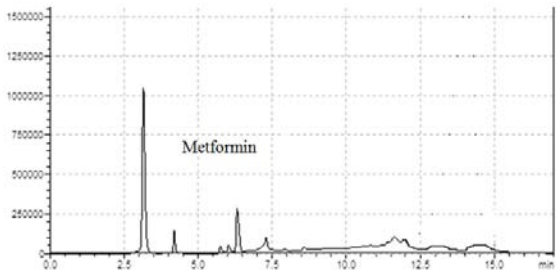

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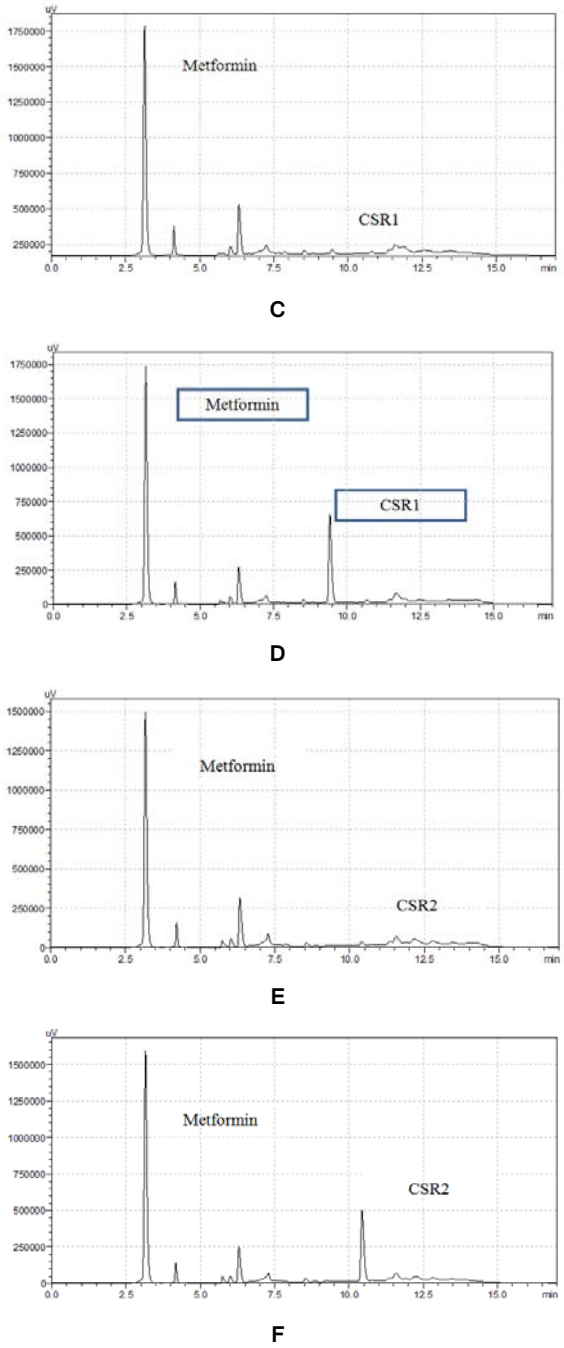

Fig. 2: Chromatogram of A) Blank sample (plasma without IS); B) A zero sample (plasma with IS); C) Plasma spiked with CSR1 (0.25 µg/ml) and D) Plasma spiked with CSR1 (20 µg/ml) E). Plasma spiked with CSR2 (0.25 µg/ml) F) Plasma spiked with CSR2 (20 µg/ml)

Metformin was chosen as the IS, given the suitable retention time and adequate recovery. Under the chromatographic conditions described, metformin, CSR1, and CSR2 eluted at 3.15, 10.44, and 9.41 min, respectively. The proposed method was found to be suitable for quantification of CSR1 and CSR2 in plasma samples. Chromatograms obtained for the blank sample (plasma without IS), zero sample (plasma with IS), and plasma samples spiked with CSR1, CSR2, and IS at lowest and highest concentrations are presented in fig. 2.

A comparison of the chromatogram of the independent blank plasma (A) with that of internal standard (B) and lower limit of quantification of CSR1 and CSR2 (C, D, E and F) illustrates the specificity and selectivity of the method. The baseline was relatively free from drift.

Calibration curve and lower limit of quantification (LLOQ)

Calibration curves were constructed using seven series of plasma samples spiked at concentration levels in the range of 0.25-20 µg/ml. A linear relationship was obtained between the peak area ratios of individual drugs to those of the internal standard at the corresponding concentration, as shown by the equation in fig. 3. The LLOQ was found to be 0.25 µg/ml, with a coefficient of variation (CV) of 10.72%. The linearity of the calibration curve for both CSR1 and CSR2 was validated by the high value of the correlation coefficient (R^2=0.999). The typical standard curve was described using the equation y= 0.0188x+0.0106 for CSR1 and y=0.0183x+0.0071 for CSR2, where y is the peak area and x is the concentration. According to the Food and Drug Administration (FDA) guidelines, the deviation for the calculated concentrations from the nominal concentration should be within 15%, with an exception of 20% for LLOQ. The accuracy and precision of calculated values at each nominal concentration in this study were within the acceptable ranges.

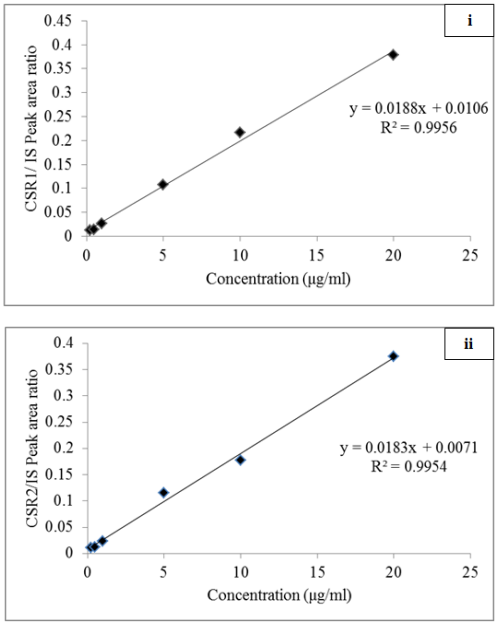

Fig. 3: The calibration curves of CSR1 and CSR2 in rat plasma

Recovery studies

Liquid–liquid extraction of CSR1 and CSR2 was performed using various solvents. Maximal recover was obtained using acetonitrile. The mean±SD recovery of CSR1 at 0.5, 5, and 20 µg/ml was found to be 90.21±2.2%, 92.53±2.5%, and 97.78±1.3%, respectively. Similarly, the mean±SD recovery of CSR2 at 0.5, 5, and 20 µg/ml was found to be 89.23±2.1%, 94.88±2.4%, and 98.21±2.3%, respectively. The mean±SD recovery of the IS at 50 µg/ml was 98.26±2.2% (table 1). The recoveries at all the three concentrations were consistent, precise, and reproducible, indicating the suitability of the liquid extraction procedure for separation of CSR1 and CSR2 from rat plasma.

Accuracy and precision

The accuracy and precision values were determined by using calibration standard curve of CSR1 and CSR2 at 0.25, 2, and 20 µg/ml in plasma on six consecutive days. Within-and between-day results are shown in table 2 with CV values<11.3% and<10.4 for CSR1 and CSR2 respectively. The error (Er) was also found to be<1.84% and<1.83% for CSR1 and CSR2 respectively. The accuracies and precisions for the tested levels were all within the defined acceptance criteria, demonstrating that the method was accurate and precise.

Table 1: Recovery study of CSR1 and CSR2

Concentration (µg/ml)	CSR1 (%)	CSR2 (%)
0.5	90.21±2.2	89.23±2.1
5	92.53±2.5	94.88±2.4
20	97.78±1.3	98.26±2.2

N=3, values are expressed in mean±SD

Table 2: Precision and accuracy estimation of CSR1 and CSR2 in spiked plasma

	CSR1			CSR2		
Concentration added (µg/ml)	**Concentration found (µg/ml)**	**CV (%)**	**Er (%)**	**Concentration found (µg/ml)**	**CV (%)**	**Er (%)**
Within-day						
0.25	0.258±0.008	11.34	1.84	0.257±0.006	10.49	1.83
2	1.960±0.073	3.53	1.03	1.762±0.037	1.76	0.98
20	20.086±0.713	5.23	0.98	19.965±0.346	3.41	1.02
Between-day (n=18)						
0.25	0.248±0.009	10.09	1.23	0.252±0.004	8.36	1.11
2	1.947±0.078	1.56	0.92	1.876±0.065	1.24	0.89
20	20.034±0.648	4.63	0.78	19.867±0.534	3.21	0.73

Within day, N=3; Between-day, N = 18; Concentration found values are expressed in mean±SD; CV=coefficient of variation; Er= Error.

Pharmacokinetic study

The validated assay method was successfully used for oral pharmacokinetic study of CSR1 and CSR2 in rats. The average plasma drug concentration vs. time profile after a single oral administration of CSR1 and CSR2 (250 mg/kg) is shown in fig. 4. The pharmacokinetic parameters (mean±SD) for CSR1 are as follows: Cmax = 12.2±1.9µg/ml, AUC_{0-12} = 65.34±0.12 µg h/ml, Tmax = 4.07±0.23 h, $t_{1/2}$ = 4.54±0.12 h; those for CSR2 are as follows: Cmax = 10.6±2.2 µg/ml, AUC_{0-12} = 62.45±0.31 µg h/ml, Tmax = 3.56±0.23 h, $t_{1/2}$ = 3.86±0.09 h. As mentioned earlier, the molecular design of each of our synthesized molecules (CSR1 and CSR2) resembles that of pioglitazone. Fujita *et al.* reported the following pharmacokinetic parameters of pioglitazone in male rats after single oral administration at 10 mg/kg: Tmax =2.8±1.1 h, Cmax = 13.6±1.9 µg/ml, and $t_{1/2}$ = 2.5±0.4 [9]. Evidently, our molecules have a similar pharmacokinetic profile. Some differences in the values could be due to the structural modifications.

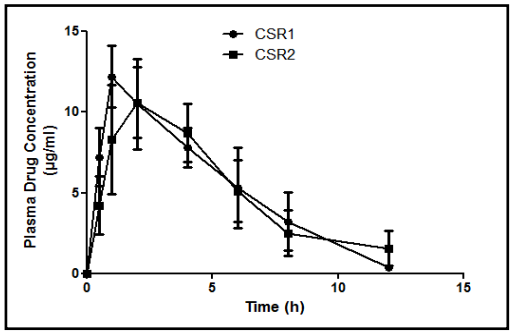

Fig. 4: Mean±SD of plasma concentration-time curve following single oral dose of CSR1 (250 mg/kg) and CSR2 (250 mg/kg) in rats (n=3)

CONCLUSION

We developed a simple and rapid HPLC method for quantification of CSR1 and CSR2 in rat plasma. All results were within the acceptable range, and clear separation of the compounds and IS, without interfering peaks, was obtained. The assay was validated and standard curves were linear over the concentration ranges investigated. The developed method was successfully applied for the analysis of plasma samples from rats for pre-clinical pharmacokinetic studies. This study provides with a better understanding of the pharmacological features of our CSR1 and CSR2, which will be helpful in further development.

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CONFLICT OF INTERESTS

Authors declare no conflict of interest

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