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Short Communication

Determination of ciglitazone in dog plasma by reversed-phase high-performance liquid chromatography

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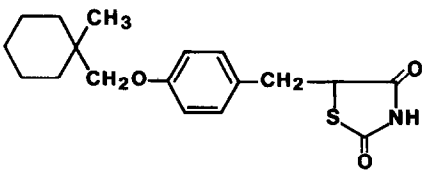
Introduction

Ciglitazone is a thiazolidinedione derivative which was synthesized by Sohda *et al.* [1] and found to have hypoglycaemic activity in insulin-resistant animal models [2, 3]. Torii



et al. [4] studied the absorption of ^{14}C-ciglitazone suspensions and found that the drug was almost completely absorbed in rats and dogs given 30 mg/kg doses. In the dogs, however, the plasma concentration–time curves showed a plateau from 1 to 10 h after dosing which could be indicative of an absorption rate problem. Hirai *et al.* [5] found a significant effect of food on the bioavailability of ciglitazone; absorption from tablets after meal administration was greater than that in the fasting state by a factor of 1.6. To permit our investigation of the biopharmaceutical properties of ciglitazone, a suitable assay for ciglitazone in dog plasma was needed. Several assays employing high-performance liquid chromotography (HPLC) have been developed for ciglitazone. Hirai *et al.* [5] developed an assay for ciglitazone in plasma but a liquid–liquid extraction step was required and an internal standard was not included. Cox and Pullen [6] developed a sensitive assay for ciglitazone and its metabolites, but the assay requires the use of column-switching technology. M.P. Cancro and L.D. Kissinger (unpublished data, Upjohn Co.) developed a simple potency assay for ciglitazone tablets, but this requires

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very long analysis times for plasma samples. The procedure discussed in this communication is simple, quick, and suitable for assaying the large number of ciglitazone plasma samples which are generated in bioavailability studies.

Experimental

Materials

Ciglitazone tablets and standards were received from Takeda Chemical Industries of Japan. Testosterone propionate was a USP reference standard. HPLC solvents were of chromatographic grade. All materials were used as received.

Chromatographic system

A modular chromatographic system was employed. The pump used was either a Beckman 110A (Beckman Instruments, Inc., Arlington Heights, IL, USA) or a Perkin–Elmer Series 4 (Perkin–Elmer Analytical Instruments, Norwalk, CT, USA). A Brownlee 3-cm guard column (Brownlee Labs., Inc., Santa Clara, CA, USA) packed with 10-μm LiChrosorb C_{18} particles was used. The analytical column was a 150 × 4.6 mm i.d. DuPont packed with 5–6-μm Zorbax C_8 (DuPont Instruments, Wilmington, DE, USA). The detector was an Anspec AN-203 fixed-wavelength UV-detector converted to detect 229 nm by a wavelength conversion kit (The Anspec Company, Ann Arbor, MI, USA). The mobile phase was acetonitrile–0.1% acetic acid (65:35, v/v) at a flow rate of 2.0 ml/min. Sample volumes of 75-μl were injected by a WISP 710B auto-injector (Waters Associates, Milford, MA, USA). Analysis time was approximately 15 min.

Procedure

A calibration curve was prepared each time samples were assayed. Ciglitazone standards equivalent to 0.4, 0.8, 2.0, 4.0, 6.0 and 8.0 μg/ml of plasma were prepared in duplicate by adding small volumes of a methanolic solution of ciglitazone (100 μg/ml) to polyethylene microcentrifuge tubes. The methanol was evaporated under nitrogen gas. Aliquots of 200-μl of suitable blank plasma were added and thoroughly mixed.

Samples were prepared by accurately pipetting 200 μl of plasma into 1.5 ml polyethylene microcentrifuge tube. To the plasma samples and standards were added 300 μl of acetonitrile containing about 4 μg/ml testosterone propionate. Samples were immediately mixed to precipitate the proteins, and the tubes were centrifuged for 2 min at 13,000*g* in a microcentrifuge (Fisher Model 235A, Itasca, IL, USA). An aliquot of 75-μl of the supernatant was injected into the chromatographic system. Each standard or sample was injected once only. Standards were randomly injected after every 6–10 samples. The ratios of peak heights for the ciglitazone standards to internal standard peak heights were calculated and plotted against ciglitazone concentration. The concentration of ciglitazone in a sample was determined by interpolation of its peak height ratio on the calibration curve.

Results and Discussion

Based upon preliminary data from Hirai *et al.* [5], ciglitazone plasma concentrations from 125 mg doses to dogs were expected to range from 0.5 to 10 μg/ml. Because of these high plasma concentrations, a plasma assay was developed that required minimal

sample preparation. Plasma proteins were quickly precipitated when the ratio of acetonitrile to plasma was at least 3:2, v/v. Because ciglitazone is strongly retained by reversed phase packings, mobile phases also contained at least 60% v/v acetonitrile. Thus the chromatographic problems anticipated due to an excessive acetonitrile concentration in the plasma supernatant did not materialize. For convenience, 200 μl of plasma was used in the assay, but a different plasma volume could be used if the acetonitrile volume were adjusted accordingly.

Initial chromatographic conditions employed a C_{18} column and yielded satisfactory chromatography. An unacceptably long run time was required with the C_{18} column, however, because of slowly-eluting plasma components. Run times were reduced by using a 15-cm DuPont Zorbax C_8 column. The internal standard used by M.P. Cancro and L.D. Kissinger (unpublished data), testosterone acetate, was found not to be resolved from ciglitazone on the C_8 column. Testosterone propionate, however, was resolved from ciglitazone and was used as the internal standard. Because of the minimal plasma sample clean-up required, a guard cartridge was employed in the assay. Chromatography with several 3-cm Brownlee C_8 guard columns proved unsatisfactory, however, and C_{18} guard cartridges were used thereafter.

Analyte detection was accomplished at 229 nm. The molar absorptivity of ciglitazone at this wavelength is approxiately 15 000 M^{-1} cm^{-1}, which is about five times greater than at 254 nm. Since the internal standard, testosterone propionate, also absorbs well at 229 nm, its concentration in the assay is in the same range as ciglitazone.

Calibration curves were linear for ciglitazone concentrations from 0.5 to 10 μg/ml. As can be seen from Table 1, there was little variation in calibration curves from day to day, the RSD in the calibration curve slope being 1.6%. When the calibration curve was constructed using water instead of plasma and compared to plasma calibration curves,

Table 1
Summary of calibration curves for ciglitazone*

Day	Matrix	Slope (μ/ml)†	Intercept	r^2‡
1	Plasma	0.166	+0.0096	0.991
3†	Plasma	0.163	−0.0084	>0.999
3	Plasma	0.161	−0.013	0.996
4	Plasma	0.163	+0.005	>0.999
5	Plasma	0.158	+0.0124	0.999
6	Plasma	0.160	+0.0029	>0.999
6	Water	0.165	0.0025	>0.999
7	Plasma	0.163	+0.0066	>0.999
8	Plasma	0.164	+0.0178	>0.999
9	Water	0.168	0.0012	>0.999
10	Plasma	0.162	+0.0039	>0.999

*The average slope for plasma calibration curves was 0.162 $(\mu g/ml)^{-1}$ with an RSD of 1.6% (reinjected curve not included). The estimated relative standard errors of individual slopes were less than 4%. The average intercept for plasma calibration curves was 0.0059, which is equivalent to 0.04 μg/ml ciglitazone (reinjected curve not included).

†This represents the reinjection of standards prepared for the curve on day 1.

‡Correlation coefficient.

there was less than a 3% difference in slopes, indicating almost complete recovery of ciglitazone from plasma proteins. Data in Table 1 also show that ciglitazone is stable in the plasma supernate over a 3-day period.

A small plasma interference with ciglitazone was observed with most blank plasma samples, but this interference averaged less than the equivalent of 0.05 μg/ml. There were no apparent plasma interferences with the internal standard. Chromatograms for blank plasma, plasma containing only internal standard and a typical plasma sample are presented in Fig. 1.

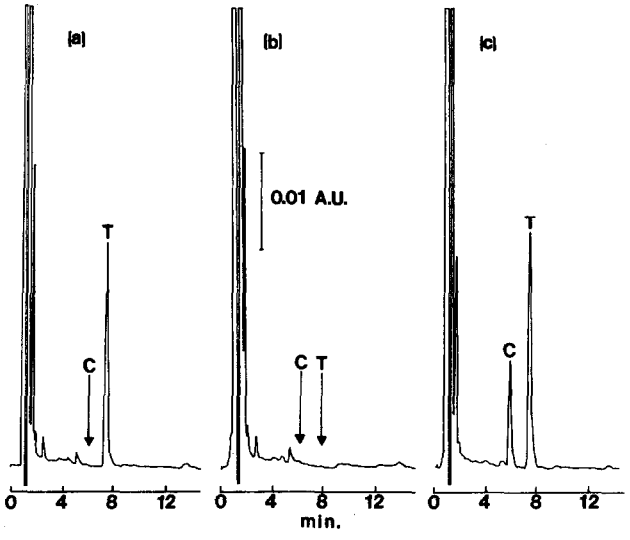

Figure 1
Chromatograms of: (a) blank plasma containing the internal standard, testosterone propionate; (b) blank plasma without added ciglitazone or testosterone propionate; (c) a plasma sample found to contain 3.0 mg/ml ciglitazone.

Precision and accuracy of the assay were evaluated by assaying spiked plasma samples in triplicate over three days. For ciglitazone concentrations of approximately 0.4, 2, 6 and 7.5 μg/ml, the pooled estimates of the relative standard deviations (RSD) were 8.5%, 8.1%, 4.0% and 2.5%, respectively. Mean absolute errors were less than the RSD values. Eight plasma samples obtained in ciglitazone dog studies were also assayed singly on each day over a 3–8 day period. For these samples, ranging in concentration from 1.7 to 7.3 μg/ml, RSD values were independent of concentration and had a mean value of 6.2%. Repeated freezing and thawing of the plasma samples had no apparent adverse effects on assay results.

The specificity of the assay was examined by studying some known metabolites of ciglitazone [7]. It was found that the metabolites eluted much more quickly than ciglitazone and did not interfere with the assay. The 3′- and 4′-hydroxylated metabolites all exhibited capacity factors of *ca* 1.0 while ciglitazone displayed a capacity factor of *ca* 6. The metabolite retention times were so short that they could not be quantitated with this procedure due to plasma interferences. It was not necessary to quantitate the metabolites, however, since it was intended that the assay be used in bioavailability studies comparing oral dosage forms of ciglitazone.

Assay suitability was assessed in ciglitazone dog bioavailability studies [8]. Following 125-mg doses to beagles, peak plasma ciglitazone concentrations were found to be 5–10 μg/ml. Even though ciglitazone levels below 0.5 μg/ml were not quantitated, the assay permitted determination of almost 90% of the total area under the plasma concentration–time curve. Representative plasma concentration–time data are shown in Fig. 2.

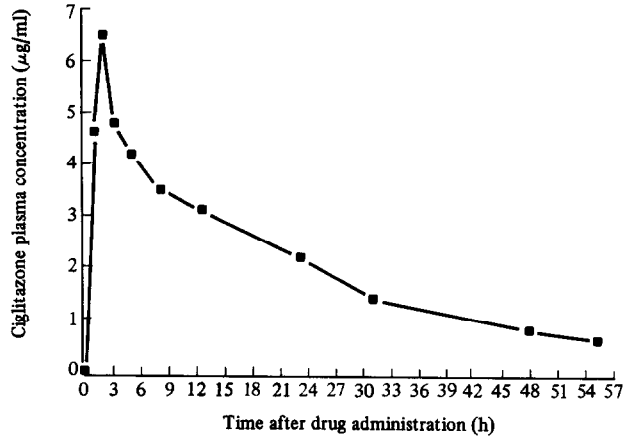

Figure 2
Plasma concentration–time curve for ciglitazone from a dog after administration of a single 125-mg ciglitazone tablet.

Because very small volumes of plasma are used in the assay, the volume of blood removed from a dog during a bioavailability study need not be large. System ruggedness was excellent and allowed the use of one analytical column for over 1000 assays.

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