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DETERMINATION OF PIOGLITAZONE IN DOG SERUM USING SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET (229 nm) DETECTION

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SUMMARY

An analytical method is described for the determination of the free base of pioglitazone hydrochloride (U72, 107A, AD-4833) in dog serum. The method used solid-phase extraction of pioglitazone from serum followed by high-performance liquid chromatographic analysis on an octadecylsilane column with an eluent of acetonitrile–water (41:59, v/v) containing 1.2 ml/l acetic acid (pH 6.0 ± 0.05). The column effluent was monitored at 229 nm. The analytical procedure has a linear range of 25 ng/ml to 20 μg/ml, a minimum quantifiable level of 25 ng/ml, absolute recovery of >90% ($n=15$), and precision of ≦8.8% ($n=45$). The method was used in a preliminary dose proportionality study in the dog.

INTRODUCTION

Pioglitazone hydrochloride, 5-{4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl}-2,4-thiazolidinedione hydrochloride salt (U72, 107A, AD-4833), is being developed as an antidiabetic agent. Pioglitazone has been shown to have beneficial effects by improving the insulin resistant state of KKAy diabetic mice and may have potential to be a glucose-normalizing agent for non-insulin-dependent diabetes mellitus (NIDDM) [1,2]. NIDDM is one of the most common metabolic diseases still lacking fully effective therapy and is characterized by subnormal tissue responsiveness to insulin resulting in excessive hyperglycemia [3]. In KKAy mice, pioglitazone produces hypoglycemia and hypoinsulinemia, strongly suggesting that the drug enhances tissue responsiveness to insulin.

To support the bioavailability and pharmacokinetic evaluations necessary

for the drug development, a high-performance liquid chromatographic (HPLC) method with a solid-phase extraction procedure has been developed for the quantitative determination of pioglitazone in serum. The method offers the advantage of simplicity with adequate sensitivity, selectivity, precision, accuracy and stability. The analytical method is being used to evaluate the pharmacokinetic and bioavailability characteristics of pioglitazone in animal models.

EXPERIMENTAL

Chemicals and reagents

Pioglitazone hydrochloride (Fig. 1) was provided by Upjohn (Kalamazoo, MI, U.S.A.). The internal standard (I.S., AD-4875) (Fig. 1) was obtained from Takeda Chemical Industries (Osaka, Japan). HPLC-grade acetonitrile and hexane were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetic acid, ammonium hydroxide, and potassium phosphate (dibasic) were of analytical reagent grade and purchased from Mallinckrodt (Paris, KY, U.S.A.). Purified water was produced by a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.).

Instrumental parameters

The HPLC system consisted of an LDC ConstraMetric III pump coupled with an LDC UV Monitor III ultraviolet detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) with a 50-μl loop mounted on an autoinjector, and a Linear Model 585 recorder (Linear Instruments, Irvine, CA, U.S.A.). The analytical column employed was an IBM ODS column (250$\times$4.6 mm I.D., 5 μm particle size) (IBM Instruments, Danbury, CT, U.S.A. or Jones Chromatography, Littleton, CO, U.S.A.) protected by a guard column (Pelliguard ODS, 50 mm$\times$2.1 mm I.D., 32 μm) (Whatman, Clifton, NJ, U.S.A.). The mobile phase for the isocratic reversed-phase chromatography was acetonitrile–water (41:59, v/v)

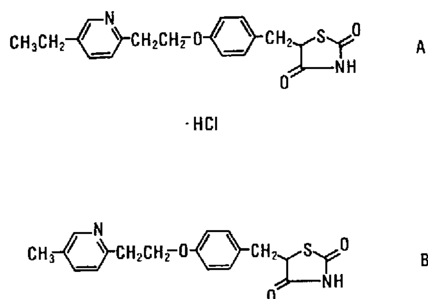

Fig. 1. Structure of pioglitazone hydrochloride (A) and the I.S. (B).

containing 1.2 ml/l acetic acid with a final pH of 6.0±0.05 [adjusted with a 40% (w/v) ammonium hydroxide solution] and was filtered and helium-degassed prior to use. The chromatographic system was operated at 21–23°C with an eluent flow-rate of 1.0 ml/min. The UV absorbance of column effluent was monitored at 229 nm with 0.02 a.u.f.s. sensitivity.

Quantification was accomplished by peak-height ratio analysis using AD-4875, an analogue of pioglitazone, as the assay I.S. Chromatographic peak heights were integrated by a Harris computer system. The standard curve, along with a statistical evaluation of standards linear fit, was computed by a linear regression program. The unknown concentrations were determined by inverse prediction against the standard curve.

Preparation of standards

Stock solution of pioglitazone was prepared by dissolving 10.5 mg of pioglitazone hydrochloride in 100 ml of acetonitrile–water (50:50, v/v) to give a concentration of 100 μg/ml pioglitazone free base. The I.S. stock solution was prepared using a similar procedure to yield a concentration of 100 μg/ml. Stock solutions were stored at 4°C.

Serum standards were prepared by aliquoting appropriate volumes of pioglitazone stock solution and 50 μl I.S. stock solution into 1 ml of control dog serum (drug-free) to produce a concentration series of 25, 50, 100, 200 and 500 ng/ml and 1, 2, 5, 10, 15, and 20 μg/ml pioglitazone and 5 μg/ml I.S. Reference standards were prepared using acetonitrile–water (30:70, v/v) to give a concentration series similar to the serum standards.

Sample preparation

Unknown samples were prepared by combining 1 ml of serum sample and 50 μl of I.S. stock solution in a disposable glass culture tube (75 mm×10 mm) and mixing briefly. A C_{18} solid-phase extraction (SPE) column (100 mg/ml, Analytichem International, Harbor City, CA, U.S.A.) was used for serum extraction. Twelve SPE columns, placed on the vacuum extraction manifold (Supelco, Bellefonte, PA, U.S.A.) were prewashed with 2 ml of acetonitrile followed by 2 ml of 0.1 *M* K_2HPO_4 solution. The serum samples and serum standards were transferred from the glass culture tubes onto individual SPE columns and were loaded on the column using slight vacuum (approximately 86.4 kPa). After the columns were vacuum-aspirated (approximately 26.6 kPa) for 5 min, 2 ml of 0.1 *M* K_2HPO_4 solution followed by 50 μl of hexane were applied to wash each column. The columns were dried with vacuum aspiration (approximately 26.6 kPa) for 10 min. Pioglitazone and the I.S. were eluted from the column with 300 μl of acetonitrile by applying a slow uniform pressure to the top of the column using nitrogen gas (about 0.2 kg/cm^2). Each eluate was mixed with 700 μl of purified water, and 50 μl of the mixture were injected onto the HPLC system for analysis.

RESULTS AND DISCUSSION

HPLC characteristics

Initial evaluations on the HPLC conditions employed an ODS column with a mobile phase of acetonitrile–0.05 *M* K_2HPO_4 (50:50, v/v). The pH of the mobile phase was found to effect both the retention time and the peak shape of pioglitazone. When the HPLC eluent was adjusted to pH 4 or 5, the retention time of pioglitazone was greater than 16 min and the peak tailed. At pH 3.0, elution was at 12 min without improvement in peak shape. At pH levels of 5.5 to 7.0, a very sharp peak with no apparent tailing was obtained, and the elution time was 6 min. A mobile phase of acetonitrile–water (41:59, v/v) containing 1.2 ml/l acetic acid (adjusted to pH 6 $\pm$ 0.05 with 40% ammonium hydroxide) was selected. This eluent gave sharp, symmetric, and well resolved peaks for pioglitazone and the I.S. with retention times ranging from 13.8 to 14.2 and 9.4 to 9.8 min, respectively.

Fig. 2 presents typical chromatograms of an extract of control (drug-free) dog serum (a), dog serum fortified with pioglitazone and I.S. (b), and a dog serum sample collected 2 h after administration of a pioglitazone oral solution (6.3 mg/kg) (c). The control dog serum chromatogram is free from extracted serum endogenous materials at the retention times of pioglitazone and I.S. Three potential drug metabolite peaks with retention times of 3.8, 4.3, and 6.2 min, respectively, were observed in the post-dose dog serum sample. These peaks did not interfere chromatographically with either the I.S. or pioglitazone.

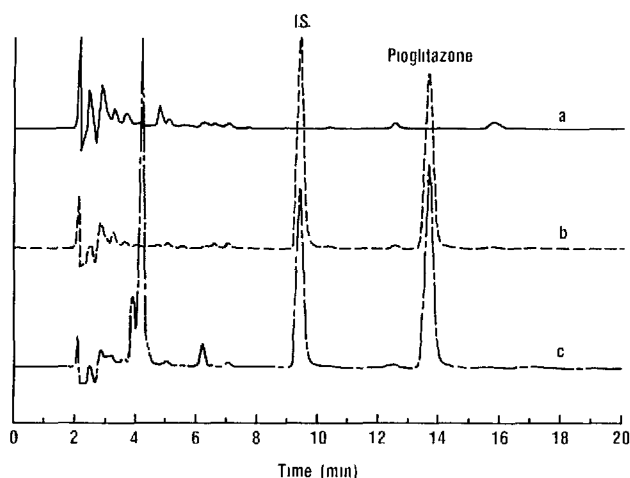

Fig 2. Typical chromatograms of extracts of control (drug-free) dog serum (a), dog serum fortified with pioglitazone and I.S. (b), and a dog serum sample collected 2 h after administration of a pioglitazone oral solution (6.3 mg/kg) (c).

Extraction efficiency

The absolute extraction recoveries were determined at concentrations of 100 ng/ml and 5 and 15 μg/ml for pioglitazone and 5 μg/ml for I.S. Samples at each concentration were extracted and analyzed in five replicates. The results were compared to the peak heights obtained from reference standards containing the corresponding concentrations of pioglitazone and I.S. The average absolute recoveries for pioglitazone and I.S. ranged from 90.2 to 99.7% (mean ± S.D. 93.2 ± 3.0%, concentration-independent, $n=15$) and 90.4 to 95.4% (mean ± S.D. 93.4 ± 2.0%, $n=5$), respectively, as shown in Table I. Several elution solvents as well as the eluate amount used for solid-phase extraction were evaluated for better recovery [4]. Acetone was the best elution solvent, i.e., highest recovery of pioglitazone and I.S.; however, it was considered unacceptable because of poor HPLC profiles of dog serum extracts. The recoveries of pioglitazone and I.S. were also increased with additional amounts of solvent; however, the elution of non-polar serum endogenous components (retention times from 20 to 30 min) occurred with the larger eluate volumes. Therefore, a 300-μl volume of acetonitrile was selected as the best compromise, giving an absolute recovery of 90% for both pioglitazone and I.S., HPLC profiles free of endogenous components at the elution position of pioglitazone and I.S., and with low levels of late-eluting compounds. The 50-μl hexane wash was found to be important for drying the SPE column, thus, improving the extraction recovery and preventing the elution of non-polar serum endogenous components.

Method validation

Linearity. Twelve dog serum standards and reference standards containing 25 ng/ml to 20 μg/ml pioglitazone were prepared and analyzed on four different days. The serum and reference standard curves were linear from 25 ng/ml to 20 μg/ml with the mean correlation coefficients greater than 0.999 ($n=4$). The regression equations for the serum and reference standard curves were $y = (1.62 \cdot 10^{-4} \pm 2.3 \cdot 10^{-6})\,x - (2.43 \cdot 10^{-3} \pm 2.74 \cdot 10^{-3})$ ($n=4$) and $y =$

TABLE I

EXTRACTION RECOVERY OF PIOGLITAZONE AND INTERNAL STANDARD

Compound	Concentration	Extraction recovery (mean ± S.D., $n=5$) (%)
Pioglitazone	100 ng/ml	93.5 ± 3.7
	5 μg/ml	93.6 ± 3.0
	15 μg/ml	92.5 ± 2.6
AD-4875 (I.S.)	5 μg/ml	93.4 ± 2.0

$(1.64\cdot10^{-4} \pm 3.0\cdot10^{-6}x + (1.62\cdot10^{-3} \pm 1.46\cdot10^{-3})$ ($n=4$), respectively. No apparent differences were observed between serum standard curves and reference standard curves. Thus, a reference standard curve can also be used for calibration. The intercepts were not significantly different ($p > 0.05$) from zero for each of the standard curves. Thus, a forced through the origin model was used for linear regression analysis.

Sensitivity. The sensitivity of the method was evaluated by analyzing serum samples at the presumed minimum quantifiable level in five replicates. Based upon the precision and accuracy results shown in Table II, a conservative minimum quantifiable level of 25 ng/ml was chosen for pioglitazone. The detection limit, based on a signal-to-noise ratio of 3:1, was 10 ng/ml. Since the method produced a clean chromatogram at the elution position of pioglitazone and I.S., the sensitivity may be improved by either decreasing the final volume or increasing the injection amount.

Precision and accuracy. The precision and accuracy of the method were evaluated at concentrations of 25, 50, and 100 ng/ml and 5 and 15 μg/ml pioglitazone in serum. The intra-assay reproducibility was determined by analyzing five fortified serum samples at each concentration on the same day. The inter-assay reproducibility was obtained by analyzing one fortified serum sample at each concentration on four days over a period of four weeks. The results are summarized in Table II and show excellent precision and accuracy as indicated by the low values of the coefficient of variation (C.V.) and the percentage deviation between theoretical and measured concentrations. The intra-assay and inter-assay precision (C.V.) ranged from 0.9 to 8.8% (mean 3.1%, $n=25$) and 1.4 to 7.3% (mean 3.7%, $n=20$), respectively. The accuracy (percentage deviation) ranged from +0.1 to 11.0% (mean +2.5%, $n=45$).

Stability. The stability of pioglitazone in serum samples maintained at 21–23°C (room temperature) for 24 h and at −20°C for one and four weeks was

TABLE II

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY DATA

Precision = coefficient of variation = S.D./mean × 100. Accuracy = percentage deviation (measured − theoretical)/theoretical × 100.

Theoretical concentration	Intra-assay ($n=5$)		Inter-assay ($n=4$)	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
25 ng/ml	8.8	+1.6	5.0	−11.0
50 ng/ml	3.2	+2.6	2.8	+4.0
100 ng/ml	1.5	+0.8	7.3	+0.1
5 μg/ml	1.1	+3.4	1.8	+0.9
15 μg/ml	0.9	+0.1	1.4	+0.6

TABLE III

STABILITY OF PIOGLITAZONE IN SERUM SAMPLE

Theoretical concentration	Measured concentration (mean ± S.D., $n=5$)			
	Fresh	24 h (22°C)	1 week (−20°C)	4 week (−20°C)
100 ng/ml	100.8 ± 1.5	101.8 ± 1.9	100.4 ± 3.6	101.3 ± 2.4
5 μg/ml	5.168 ± 0.058	5.011 ± 0.122	5.057 ± 0.123	5.066 ± 0.048
15 μg/ml	14.99 ± 0.13	15.16 ± 0.14	15.14 ± 0.07	15.00 ± 0.11

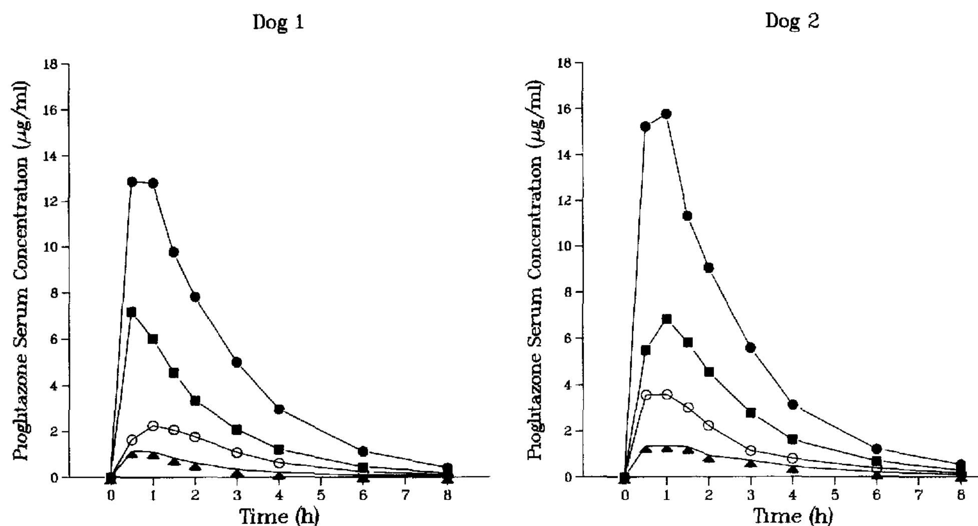

Fig. 3. Pioglitazone serum concentration-time profiles obtained in two dogs receiving a 100-mg (●), 50-mg (■), 25-mg (○), and 10 mg (▲) single oral dose of pioglitazone given as 50 ml of oral solution (using 0.5 *M* citric acid as vehicle).

evaluated. No significant decrease in concentrations was detected for pioglitazone as illustrated in Table III. Reference standards and extracted serum samples were stable for at least three weeks when stored at 4°C.

Application

The developed method has been successfully applied for the determination of pioglitazone serum concentrations in samples obtained from beagle dogs in a preliminary dose proportionality study. Two dogs each received four dose levels (100, 50, 25, and 10 mg) of a pioglitazone oral solution (50 ml) with at least a two-day washout period between doses. Blood samples (5 ml) were collected by venipuncture at 0 (predose), 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 h after dosing. The serum was harvested by centrifugation (4°C) at 1250 *g* for

TABLE IV

SELECTED PHARMACOKINETIC PARAMETERS

Dog No.	Dose (mg/kg)	C_{max} (μg/ml)	T_{max} (h)	AUC (μg·h/ml)	AUC/dose[a]	k (h^{-1})	$t_{1/2}$ (h)
1	6.30	12.88	0.5	36.51	5.80	0.49	1.42
	3.21	7.20	0.5	16.63	5.18	0.52	1.34
	1.60	2.25	1.0	7.02	4.40	0.49	1.41
	0.62	1.16	0.5	2.90	4.68	0.51	1.35
2	7.20	15.76	1.0	42.23	5.86	0.48	1.43
	3.66	6.84	1.0	19.94	5.45	0.46	1.49
	1.90	3.58	1.0	10.33	5.44	0.44	1.55
	0.70	1.37	1.0	4.03	5.75	0.40	1.71

[a]AUC/dose = dose-normalised AUC.

20 min. The pioglitazone serum concentration–time curves for each dose level in two dogs are shown in Fig. 3. Selected pharmacokinetic parameters were calculated using non-compartmental techniques [5] and are listed in Table IV. The relationships between area under the serum concentration–time curve from time zero to infinity (AUC) and dose (mg/kg) were represented by equations of $y = 6.01x - 1.87$ and $y = 5.90x - 0.74$ with correlation coefficients of 0.998 and 0.999 for dog 1 and 2, respectively. For the four doses, AUC values were approximately proportional to the dose administered based on the similar dose-normalized AUC values (AUC/dose value ranged from 4.4 to 5.8 for dog 1 and from 5.4 to 5.9 for dog 2) as indicated in Table IV. Dose level also had no apparent effect on the time (T_{max}) to reach the highest pioglitazone serum concentration (C_{max}), and the apparent terminal disposition rate constant (k) and its half-life ($t_{1/2}$) as shown in Table IV. Thus, the disposition profiles are apparently independent of the dose over the evaluated range (10–100 mg).

CONCLUSION

An analytical method for the quantitative determination of pioglitazone in serum has been developed and validated. The method has good chromatographic resolution of the parent drug and I.S. from endogenous serum components and potential metabolites, adequate sensitivity, accuracy, and reproducibility as well as simplicity and rapidity in sample preparation. The method has been successfully employed for the analysis of serum samples obtained from dogs in a preliminary dose proportionality study. Results suggest that orally administered pioglitazone hydrochloride is absorbed into the systemic circulation, displays dose proportionality over a dose range of 10–100 mg, and has dose-independent disposition characteristics. The developed method will

be used to support definitive pharmacokinetic and bioavailability evaluations of pioglitazone hydrochloride.

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