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Automated high-performance liquid chromatography method for the determination of rosiglitazone in human plasma

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Abstract

A robust, fully automated assay procedure for the determination of rosiglitazone (**I**, BRL-49653) in human plasma has been developed. Plasma concentrations of **I** were determined using automated sequential trace enrichment of dialysates (ASTED) coupled to reversed-phase high-performance liquid chromatography. Sequential automated dialysis of human plasma samples was followed by concentration of the dialysate by trace enrichment on a C_{18} cartridge. Drug and internal standard, SB-204882 (**II**) were eluted from the trace enrichment cartridge by mobile phase (0.01 *M* ammonium acetate, pH 8–acetonitrile, 65:35, v/v) onto the HPLC column (a Novapak C_{18}, 4 μm, 100×5 mm radial compression cartridge) protected by a Guard-Pak C_{18} cartridge. The compounds were detected by fluorescence detection, using an excitation wavelength of 247 nm, and emission wavelength of 367 nm. The lower limit of quantitation of the method was 3 ng/ml (200 μl aliquot) with linearity demonstrated up to 100 ng/ml. Within- and between-run precision and accuracy of determination were better than 10% across the calibration range. There was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles and samples can be safely stored for at least 7 months at −20°C. This method has been successfully utilised to provide pharmacokinetic data throughout the clinical development of rosiglitazone. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Rosiglitazone

1. Introduction

Rosiglitazone (**I**, BRL-49653) (Fig. 1), an aminopyridyl thiazolidinedione, is a very potent synthetic peroxisome proliferator-activated receptor (PPAR)-γ agonist and effective antidiabetic agent [1,2]. It exerts its glucose-lowering effects by increasing insulin sensitivity in liver and peripheral tissues. Rosiglitazone has received regulatory approval for the treatment of type 2 diabetes as both monotherapy (USA) and in combination with other oral antidiabetic agents (USA and Europe). The drug is highly bound to plasma proteins (99.8%) and is primarily eliminated via metabolism in the liver by cytochrome P450 isoenzyme 2C8. Following oral administration, rosiglitazone is rapidly absorbed (T_{max} 1 to 2 h) with an elimination half-life of approximately 3 to 5 h [3–5]. Rosiglitazone has a pK_{a1} value of 6.08 and pK_{a2} value of 6.80.

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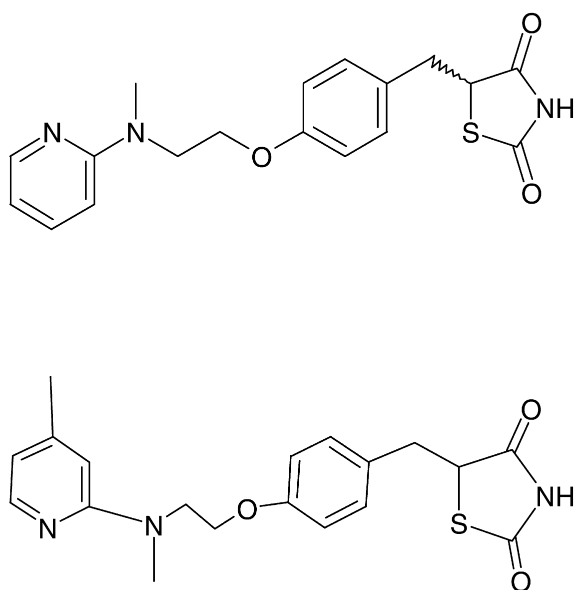

Fig. 1. Chemical structures for rosiglitazone (**I**, upper) and the internal standard SB-204882 (**II**, lower).

The early method for the analysis of **I** in human plasma involved isolation of the analyte through manual solid-phase extraction, followed by analysis with high-performance liquid chromatography (HPLC) using fluorescence detection [6]. Whilst this method was suitable for initial clinical support where the number of samples for each study did not typically exceed 300, it was considered too resource intensive for application to a complete clinical development program. This paper describes a fully automated method for quantitative analysis of **I** in clinical studies using on-line dialysis and concentration of the dialysate by trace-enrichment on a reversed-phase HPLC cartridge. Trace enrichment is a well known technique for the isolation of analytes from biological fluids, described first in 1985 [7,8] and in subsequent reports [9–15]. It was made commercially available by Gilson as the ASTED (automated sequential trace enrichment of dialysates) system in the 1980s. This paper describes the development and validation of this automated analytical procedure for **I** in human plasma. The method exhibited excellent ruggedness and was successfully applied to the analysis of clinical samples from healthy volunteers and patients.

2. Experimental

2.1. Chemicals and reagents

Analytical-reagent grade ammonia (ca. 25%, w/v), ammonium acetate and glacial acetic acid were obtained from BDH (Poole, UK). Acetic acid (special for chromatography grade) and GPR-grade dimethylsulfoxide and hydrochloric acid were also purchased from BDH. Acetonitrile (Super Purity 210 grade), methanol (Super Purity 205 grade) and toluene (Super Purity grade) were purchased from Romil (Loughborough, UK). Ultra-pure water, Elgastat Maxima, was obtained from Elga Products (High Wycombe, UK). SurfaSil was purchased from Pierce (Rockford, IL, USA). Rosiglitazone, {5-(4-[2-(*N*-methyl-*N*-(2-pyridyl)amino)ethoxy]benzyl)-thiazolidine-2,4-dione} (**I**, Fig. 1), and the internal standard (I.S.) SB-204882 {5-(4-[2-(*N*-methyl-*N*-(4-methyl-2-pyridyl)amino)ethoxy]benzyl)-thiazolidine-2,4-dione} (**II**, Fig. 1) were supplied by SmithKline Beecham, UK.

2.2. Preparation of calibration standards and validation samples

Calibration standards ranging from 1 to 100 ng/ml were prepared daily by spiking 1 ml aliquots of control human plasma with working solutions of **I**. Validation samples were similarly prepared from independent stock solutions (separate weighing) at nominal concentrations of 3, 10, 50 and 100 ng/ml. The validation samples were thoroughly mixed, centrifuged and dispensed into 200 μl aliquots in polypropylene microvials and were stored frozen (−20°C) until analysed.

2.3. Instrumentation

The Gilson ASTED system (Anachem, Luton, UK) comprised of a 231 autosampling injector and two 401 dilutors fitted with 1 ml syringes. The dialyser block, 370 μl donor chamber size PCTFE (Kel-F), was used with premounted cellulose (Cuprophan) dialysis membranes, 15 000 molecular mass cut-off (Anachem). Stainless steel trace enrichment cartridges, 5.8×4.6 mm, 70 mg packing, 10 μm particle size, ODS (Anachem) replaced the injector

loop of a Rheodyne 7010 injection valve (Rheodyne, Cotati, CA, USA). The HPLC system consisted of a Merck–Hitachi Model L-6000 pump (Hitachi, Tokyo, Japan) operated in isocratic mode at a flow-rate of 1.0 ml/min, and a Perkin-Elmer Model LC-240 fluorescence detector (Beaconsfield, UK). Communication with the HPLC and ASTED system, and integration of the chromatographic peaks was carried out by the Waters 860 data acquisition system using Expert Ease v3.1 software (Waters, Watford, UK).

2.4. Optimised ASTED sample preparation

Prior to analysis, plasma samples were centrifuged (5 min, approximately 2000 *g*). Calibration standards, control blanks and validation samples were spiked with 25 μl of the working solution of the internal standard **II**. Each sample (200 μl) was acidified with 200 μl of 0.05 *M* HCl, and the samples were capped and thoroughly vortex-mixed. Analysis was completely automated using the ASTED system. Each sample was mixed on-line with 80 μl of acetonitrile, and 490 μl of the total volume was loaded into the donor chamber which was equilibrated with ammonium acetate buffer (0.01 *M*, pH 2.7). The sample was held static while a total of 4000 μl of recipient buffer (0.01 *M* ammonium acetate, pH 3.7) was continuously moved through the dialysis chamber and onto the trace enrichment cartridge (TEC). The analytes were eluted from the TEC to the analytical column using the HPLC mobile phase. As the analysis was proceeding on the analytical column, the TEC was re-equilibrated with 500 μl of the recipient buffer, and the dialysis cells and tubing were flushed with 8000 μl of the respective donor and recipient buffers.

2.5. Optimised chromatography

The chromatographic analysis was performed at ambient temperature on a Novapak C_{18} radial compression cartridge (4 μm particle size, pore size 60 Å, 100×5 mm) fitted with a Novapak C_{18} Guard Pak insert (4 μm particle size, 5×5 mm) (Millipore, Milford, MA, USA). The drug and its internal standard were eluted with mobile phase composed of ammonium acetate (0.01 *M* adjusted to pH 8.0)–acetonitrile (65:35, v/v). The mobile phase was degassed with helium before use. Typical retention times for **I** and **II** were 5.0 min and 6.5 min, respectively (Fig. 2), from the time the TEC was switched on-line. Peaks were detected using fluorescence at an excitation wavelength of 247 nm and an emission wavelength of 367 nm.

2.6. Quantification and validation

Calibration standards were analysed at the beginning of each run. Based upon analysis of residuals, a weighted fit (1/peak area ratio) linear regression was used to construct a calibration line for the peak area ratio of analyte to I.S. versus analyte concentration. The calibration lines were used to calculate test analyte concentrations by interpolation.

Four pools of human plasma containing 3, 10, 50 and 100 ng/ml were prepared from a fresh stock solution of **I**. Six replicate standards from each pool were extracted and analysed in each of three separate analytical runs. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. The within-run precision was estimated using the mean and the standard deviation (S.D.) of the six replicate results. The between-run precision was estimated by calculating the ratio of the standard deviation of the within-run means to the average of the within-run means, and expressed as a percentage. Accuracy was estimated as the ratio of the mean concentration by analysis to the nominal concentration, and expressed as a percentage. Overall accuracy was reported as the average of the individual run accuracy estimations.

3. Results and discussion

3.1. Method optimisation

The HPLC–fluorescence conditions described here were previously developed for the earlier solid-phase extraction-based method [6] for the determination of **I** in plasma, and were found to be robust and reliable. Development work indicated that UV absorbance (247 nm) and fluorescence (excitation 247 nm, emission 367 nm) both gave similar sensitivity

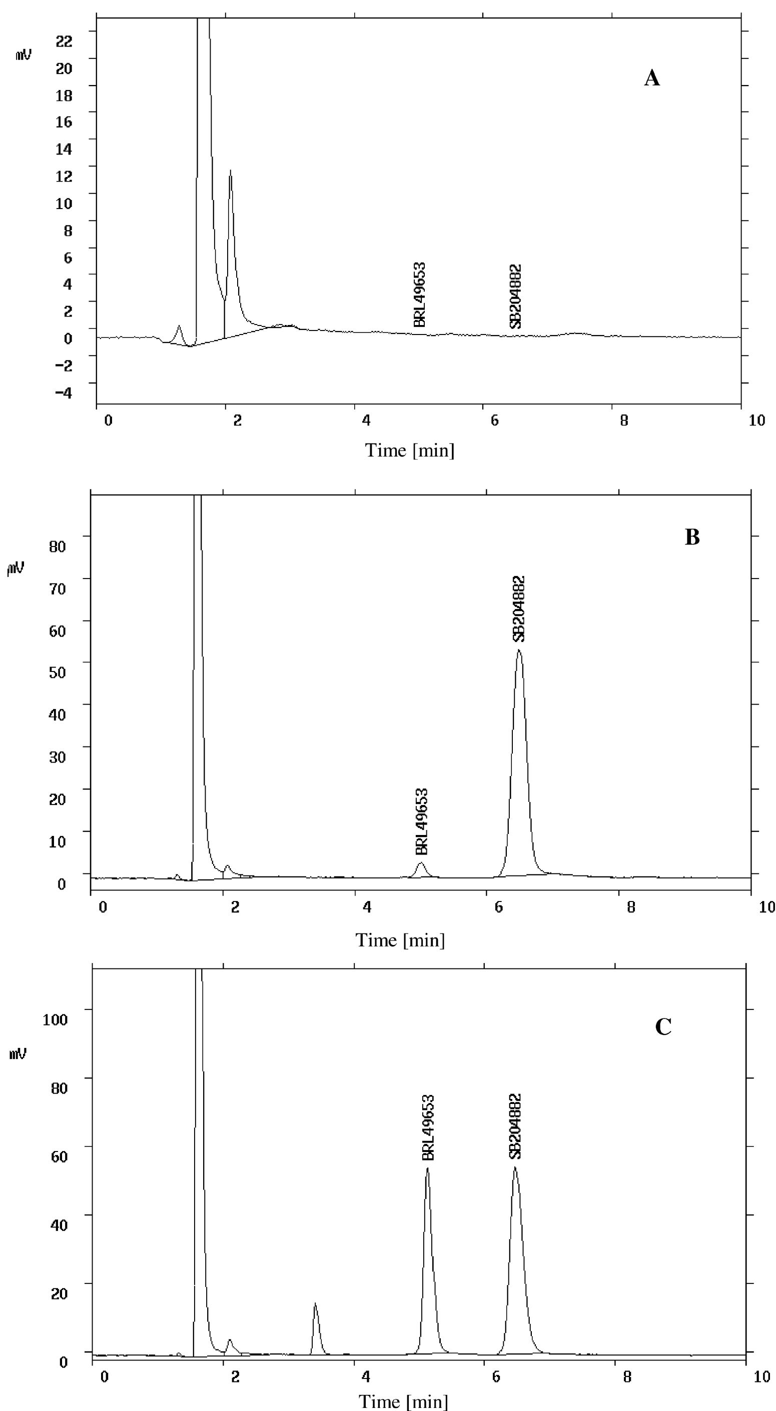

Fig. 2. Representative chromatograms: (A) drug-free control plasma depicting the respective retention times of rosiglitazone (BRL-49653) and SB-204882; (B) control plasma fortified with 3 ng/ml of rosiglitazone (the LLOQ) and 125 ng/ml of the internal standard, SB-204882; (C) a clinical plasma sample taken from a volunteer 2 h after a 1 mg oral dose of rosiglitazone (quantitated to be 52.2 ng/ml). A small peak from a putative metabolite is observed at ca. 3.5 min.

for detection of **I**. However, fluorescence was chosen because of its greater selectivity. The fluorescent response of **I** was induced at pH 8, and mobile phase of this pH also gave good chromatographic separation.

The manufacturers (Millipore) of the HPLC column recommend a mobile phase of pH 3.5 to 6.5 to maintain column longevity. However, in practice it was found that column life at pH 8.0 was generally sufficient to allow one column to be used for a typical clinical study (200–300 samples; approx. 5–6 analytical batches).

Ammonium acetate (0.01 *M*, pH 8) was initially selected as the recipient buffer in the ASTED system, for its compatibility with the mobile phase. By evaluating a range of pH values for the donor buffer it was found that lower pH gave the best recovery of **I**. The packing chosen for the TEC was C_{18}, as it was known that **I** had affinity for this phase and would also be efficiently eluted by the mobile phase.

Equilibrium dialysis is frequently used to study protein binding and determine free analyte concentrations in biological samples [16]. In human plasma, **I** is known to be highly protein bound (99.8%) mainly to serum albumin. It was therefore important to recognise that this could affect the diffusion rate in different matrices across the semi-permeable membrane. In order to minimise this possibility, and improve recovery, both trichloroacetic acid and acetonitrile were evaluated for their ability to disrupt the protein binding but not cause sufficient precipitation to result in the blockage of the needle or the tubing. Acetonitrile gave the best recovery and a small volume (80 μl) was added to all samples.

Contamination by carry-over was initially a significant problem, especially after the injection of aqueous standards. Increasing the period of flow of mobile phase through the TEC had no effect, hence it was believed that the problem was partially due to the accumulation of the drug on the membrane and tubing.

Acidification of the sample by addition of HCl (0.05 *M*), as well as the acetonitrile, was found to dramatically reduce the carry-over effect and also improve recovery of the analyte. Subsequently, it was found that decreasing the pH of the donor buffer to pH 2.7 resulted in almost total elimination of the carry-over (>0.5%). However, the recipient buffer could only be lowered to pH 3.7 before deterioration of the chromatography occurred.

The 370 μl dialysis block was chosen, rather than the 100 μl dialyser block in order to be able to dialyse an adequate sample volume to reach the level of sensitivity required. The mode of dialysis chosen was to keep the sample static in the donor channel, while continuously moving the recipient buffer in order to maintain the concentration gradient and maximise recovery by dialysis. The volume of recipient buffer used (4000 μl) was optimised for maximum recovery while avoiding breakthrough of the compound from the TEC. Likewise the speed of dialysis (610 μl/min) was optimised to allow sufficient dialysis while keeping the sample processing time to a practical length. The methods used for optimisation of dialysis and trace enrichment have been previously discussed in this journal [10] and will not be described here.

In order to maintain good method performance, the membrane and TEC were replaced regularly (at least weekly). After every study, or if the system was to be unused for more than a couple of days, the system was purged with sodium hydroxide (2 *M*) in order to remove built up contamination. Tubing was replaced at regular intervals (2–3 monthly) or whenever a build up of material was observed on the inner surfaces of the tubing.

3.2. Method validation

Since the method was required to support clinical pharmacokinetic studies, the assay had to be precise with a relative standard deviation (RSD) less than 20% at the lower limit of quantification (LLOQ, 3 ng/ml) and better than 15% throughout the higher concentration range. Accuracy (±15% bias) and linearity had also to be demonstrated throughout the required range. Recovery of **I** and its internal standard **II** from human plasma were estimated to ensure reproducibility of the dialysis process. The assay was shown to be linear from 1 to 100 ng/ml using 200 μl aliquots of human plasma. Back-calculated calibration concentrations showed accuracies ranging from 97.9 to 105.3% using a weighted linear regression model (1/peak area ratio) with a correlation coefficient (*r*) typically greater than 0.997. Specifically, the

Table 1
Summary of pre-study within- and between-run precision, and accuracy for rosiglitazone

Nominal concentration (ng/ml)		Day 1	Day 2	Day 3	Average within-run precision (%)	Between-run precision (%)	Average accuracy (%)
3	Mean	2.94	3.03	3.29	8.1	5.9	102.9
	SD	0.37	0.21	0.15			
	RSD (%)	12.4	7.1	4.7			
	Accuracy (%)	98.1	101.0	109.7			
	n	6	6	6			
10	Mean	8.72	10.00	10.15	3.5	8.1	96.2
	SD	0.45	0.34	0.19			
	RSD (%)	5.2	3.4	1.9			
	Accuracy (%)	87.2	100.0	101.5			
	n	6	6	6			
50	Mean	47.46	51.15	50.51	2.8	4.0	99.4
	SD	2.45	0.62	1.05			
	RSD (%)	5.2	1.2	2.1			
	Accuracy (%)	94.9	102.3	101.0			
	n	6	6	6			
100	Mean	86.62	103.71	103.58	3.0	10.0	98.0
	SD	2.99	2.76	2.83			
	RSD (%)	3.5	2.7	2.7			
	Accuracy (%)	86.6	103.7	103.6			
	n	6	6	6			

within- and between-run precision values for the assay were better than 10% at all concentrations studied (Table 1). Mean accuracy was also better than 10% of nominal at the concentrations studied (Table 1).

The mean absolute recoveries of **I** from 200 μl aliquots of human plasma at concentrations of 3 and 100 ng/ml were determined to be 66.3% and 61.7%, respectively, with no evidence of concentration dependent recovery. The mean absolute recovery for the internal standard **II** was found to be 57.9%. Based on the analysis of control drug-free samples ($n > 100$), endogenous components of plasma did not interfere with either the drug or the internal standard (Fig. 2A). The retention times for both **I** and **II** were very reproducible, even over the course of studies as large as 200 to 300 samples using the same analytical column. For example, over six runs the mean retention time (±SD) for **I** was 4.8 min (±0.3) and for **II** was 6.0 min (±0.5).

Stability of **I** in unextracted human plasma stored at ambient room temperature has been demonstrated with less than a 5% change in concentration over a 24 h period. Similarly, the compound has been shown to be stable through three freeze–thaw cycles and for up to 7 months at approximately −20°C.

3.3. Application of the assay

Using the ASTED system, approximately 7 samples/h can be processed with minimal manual intervention running unattended for most of a 24 h period. Centrifugation of the plasma samples was found to enhance the ruggedness of the assay by reducing the instances of plugging of the ASTED system from particulates and debris in plasma. A representative chromatogram from a clinical subject administered **I** orally is shown in Fig. 2C. A putative drug related peak eluting approximately 1.5 min earlier than **I** (Fig. 2C) was typically observed in clinical samples from subjects dosed orally with **I**. This peak was always completely resolved from the peaks of interest, and its peak area appeared to be proportional to the administered dose. No equivalent

peak was observed in ex vivo spiked plasma samples, even after lengthy storage. At the time the assay was first used to support clinical studies, LC–MS and LC–MS–MS data had identified one major metabolite circulating in human plasma, namely SB-237216, the *n*-demethylated metabolite of rosiglitazone {(±)-5-[(4-{2[pyridinylamino]ethoxy}phenyl)methyl]-2,4 thiazolidinedione}. When analysed under the same assay conditions using fluorescence detection, an aqueous standard of SB-237216 was found to have the same retention time as the unknown peak obtained in the authentic plasma samples, therefore it is possible to speculate that this is the identity of the analyte peak observed. As the *n*-desmethyl metabolite was shown to be approximately 100-fold less potent in vivo than rosiglitazone, concentrations of SB-237216 were not determined in clinical samples.

Representative plasma concentration versus time profiles following oral (8 mg) administration of **I** to two subjects are presented in Fig. 3. Samples above the upper limit of quantification (ULOQ, 100 ng/ml) were diluted into the linear range of the assay using drug-free human plasma. Subsequently this method was successfully revalidated over an extended linear range of 5 to 1000 ng/ml, by simple modification of the sample: internal standard volume ratio. Precision and accuracy over the extended range, determined from validation quality control samples during a three-run validation were 4.2% and 99.5%, respectively at the LLQ (5 ng/ml) and 1.9% and 96.1%, respectively at the ULOQ (1000 ng/ml). The assay demonstrated appropriate sensitivity and robustness to adequately determine pharmacokinetic parameters in a range of clinical studies.

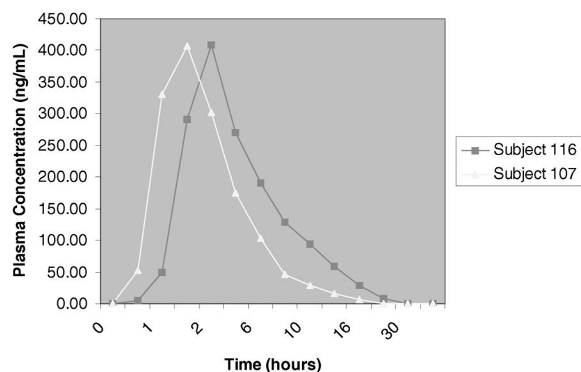

Fig. 3. Plasma concentration versus time profiles for two subjects administered an 8 mg oral tablet of rosiglitazone.

4. Conclusions

An automated HPLC–fluorescence assay procedure for the determination of **I** in human plasma has been developed. It has been shown to be accurate, precise and reliable in routine use. The method has been successfully used to provide pharmacokinetic data in support of a large number and range of clinical pharmacokinetic studies. There was no evidence of instability of **I** in human plasma following three freeze–thaw cycles or after long term storage at −20°C.

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