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Factorial design for the development of automated solid-phase extraction in the 96-well format for determination of tesaglitazar, in plasma, by liquid chromatography–mass spectrometry

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Abstract

An analytical method was developed for the determination, in blood plasma, of a novel peroxisome proliferator-activated receptor (PPAR) agonist drug, tesaglitazar. The drug and the isotope labelled internal standard were isolated by solid-phase extraction (SPE) on hexylsilica, separated by reversed-phase liquid chromatography and quantified by tandem mass spectrometry. Factorial design and a robotic sample processor were employed in the exploration and optimisation of the SPE procedure in the 96-well format. This allowed rapid development of the method, notably limiting the process to four experiments before validation. The detectability was greatly improved by utilising the formation of sodium adducts in atmospheric pressure positive ionisation mass spectrometry. Absolute recovery was more than 95% with a coefficient of variation of 5% at a level of 8.7 n*M*. The accuracy and precision of the automated SPE method presented here matched the excellence of the previously used method based on manual liquid–liquid extraction. Furthermore, the method resulted in an increased sample throughput.

Keywords: Factorial design; Tesaglitazar

1. Introduction

Tesaglitazar is a novel peroxisome proliferator-activated receptor (PPAR) α/γ agonist, that binds and activates both PPARα and PPARγ. It is being developed to target insulin resistance-related glucose and lipid abnormalities associated with type 2 diabetes and insulin resistance syndrome. Tesaglitazar is a dihydrocinnamate derivative and is structurally different to the thiazolidinediones (a class of drugs which includes the PPARγ agonists troglitazone, pioglitazone and rosiglitazone). For our preclinical investigation studies of tesaglitazar, the sample work-up employed was manual liquid–liquid extraction (LLE). The quantitative determination was performed by liquid chromatography (LC) with fluorometric detection, but we switched to mass spectrometry (MS) detection during the clinical phase in order to enhance selectivity and sensitivity.

Looking to the future, and the prospect of large clinical studies, automation of the sample preparation process became important and solid-phase extraction (SPE) in a 96-well format was evaluated as a means to improve sample throughput. In recent years the automation of SPE methods has been frequently used

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and thoroughly investigated [1–4]. Similarly, LLE methods in 96-well format have also been presented as exemplified in Refs [5–7]. In our opinion the most advantageous feature of SPE relative to LLE is that the mixing of two immiscible liquid phases with subsequent phase separation, which is not as easy to automate efficiently, is avoided. The advantages of the 96-well format over the single cartridge SPE format are that (1) the 96 parallel extractions enhance the sample throughput of the SPE substantially and (2) the 96-well columns are of smaller dimensions, giving less void volumes in frits and spigots and therefore less band broadening in the elution step. This makes it worthwhile to use low sorbent bed masses. However, the development of SPE methods can be tedious and labour intensive, with a great deal of trial and error involved [8] since usually only one parameter is optimised in each experiment.

Method development is much faster if many parameters can be optimised simultaneously, as in factorial design. This is a means to more easily find optimal conditions when many parameters, possibly interconnected, affect the results. In factorial design, two or more values are chosen to represent the range of variation of each factor and all possible combinations of these values are applied. Hannah et al. [9] and Wells et al. [10] have used this approach to optimise SPE for determination of pesticides. In the present study, we applied larger factorial-design experiments than Hannah et al. and the design was used to optimise SPE for determination of a drug in blood plasma. Factorial design may result in an extensive number of experiments, and is therefore often reduced to make the experiments more feasible to perform [11–14]. The extensive amount of experiments in full factorial designs and the expert knowledge needed to reduce them may explain why factorial design has not been used more frequently in the development of SPE methods. However, the problem of performing a large number of experiments is less demanding if appropriate automation equipment is utilised during the method development. This will also avoid the additional work required to automate an existing method initially optimised for manual use [15,16].

In the present study, a robotic sample processor with eight pipetting needles was used in combination with the recently introduced modular 96-well format in which different SPE materials can be arranged as desired. Others have discussed the benefits of modular 96-well plates [17,18]. The reproducibility in the SPE process was enhanced by the use of a recently published method of flow control using a small pressure difference [19]. The modular plate enabled simultaneous optimisation of sorbent choice and SPE process variables. The high pipetting capacity of the robotic sample processor made it possible to utilise full factorial designed experiments with complex pipetting schemes in the development of an automated SPE method for the determination of tesaglitazar in plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The detectability in the MS/MS was promoted by the choice of a suitable LC mobile phase composition, which gave rise to sodium adducts as precursor ions and product ions, as previously reported [20–23]. The LC–MS/MS method was used for hundreds of plasma samples from preclinical and early clinical studies.

2. Experimental

2.1. Chemicals, reagents and materials

Tesaglitazar, *S*-enantiomer ($M_r = 408.5$), (Fig. 1) and the isotope labelled internal standard AR-H040156XX-[D_5], as a racemic mixture, were synthesised at Medicinal Chemistry, AstraZeneca R&D (Mölndal, Sweden). Acetonitrile and methanol were of HPLC grade (Rathburn, Walkerburn, UK). Formic acid, hydrochloric acid (Titrisol), ammonium acetate (NH_4Ac), NaH_2PO_4, and Na_2HPO_4, were of analytical grade (Merck, Darmstadt, Germany). Sodium acetate was Sigma ultra grade (Sigma–Aldrich, St. Louis, MO, USA). Water was purified with an Elga system (Elga, Bucks, UK). SPE columns were

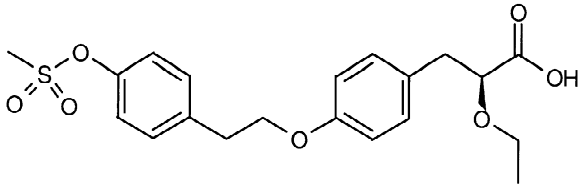

Fig. 1. Structure of tesaglitazar and the isotope labelled internal standard.

Isolute Array® 1 ml 25 mg C_2, C_4, C_6, C_8, and the columns containing resin based sorbents, namely ENV+, a hydroxylated polystyrene–divinylbenzene resin, and 101, a polystyrene–divinylbenzene resin (Int. Sorbent Techn., Mid Glamorgan, UK).

2.2. Standard solutions

Stock solutions of tesaglitazar and the internal standard AR-H040156XX-[D_5] were prepared in phosphate buffer, pH 7.0, $I = 0.1$ (I = ionic strength). Working standard solutions were obtained by further dilution of the stock solutions with phosphate buffer pH 7.0, $I = 0.1$, and were used for the preparation of plasma standards. Stock solutions were stable for up to 12 months if refrigerated.

2.3. Chromatographic system

The chromatographic system consisted of a Perkin-Elmer 200 Series LC-pump and autosampler (Norwalk, CT, USA), equipped with a 200-μl loop, and an Applied Biosystems API-365 (Ontario, Canada) quadrupole mass spectrometer with turbo ion spray interface. The mobile phase was a mixture of 500 ml acetonitrile, 1.0 ml formic acid, 0.50 ml 0.050 *M* aqueous sodium acetate, and 20 ml aqueous 0.10 *M* ammonium acetate diluted to 1000 ml with water. Analytes were separated on a Zorbax SB-CN 3.5 μm 50×4.6 mm (Agilent Techn., Palo Alto, CA, USA) maintained at room temperature, with a mobile phase flow of 0.75 ml/min. A guard column, Optiguard CN 15×1 mm (Optimize Techn., OR, USA), was used to protect the analytical column.

2.4. Mass spectrometric conditions

The effluent from the chromatographic column was split in the interface so that the liquid flow to the ion spray was about 100 μl/min (turbo ion spray). For API 365 the orifice voltage was set at 120 V, the collision energy (Q0-R02) at 45 V and the CAD gas at 3. Other settings were used as obtained during routine optimisation of the instrument (Q2 purge on). The analytes were detected by MRM (multi reaction monitoring) of precursor ions 453 and 458 m/z and product ions 267 and 272 m/z for tesaglitazar and its deuterated internal standard, respectively. This corresponds to the disodium adduct ions as discussed below.

2.5. LLE method

Frozen plasma samples were thawed at room temperature, homogenised and centrifuged for 5 min at 1400 *g*. A mixture of 500 μl sample plasma, 50 μl internal standard solution (4.8 μ*M*) and 500 μl aqueous HCl 0.15 *M*, giving a final pH of about 2, was extracted with 5.0 ml of dichloromethane–hexane (45:55, v/v) by shaking for 20 min. After centrifugation at 1400 *g* for 5 min, the aqueous phase was frozen and the organic phase was transferred to a conical tube and evaporated until dryness under nitrogen flow at 30 °C. The residue was dissolved in 250 μl of 30% acetonitrile in 0.015 *M* aqueous ammonium acetate, and then transferred to a sample vial from which 60 μl was injected onto the chromatographic system. The plasma standards, 500 μl drug-free plasma and 50 μl working standard solution, for daily calibration, were treated as the genuine plasma samples.

2.6. SPE method

The SPE system consisted of a robotic sample processor, Genesis RSP 150 (Tecan, Hombrechticon, Switzerland), a vacuum control box and manifold (Tomtec, Hamden, MA, USA) and an on-site constructed vacuum restricting device [19]. The extraction was performed on Isolute Array® 1 ml, C_6 25 mg SPE columns. The plasma samples were thawed at room temperature, homogenised by vortex-mixing and spun in a centrifuge for 5 min at 1400 *g*. The robotic sample processor prepared plasma standards by pipetting 250 μl drug-free plasma to separate tubes followed by 50 μl working standard solution and an additional aliquot of 250 μl drug-free plasma. This sequence was used to ensure complete mixing of the working standard solution with the entire volume of plasma in the dispensing process. These plasma standards were treated as the authentic plasma samples, and a 400-μl sample was used in the following extraction. The pressure level in the manifold was adjusted to 10 mbar below ambient pressure. The robotic sample processor

Table 1
Schedule for proposed SPE method

Process	Reagents	Volume (μl)	Time[a] (min)
Activation	Methanol	500	>4.6
Conditioning	Water	250	>2.3
pH adjustment	0.1 *M* HCl (aq.)	400	
Sample	Plasma	400	
Internal standard	Phosphate buffer pH 7, $I = 0.1$ (aq.)	50	>7.5
Wash 1	50% Methanol in HCl 0.01 *M* (aq.)	400	>5
Wash 2	Water	100	>4
Wash 3	30% Acetonitrile in 14 m*M* NH_4Ac (aq.)	50	Plate exchange
Elution	30% Acetonitrile in 14 m*M* NH_4Ac (aq.)	2×100	

[a] Time elapsed until next pipetting in the same well.

distributed the liquids in the activation, conditioning, adsorption and washing steps according to Table 1. After the second washing step the underpressure was released to ambient, the waste plate removed manually and a collection plate inserted in the manifold. The pressure was again adjusted to 10 mbar below ambient pressure and the robotic sample processor delivered the eluent. The collection plate was sealed, mixed and spun in a centrifuge for 1 min at 1400 *g*. Thereafter, 60 μl of the collected extract was injected onto the chromatographic system.

2.7. Experimental design and evaluation

The four full factorial experimental designs comprised the factors and chosen levels, as specified in Table 2. The role of the first experiment was to examine loading conditions, the second to determine the suitable sorbent and to examine washing and elution conditions, and the third and fourth to finalise the method. The chosen levels of each factor were arranged along the borders of the 96-well plate so that there were blocks, or at least rows, to which the same liquid should be dispensed (Fig. 2). Blank plasma spiked with the tesaglitazar was used as a test sample and the robotic sample processor distributed this and the other liquids to the SPE columns. The flow through the SPE columns was driven by a pressure difference of 10 mbar applied during the entire process. Experimental variability was estimated via a duplicate row. A spreadsheet software package (EXCEL, Microsoft software) was used to

Table 2
Set of SPE conditions used in experiments

Variable	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Sorbent	CN, C_2, C_8, 101 or ENV+	C_6, C_8, 101 or ENV+	C_6 or C_4	C_6
Activation liquid, 500 μl	Methanol or acetonitrile	Methanol	Methanol	Methanol
Conditioning liquid, 250 μl	Water or 0.01 *M* HCl (aq.)	Water	Water	Water
Sample volume also used for sample additive	200 μl	200 μl	200 μl	400 μl
Sample additive	None, 0.1 *M* HCl (aq.) or 0.1 *M* $TBANaSO_4$	0.1 *M* HCl (aq.)	0.1 *M* HCl (aq.)	0.1 *M* HCl (aq.)
Wash 1, 400 μl 0.01 *M* HCl (aq.) with % of methanol	0%	0, 15 or 30%	30, 40 or 50%	50%
Wash 2, 100 μl water				
Wash 3, volume 30% ACN in 14 m*M* NH_4Ac (aq.)	N.a.	N.a.	N.a.	25, 50 or 75 μl
Elution volume 14 m*M* NH_4Ac (aq.) with percentage of ACN	2×250 μl 60%	2×100 μl 30%, 2×250 μl 30% or 2×250 μl 60%	2×50 μl 2×100 μl or 2×150 μl 30%	2×100 μl 30%

		Methanol						Acetonitrile					
WATER	A1	1	1.6	18.7	44.3	32.1	A7	0.9	1.6	18.1	45.7	32.8	200 µl plasma
	B1	1.7	3.9	18.3	41.5	30.4	B7	2.1	3.8	16.9	39.2	31.6	200 µl plasma + 200 µl TBA
	C1	18.8	**72.3**	**115**	**115.2**	**112**	C7	20.3	**72.5**	**115**	**114.3**	**102**	200 µl plasma + 200 µl HCl 0.1 M
	D1	21.9	68.6	**111**	**116.3**	**109**	D7	22.7	**73.7**	**108**	**115.1**	**100**	200 µl plasma + 200 µl HCl 0.1 M
0.01 M HCl	E1	1	1.6	14.7	40.6	29.9	E7	1.3	1.8	14.3	36.6	23.1	200 µl plasma
	F1	2.2	2.8	15.6	30.4	21.2	F7	1.9	3.5	16.4	31.1	23	200 µl plasma + 200 µl TBA
	G1	20	63.1	**111**	**115.8**	**104**	G7	24.8	58.3	**107**	**113.7**	**101**	200 µl plasma + 200 µl HCl 0.1 M
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	
		CN	C2	C8		101 ENV+		CN	C2	C8		101 ENV+	

duplicate differances	-3.1	3.7	4.1	-1.1	2.8		-2.4	-1.2	7	-0.8	1.6	sum of squares:	109
square of diff.	9.61	13.69	16.81	1.21	7.84	0	5.76	1.44	49	0.64	2.56	varians:	12.1
												"standard deviation":	3.5

Fig. 2. Experiment layout and result visualisation, experiment 1. The well numbers A1 to H12 indicate the 96-well format; the grey marked wells were not used. The SPE columns, with the sorbent indicated below, were activated with the liquids indicated at the top. The entire rows of SPE columns were conditioned with the liquids indicated to the left and the plasma sample treatment used indicated to the right. The numbers in the columns represent the recovery of the analyte with that treatment. Standard deviation was calculated as shown at the bottom of the table.

evaluate the influence of each factor by calculating the mean response for the levels of each factor. The responses were also scrutinised individually to deduce single optimal values. As per calculations for standard deviation, the duplicates were used to estimate variability.

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Our initial studies on the detection properties of the mass spectrometer for tesaglitazar using a mobile phase without either sodium or potassium acetate did not show the ion for the proton adduct or for the ammonium adduct expected for this kind of molecule. Instead we found ions at 453, 469 and 485 m/z (Fig. 3A). The delta mass between these ions is 16 amu (atomic mass unit), which was tracked to the presence of sodium and potassium adduct formation. The loss of 1H and addition of 2Na, 1Na + 1K, and 2K, respectively will result in these m/z values. By modifying the concentration of sodium and/or potassium ions in the mobile phase we were able to influence the equilibrium between formation of different adduct ions in the ion source and also the extent of the adduct ions present in the mass spectrum (Fig. 3A and B). Fig. 3B is representative of the established method.

It was possible to direct the equilibrium towards sodium adducts at the expense of potassium adducts by using a sodium ion concentration of 20 µ*M* in the mobile phase. A much higher concentration of sodium ions relative to potassium ions was required to form sodium adducts than to form potassium adducts. This is probably due to the fact that the ability to form adducts increases in the order lithium < sodium < potassium. The potential difficulty in achieving product ions with a high abundance when using sodium and potassium adducts as precursor ions is probably due to the strong interactions between sodium or potassium and the molecule. Accordingly we found that rather high collision energy was needed to create any product ions at all from the potassium adduct, but using the corresponding sodium adduct product ions were created at low collision energies. When a potassium adduct was

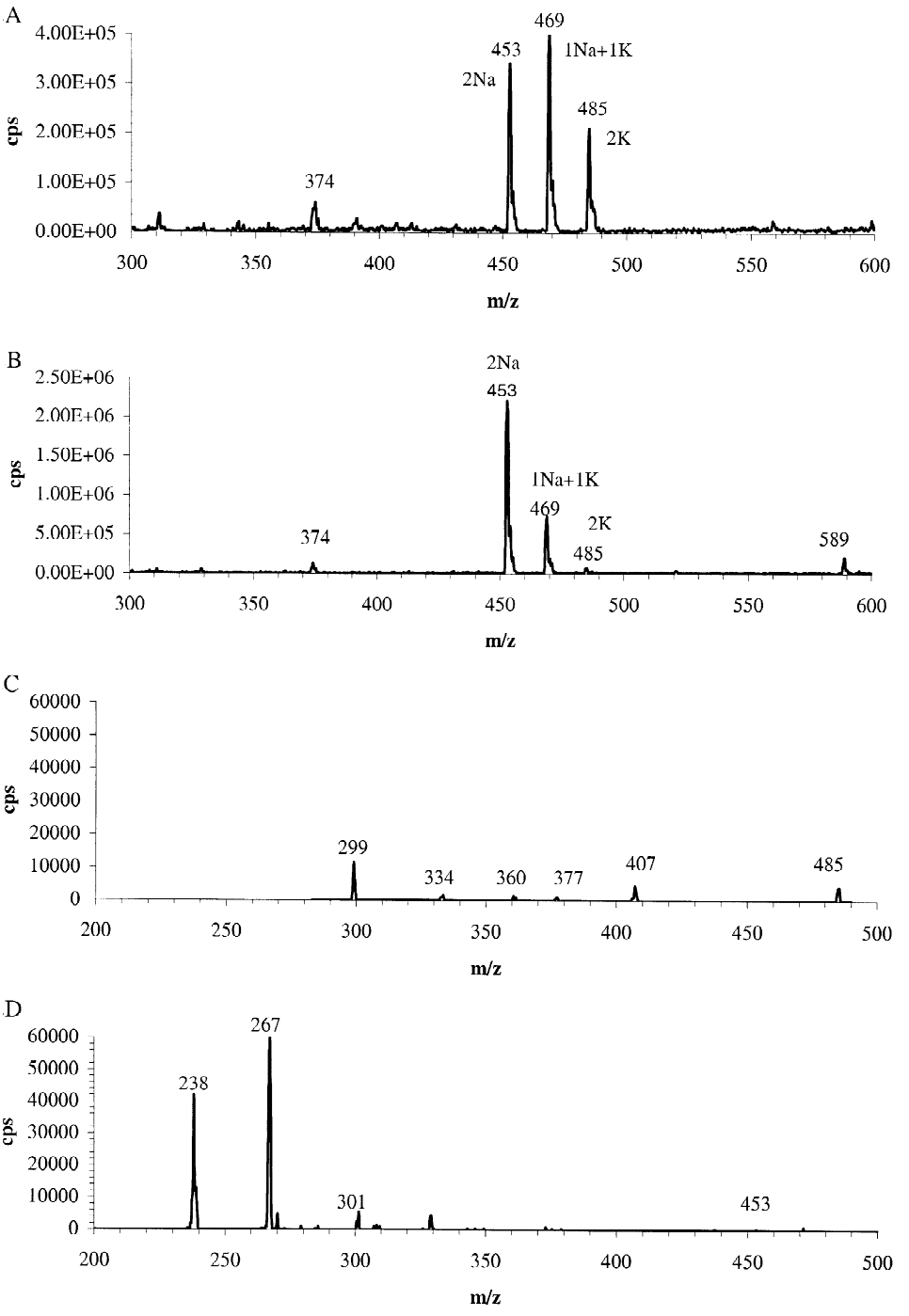

Fig. 3. (A) Q1 scan for tesaglitazar without either sodium or potassium acetate in the mobile phase. The adducts formed by removing 1H and adding 2Na, 1Na + 1K, and 2K are marked accordingly. (B) Q1 scan with addition of 20 μM sodium acetate to the mobile phase (note that this is a lower concentration than is used in the now established method). (C) Product ion scan, precursor ion 485 m/z, corresponding to dipotassium adduct formation of tesaglitazar. Collision energy 40 eV. (D) Product ion scan, precursor ion 453 m/z, corresponding to disodium adduct formation of tesaglitazar, product ion shown at m/z 267. Concentration of tesaglitazar is three times lower than in Fig. 3C. Collision energy 50 eV.

used as the precursor ion, many too-low abundant fragments were seen. The best of these product ion scans is shown in Fig. 3C. When a sodium adduct was used, a few fragments with high abundance were seen and the abundance of the 267 fragment was maximised at a collision energy of 50 eV (Fig. 3D). A somewhat lower collision energy was used in the established method.

3.2. Factorial design in SPE method development

The use of full factorial design facilitated the optimisation of conditions for the SPE process and choice of sorbent. However, there are many different types of sorbents available and the entire SPE process has to be customised for each of the sorbents to enable their evaluation and comparison. Factorial design and a robotic sample processor make it possible to evaluate several representative sorbents simultaneously. Factorial design includes three major steps: experimental design, performing the experiments and evaluation of experiments. The experimental design should preferably be based on previous experience and aimed towards a clear objective. In this study the objective was to establish an SPE method competitive with the present LLE method regarding sample volume, precision and limit of quantitation (LOQ) with the same separation and quantitation technique, LC–MS/MS, as in the LLE method. Our aim for SPE was to achieve a recovery above 90% and a clean extract without significant ion suppression effects. In order to reach this goal, the sorbent had to be as weakly retarding as possible and the efficiency of the washing conditions maximised. If the extract is to be injected directly onto the chromatographic system without prior evaporation and reconstitution, the elution liquid should be similar to the mobile phase of the chromatographic system.

The potential factors for inclusion in the factorial design are summarised in Table 2. Accordingly, there were eight factors that required evaluation at a minimum of two levels, resulting in a factorial design including 256 experiments. As this was too large, even for a robotic sample processor, we decided to perform the experiment in parts, firstly by optimising the activation, conditioning and loading conditions, using an elutropically strong elution solvent and a weak washing solvent. The washing and elution conditions were then optimised. Out of the chosen sorbents in the first experiment, the C_2 sorbent and C_8 sorbent represented the range of alkyl substituted silicas. The CN sorbent was chosen because a CN modified silica stationary phase was used in the chromatographic system; the 101 sorbent and the ENV+ sorbent were chosen on the basis that they possibly should retain the drug in a charged state. These resin-based sorbents are highly hydrophobic but the ENV+ also has some hydrophilic properties. Methanol and acetonitrile were used as activation liquids since they interact somewhat differently with the sorbent surface.

We explored the use of undiluted plasma sample and two plasma sample treatments to promote retention during the loading step: (1) acidification of the plasma samples to protonate the carboxylic acid functionality and (2) addition of an ion-pairing agent to the plasma samples. Both water and aqueous hydrochloric acid were examined as conditioning liquids. In total $5\times2\times2\times3=60$ combinations of experimental conditions were applied in the first experiment. In the second experiment (Table 2, experiment 2), with $4\times3\times3=36$ combinations, the sorbent choice was somewhat extended and the washing and elution conditions were examined. Experiments 3 and 4 were performed for final optimisation.

Executions of the experiments utilising the robotic sample processor were fast and convenient. The programming of the robotic sample processor was simple due to the regular blocks and rows to which the different liquids were dispensed (Fig. 2). The evaluation of the experiment was simple. A good overview of the results was achieved by typing the recovery into the experimental layout (Fig. 2) and then the evaluation was completed with spreadsheet calculations. Only 1.5 working weeks were required for the entire method development process, compared with the 3–4 working weeks required for the traditional development process.

3.3. SPE method

The findings from the four experiments resulted in the proposed method specified in Table 1. The liquid for pH adjustment and the sample were aspirated consecutively in the robotic sample processor needle and delivered as one portion into the SPE column.

This ensured that thorough mixing was completed through the action of dispensing. The needles were washed immediately and the internal standard distributed into the sample, which still remained, to a great extent, above the extraction bed. This method of internal standard addition was found to be equally as good as mixing the sample with internal standard prior to addition to the SPE column. The SPE method was completed within 2.5 h, compared with the 5 h needed for the manual LLE method. This 2.5 h includes all aspects of the experiment, from thawing of samples to placing extracts onto the autosampler. The operator intervention time was less than 40 min for the SPE method and about 180 min for the LLE method.

The four factorial designed experiments provided detailed knowledge about the SPE process. In the first experiment, the recovery of the drug as a percentage of added amount was used to calculate the result. The C_8 and both the resin-based sorbents, ENV+, and 101, retained the analyte satisfactorily provided the sample was acidified to promote retention, row C, D and G relative to A, B, E and F (Fig. 2). However, the C_2 sorbent gave a recovery of only 60%. It is possible that the alkyl substituted silica sorbents could be further optimised using the C_4 and C_6 sorbents, which may have more favourable characteristics. The CN sorbent gave very poor recovery, only 20%. Even though the resin-based sorbents, ENV+, and 101, retained the charged drug better than the silica sorbents, the retention was not sufficient and the ion-pairing reagent did not enhance the extraction either. It should be noted that the recovery values are only valid as comparators within the experiment, since there were no exact measures of elution volumes to ensure accurate recovery determinations within experiments 1–4. No significant differences were observed between methanol and acetonitrile as activating solvent. Both water and hydrochloric acid (0.01 *M*) were suitable as the conditioning liquid.

The second experiment examined recovery and degree of ion suppression. Ion suppression was evaluated by comparing peak heights from internal standard spiked to extracts and to elution liquid, respectively. The relative standard deviation of replicates was below 5% for both the responses. The C_6 sorbent was most effective despite the fact that the C_8 and the resin-based sorbents, ENV+, and 101, retained the analyte more than the C_6 sorbent. The C_6 and C_8 sorbents gave a clear advantage by enabling the analyte to be eluted with an elution liquid having an organic content lower than the mobile phase. This provides two benefits: (1) the eluted extracts can be injected onto the chromatographic column without preceding dilution or evaporation and dissolution, (2) a reduction of ion suppression was observed when the acetonitrile content in the eluent was reduced from 60 to 30%, this is important when selecting appropriate sample preparation conditions for an LC–MS method. However, C_6 was the only sorbent that gave high recovery despite the elution volume being decreased to two aliquots of 100 μl. No significant decrease in the recovery was observed when the content of methanol in the wash liquid (15 or 30%) was changed.

The results from experiments 3 and 4 showed that C_6 was superior to C_4, 50% methanol could be used in the washing liquid, and that the minimum volume of eluent was 2×100 μl. Experiment 3 showed that at least 2×100 μl elution were needed for complete elution for both the C_6 and the C_4 sorbent, but the recovery for the C_4 sorbent did decrease when 40 and 50% methanol were used in the washing liquid. Thus, the C_6 sorbent was chosen for this method. An elution profile was established and it was investigated whether a smaller elution volume could be used by collecting 10 eluate fractions of 25 μl each. Elution profiles for 200, 300, and 400 μl sample were found equivalent and Fig. 4 shows that the major part of extracted analyte was eluted in 50–250

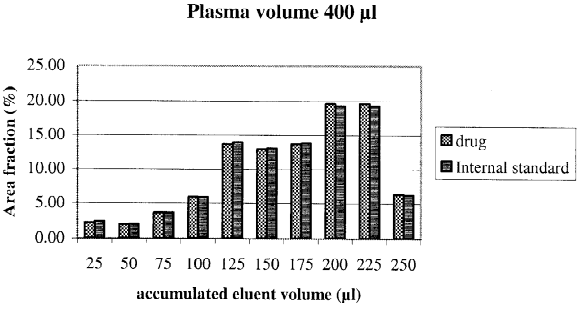

Fig. 4. Fraction of total amount of analyte in consecutive aliquots of eluent after extraction of spiked plasma samples with the proposed method.

μl eluent. We therefore concluded, and experiment 4 confirmed, that up to 50 μl eluent can be used as a third washing step without any significant loss of recovery and that 400 μl is an appropriate sample volume.

3.4. Method performance

The absolute recovery of tesaglitazar and the internal standard from the SPE and the LLE procedures, and the precision of the analytical method

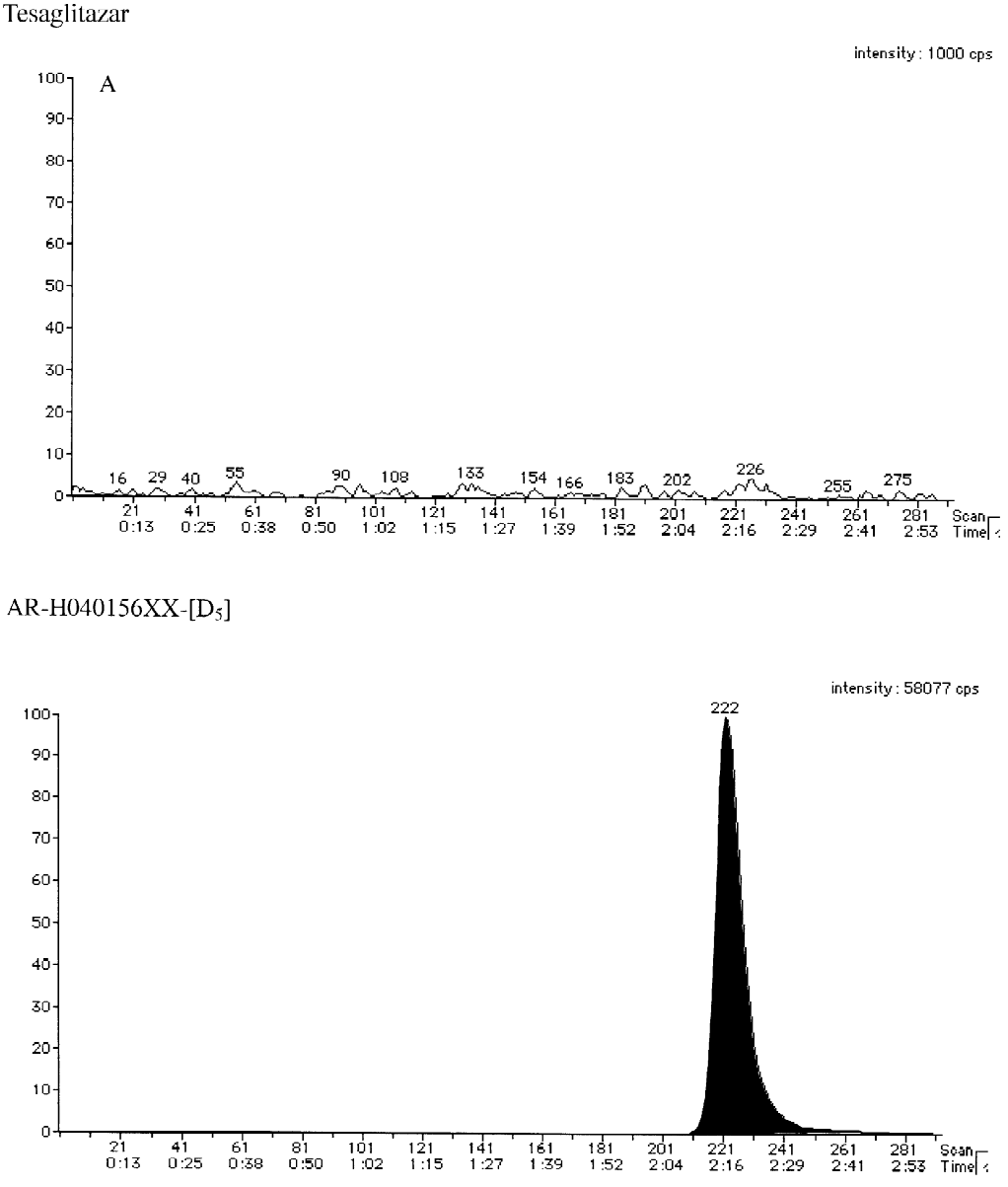

Fig. 5. (A) Chromatogram from a blank sample containing 618 n*M* of the internal standard AR-H040156XX-[D_5], extracted with the proposed method. (B) Chromatogram from an authentic plasma sample containing tesaglitazar, 8.0 n*M* and AR-H040156XX-[D_5], 618 n*M*, respectively, extracted with the proposed method.

were estimated at three different concentrations for both the SPE and the LLE method. The absolute recoveries were above 95% for the drug and the internal standard and equal for all three batches of SPE columns tested. The corresponding value estimated relative to a directly injected solution was 94.5% for the LLE method. Both the SPE and the LLE method showed linearity between 3 and 5000 n*M* with the limit of quantitation set at 3 n*M* (RSD< 20%). The coefficient of variation was below 5% at concentrations higher than 10 n*M*. A chromatogram from a blank plasma sample and a sample with added drug, using the SPE method, are shown in Fig. 5A and B, respectively. Repeated determination of the concentration of tesaglitazar in 37 authentic plasma samples, previously assayed by the LLE method, did not show any significant differences (Fig. 6).

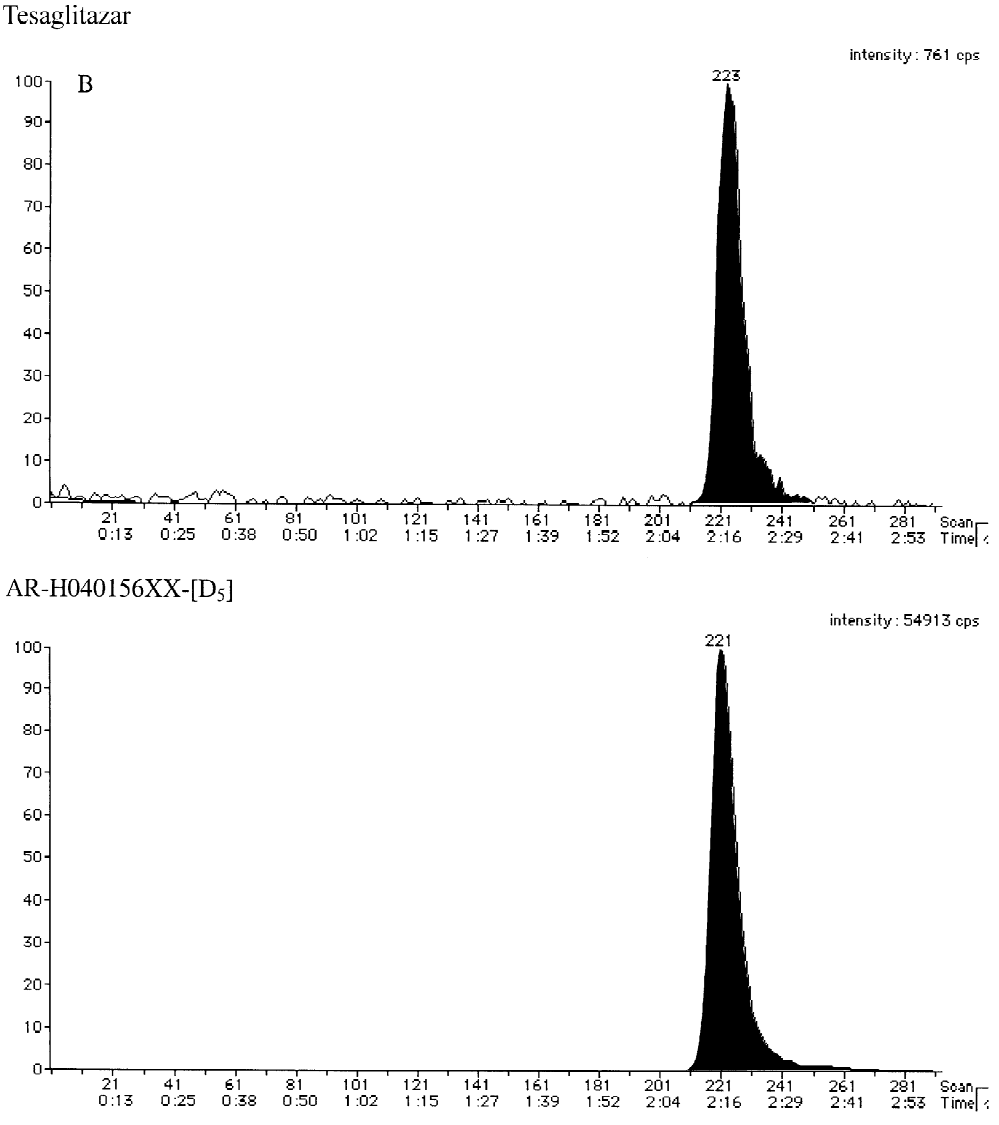

Fig. 5. (*continued*)

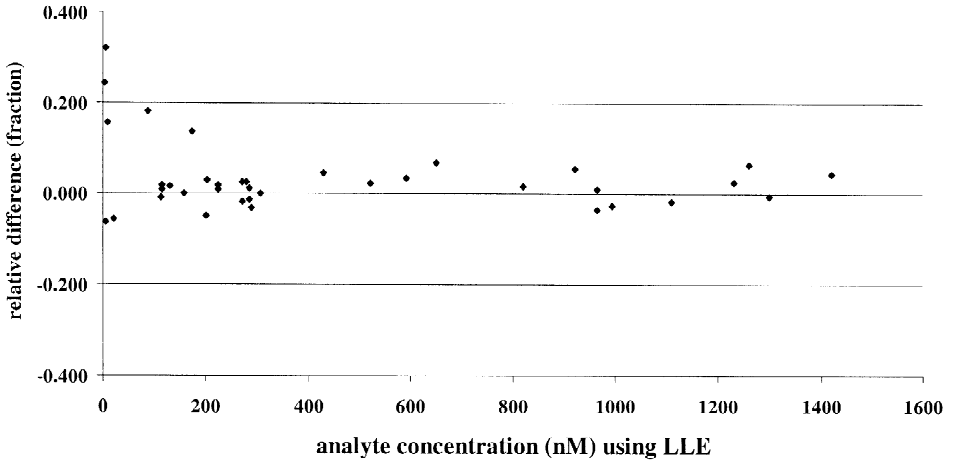

Fig. 6. Authentic samples ($n=37$) analysed with the manual LLE method and with the automated SPE method. The concentration results obtained for tesaglitazar with the SPE method were subtracted from the concentration results from the LLE and the difference divided by the LLE results. The relative differences are plotted above vs. the LLE results, the mean relative difference is 3.4%.

4. Conclusion

Factorial design was used successfully in the development of a SPE method for tesaglitazar, a novel PPARα/γ agonist, in human plasma. The development process for this type of extraction is usually time consuming. However, our approach shortened the development process to just four experiments. The optimised extraction method employed Isolute array C_6 25-mg plates with use of minute levels of underpressure.

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