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Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 573–580
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Supercritical fluid chromatography–tandem mass spectrometry for fast bioanalysis of *R*/*S*-warfarin in human plasma

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Received 16 August 2005; received in revised form 10 May 2006; accepted 12 May 2006
Available online 27 July 2006

Abstract

Chiral separation for the analysis of enantiomers in biological fluids by HPLC often takes relatively long chromatography time compared to achiral analysis. The advantage of fast mass transfer in packed-column supercritical fluid chromatography (pSFC) and the high-flow compatibility of APCI-MS/MS were applied to develop a fast bioanalytical method for *R*/*S*-warfarin in human plasma. Presented here are the main challenges encountered during method development of a semi-automated liquid extraction SFC-MS/MS method. The selection of internal standard, robustness of the SFC equipment, and carryover issues are discussed. The method has high-throughput: the chromatography time is at least two-fold faster than the our fastest previous method; and the liquid/liquid extraction time of 96 samples is less than 20 min using a Tecan Genesis® RSP 100 pipetting station and a Tomtec Quadra-96® workstation. The standard curve range was 13.6–2500 ng/ml. Precision of QC concentrations from four validation runs was 7.0% for *R*-warfarin and 6.0% C.V. for *S*-warfarin; and the bias was 3.7 and 3.2% R.E., respectively. The method is sensitive, accurate, selective and robust, and was applied to a drug-interaction clinical study with rapid turnaround of sample analysis.

Keywords: Warfarin; Supercritical fluid chromatography; Automation; Bioanalysis; LC–MS/MS

1. Introduction

Bioanalysis of enantiomers are required to provide data in support of pharmacokinetic studies when the enantiomers possess different pharmacological activities and/or are metabolized at a different rate. For example, *S*-warfarin is two to five times more potent than the *R*-form [1]. Also, the elimination rates of the enantiomers are different. The *S*-warfarin enantiomer is selectively metabolized by the hepatic enzyme P450 2C9 (CYP-2C9), which has six distinct single-base pair substitution polymorphisms [2,3]. Besides polymorphism, the elimination rates are affected by other factors such as age, alcohol use and drug interaction. Chiral bioanalytical methods for warfarin have been reported by several authors [4–6]. However, chiral separation for the analysis of enantiomers in biological fluids often requires relatively long chromatography time to achieve sufficient resolution. For example, the methods previously developed in our laboratory for *R*/*S*-warfarin had run times of 6 min for an LC–MS/MS and 28 min for an HPLC–UV method.

Packed-column supercritical fluid, subcritical fluid, and enhanced fluidity liquid chromatography has gained popularity in the analytical application of drug substance and food compounds. The advantage of pSFC on chiral separation lies in the low viscosity and high diffusivity of the mobile phase with CO_2. Very high flow rates of 5–10 ml/min, can be used to decrease chromatography time while maintaining the separation efficiency. However, there are only a couple bioanalytical applications for pSFC plasma sample matrix reported in the literature [7,8]. The authors reported a faster turnaround time compared to HPLC methods of at least three-fold as a result of the higher flow rate of the mobile phase used with the increased diffusion coefficient, and optimized velocities.

The application of a bioanalytical method requires a robust system that can endure the stress of a large number of injections per batch and intense use of the instrument to meet the demand of the clinical high-throughput assay environment. Therefore, judicious system maintenance and method robustness should be considered during the method validation using pSFC intended

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doi:10.1016/j.jpba.2006.05.025

for bioanalytical applications. The high sensitivity allowed the use of a small sample volume of 0.2 ml of human heparinized plasma.

Here we presented the challenges encountered in the assay development and the results of method validation of a semi-automated liquid extraction pSFC-MS/MS method that is at least two-fold faster than our previous methods.

2. Experimental

2.1. Chemicals and reagents

Racemic warfarin was from United States Pharmacopeia (Rockville, MD, USA); d_8-warfarin was synthesized in-house. *R*-(+)-Warfarin and *S*-(−)-warfarin enantiomers were supplied by Ultrafine Chemicals (Manchester, UK), with purity of 99.7 and 100%, respectively. Ethyl ether was from Fisher (St. Louis, MO, USA). Acetontrile (HPLC grade), formic acid and glacial acetic acid were from Mallinckrodt (Irvine, CA, USA). Methanol (HPLC grade) and sulfuric acid were from EMD (Gibbstown, NJ, USA). Absolute ethanol was from Sigma–Aldrich (St. Louis, MO, USA). Control human heparinized plasma was from Biochemed (Winchester, VA, USA). Liquid CO_2 (SFC/SFE grade, with dip-tube, not capped with helium) was from Air Liquide (Grande Prairie, TX, USA). Water was deionized in-house using a Barnstead NANOpure® system (Dubuque, IA). The 1.6 ml 96-well collection plates were from Axygen (Union City, CA), and 1.2 ml 96-well plates from Marsh Biomedical Products (Rochester, NY, USA). Filtration plates (96-well) were Millipore-Multiscreen-BV® 1.2 μm pore size (Millipore, Bedford, MA, USA).

2.2. pSFC-MS/MS

The pSFC/MS/MS system set up is depicted in Fig. 1. It consisted of a Jasco PU-1580 CO_2 and PU-980 solvent pumps (Tokyo, Japan), a CTC-PAL autosampler (Zurich, Switzerland), and an Applied Biosystems/MDS Sciex API 4000 Triple Quadrupole Mass spectrometer (Concord, ON, CA) with an APCI source. A Chiralpak AD-H guard column of 1.0 × 0.4 cm and a Chiralpak AD analytical column of 250 mm × 4.6 mm (both from Chiraltech, West Chester, PA, USA) were used. The injection volume was 5 μl; the injection cycle was 3.0 min; and the total flow rate was 7.0 ml/min. The mobile phase of ethanol–CO_2 (30:70) was generated by pump A delivering liquid CO_2 at 4.9 ml/min and pump B delivering absolute ethanol at 2.1 ml/min into a 50 μl mixing valve, which was plumbed into the injector port of the autosampler. The column pressure was measured as 24 MPa. The column was kept at room temperature.

The analytical column effluent was introduced via PEEK tubing (0.127 mm in diameter and 610 mm in length) to the MS with an APCI interface operated in positive ion mode with multiple reaction monitoring (MRM). The following mass/charge ratios (*m*/*z*) were used to monitor precursor → product ions: 309.1 → 163.0 for warfarin and 317.0 → 163.0 for d_8-warfarin.

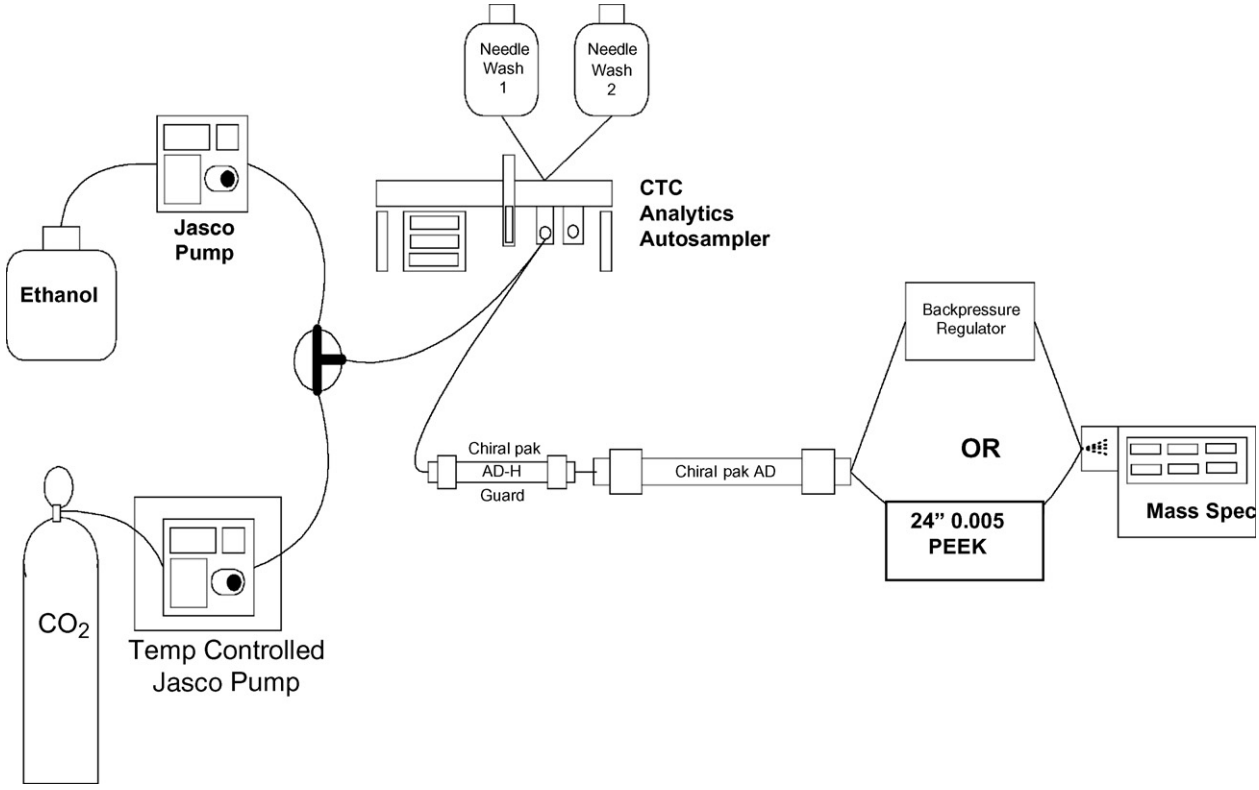

Fig. 1. Diagram of a pSFC-LC–MS/MS system.

The source was operated with a needle current of 3.00 μA, and a source temperature of 450 °C. The fragmentation occurred at collision energies of −21 and −23 eV for warfarin and d_8-warfarin, respectively. Nitrogen was used as collision gas. The dwell time was 400 ms for the analytes and 200 ms for the internal standard (I.S.).

Chromatograms were integrated using AB/MDS Sciex Analyst™ v1.4 (Innaphase, Philadelphia, PA) and the data transferred to Watson™ LIMS for standard curve regression, curve fitting and data management. A linear regression weighted 1/concentration^2 was used to determine slopes, intercepts and correlation coefficients (r^2). The resulting parameters (y-intercept and slope) were used to calculate concentrations from the equation: concentration = [ratio − (y-intercept)]/slope, where "ratio" is the ratio of the compound peak area to the I.S. peak area.

2.3. Calibration standards and quality control samples

All preparation and handling of solutions and samples with warfarin was conducted under UV-shielded lighting. Two primary stock solutions of racemic warfarin prepared from separate weighings were used for the preparation of standard solutions and quality control samples (QCs). The stock solution was 1000 μg/ml in methanol, and 10× concentrated solutions of the standards were prepared from the stock solution by serial dilution with methanol. Working standards were prepared fresh daily by spiking 20 μl of the 10× concentrated solutions into 0.2 ml of blank control human heparinized plasma. The final concentrations in plasma calibration standards were 13.6, 27.1, 50, 100, 250, 542, 1000, 2000, and 2500 ng/ml for each enantiomer (*R* or *S*) of warfarin. The I.S. stock solution (1000 μg/ml) in methanol was diluted into a working solution of 5.0 μg/ml. Three levels of QCs in human plasma, at concentrations of 37.5, 150 and 1880 ng/ml were prepared, aliquoted, and stored frozen at −20 °C. An additional QC of 5000 ng/ml was prepared and diluted 10-fold with blank control plasma before analysis to reflect the dilution validity of study samples with concentrations higher than the upper limit of quantitation (ULOQ).

2.4. Sample preparation

A 0.2 ml aliquot of plasma sample and 20 μl of I.S. working solution were added into a 1.6 ml 96-round-well plate and mixed. The sample plate was placed onto the deck of the Tomtec Quadra-96® along with the reagents for the extraction. The workstation subsequently added 150 μl of 1N sulfuric acid to all samples, mixed, and then added 1.0 ml of ethyl ether. The plate was capped tightly and vortexed vigorously for 10 min and centrifuged at 5200 rpm for 3 min. The plate was placed onto the Quadra-96®, which transferred 0.4 ml of the ether supernatant into a clean 1.2 ml 96-well plate. The samples were evaporated to dryness and reconstituted with 0.2 ml methanol. The plates were capped with a self-sealing mat to be injected onto the LC/MS/MS.

2.5. Validation of the LC–MS–MS method

The method was validated by four analytical batches on separate days. Each validation batch contained a single set of nine concentrations of calibration standards, six replicates of QCs at each of three concentrations, and six replicates of control plasma spiked at the concentration of the low limit of quantitation (LLOQ). Each batch also contained test samples such as spike-recovery, stability samples of processing and storage, or dilution QCs. Quality controls were evenly spaced through the batch among the samples. Each validation batch contained 96 samples to simulate the anticipated length of a clinical sample analysis batch.

Analyte stability was tested by subjecting QCs through multiple freeze/thaw cycles (F/T cycles), extended time on bench at room temperature (short-term), or storage in a freezer set at −20 °C. Post-extraction analyte stability (at 4 °C) was determined by comparing the results of previously extracted to those of freshly extracted calibrators. Post-extraction batch integrity was determined by batch reinjection.

Recoveries of the analytes were determined by comparing the peak areas of the extracted analytes from the plasma (spiked before extraction) with those of the unextracted analytes, which were spiked into blank plasma extracts (spiked after extraction). The recovery testing was performed at three concentrations (at the ULOQ, a middle standard and the LLOQ). The presence of matrix effect (suppression or enhancement) was determined by peak area comparison of samples spiked after extraction to samples prepared at the same concentrations in methanol.

Warfarin was spiked into samples of each of 10 lots of human plasma at a concentration equal to the LLOQ and high QC level to determine the consistency of between-lot quantitation and the presence of matrix effects. Extraction of the 10 lots spiked only with the I.S. (standard zero) and unspiked (blank) was carried out to demonstrate method selectivity. The method robustness was evaluated from validation batches performed by multiple analysts, on two different MS–MS instruments, with several different APCI sources and with different lots of the analytical columns. A test of the potential for cross-well contamination using the Quadra-96® was performed by processing samples spiked at the ULOQ in wells adjacent to unspiked samples.

3. Results and discussion

3.1. Mass spectrometer ion source

At our laboratory the potential for SFC-MS/MS for bioanalytical sample analysis was explored over a period of nearly 2 years. Experimentation with different types of drug compounds to learn about the applicability and the advantages of this technique for high-throughput bioanalysis was conducted against the conventional methods. The experiments were conducted on different LC–MS platforms and various ion sources. The development of the *R*/*S*-warfarin method is an example of these experiments.

The initial feasibility investigation of the warfarin enantiomers' separation and sensitivity was performed with a PE Sciex API 365 using an atmospheric pressure photoionization

(APPI) source (Applied Biosystems/MDS Sciex), which had shown promising results for other analytes, such as estrogen and androgen compounds and methylphenidate. Several solvents, such as toluene and acetone were tested as dopants for the APPI source to optimize sensitivity. Toluene provided the highest sensitivity when added post-column at a flow-rate of 0.1 ml/min. Sensitivity was sufficient to accurately quantitate the LLOQ of *R*/*S*-warfarin in extracted plasma samples. However, it was found that the signal intensities from the APPI source were not stable for these particular applications from day-to-day.

Next, electrospray ionization (ESI) was tested on an Applied Biosystem/MDS Sciex API 3000 platform to investigate whether ESI could provide better sensitivity. The results indeed showed ample sensitivity, but with significant signal drift within a study-length sample batch. The relatively high flow rate used at 5 ml/min caused concern that the ESI signal stability would not be robust enough for bioanalytical application. The APCI source, which performs gas-phase ionization, is amenable to higher flow-rates than ESI. The APCI's ability to ionize is dependent upon the evaporation of the mobile phase in the quartz/ceramic tube. When using a gaseous mobile phase as CO_2 with a small amount of volatile modifier, such as ethanol, very high flow-rates are easily tolerated while maintaining high ionization efficiency. We observed an approximately 10-fold increase in response than with the ESI. However, both enantiomer peaks showed tailing that increased gradually over time and was accompanied by a decrease in response. The API 3000 APCI source needed an unacceptable frequency of maintenance to restore acceptable peak shape and response. Generally, this involved changing the quartz tube. Because of the improved design of the API 4000 APCI, the assay was switched to that instrument to continue the use of the APCI source. The API 4000 has a ceramic tube that resulted in less tailing and memory effect than that of the API 3000. Thus, a stable signal and the highest response were achieved. Signal intensity increased approximately eight-fold over the API 3000 instrument. The dual-heater in the API 4000 Turbo V source provided greater ionization efficiency, and the improved gas-dynamics gave greater sensitivity over the API 3000 source.

3.2. Chromatographic system

A common application for pSFC is chiral separations. In general, enantiomers are readily separated on chiral columns with liquid CO_2 [9]. We tested a variety of chiral columns using an initial experimental condition of 15% methanol with liquid CO_2 and a total flow rate of 5 ml/min. Although separation was obtained on other columns tried (Chiralpak AD-H, Chiralpak OD), the Chiralpak AD, 250× 4.6 mm, was selected because of the optimal column resolution, peak shape, and lower backpressure.

The low viscosity and high diffusivity of the pSFC mobile phase enabled the use of a high flow rate and long columns to increase the numbers of theoretical plates to resolve any interference from potential matrix components and structurally similar compounds. The use of ethanol in the mobile phase produced a sharper peak shape than that of methanol and with better column retention. Isopropanol as the organic modifier resulted in even longer retention times, however, with broader peak shapes. The solvent modifier was finally optimized to 30% ethanol and the total flow rate to 7 ml/min to facilitate a fast chromatography time. The use of the modifier increased the backpressure to approximately 22.3 MPa.

The column outlet was plumbed into a valve-actuated backpressure regulator that was set at 110 bar. The purpose of this was to keep the CO_2 and modifier in one phase. The PEEK tubing of diameter 0.127 mm from the backpressure regulator was directly connected to the MS source.

The k' of *R*-warfarin was 1.7 and 4.2 for *S*-warfarin. The separation factor (α) was 2.5 for the enantiomers, which was more than twice of the α of 1.2 in our previous LC–MS/MS method. The higher α was important for method robustness considering that patient samples might have different polymorphism of the CYP-2C9 enzyme, with disproportionate concentrations of one over the other, and potentially resulting in overlapping of the peaks. Additional method robustness was shown by the similar and reproducible chromatographic performance from analytical columns with different lot numbers of packing material.

One of the concerns about using SFC equipment for a bioanalytical method was the robustness of the equipment. Equipment ruggedness is a critical factor in the development of an assay to support clinical trials. Despite the apparent advantages of pairing pSFC with MS/MS, very few bioanalytical methods have been developed. This was partly due to the low availability of quality equipment [10]. After using one Jasco PU-1580-CO_2 pump for nearly a year in method development work on pSFC bioanalysis applications, the pump began to cavitate on an irregular basis. It was unable to be repaired, and had to be replaced. It was also discovered that with high daily usage simulating that of a bioanalysis production environment, the pump seals would eventually freeze up and had to be replaced approximately every 2 weeks. A proactive approach of preventive maintenance, such as routinely changing the pump seals biweekly, prevented such occurrence during an analytical run.

Carryover was a significant issue in the method development of the project. We identified the sources of carryover coming from both the injection valve and the backpressure regulator. Using the CTC-PAL autosampler, two needle wash solutions were used to minimize the carryover at the injector port: an initial rinse with water–methanol–acetic acid (94:5:1, v/v/v), followed by a wash with acetonitrile–formic acid (100:1, v/v). We found that the use of a Teflon rotor seal in the Valco valve also contributed to carryover; it also became brittle from the cold temperature of the mobile phase and could shatter over time. This rotor seal was replaced with a Hastaloy seal (Microliter Analytical Supplies, Suwannee, GA). Carryover was reduced to an acceptable level ($\leq$0.2%) when measured following a ULOQ sample.

After the injection valve carryover problem was resolved, it was noticed that approximately one out of every 96 samples would have an unexpected high concentration. This was traced to the backpressure regulator, which is a rotating needle valve designed to keep pressure on the system to maintain the mobile phase as one homogenous phase. Since the flow rate was so high

it was possible that sample might overflow to an area where it would eventually leech out to contaminate other samples. The backpressure regulator was set for 110 bar, and the set pressure and actual pressure were displayed on a digital display. However, given the high flow rate of the system and the narrow bore PEEK tubing used to feed into the APCI source, the actual pressure readout was at 132 bar from the regulator. Therefore, the backpressure regulator was probably not adequately serving its function to regulate the pSFC pressure. In addition, column pressure may not be important for enantiomer resolution, at least on this particular column. The separation of enantiomer pairs of four drug compounds (monitored as $\ln \alpha$) on a Chiralpak AD column were shown to be insignificantly affected by column pressure [9]. To eliminate the major contributing factor to the carryover problem, the backpressure regulator was removed. Indeed, the sporadic carryover was not observed anymore, with no change in the chromatography of the enantiomers, such as retention times and peak shape. While the narrow bore PEEK tubing was sufficient to maintain the mobile phase homogeneity, the tubing was measured at 610 mm for consistency and reproducibility of the system configuration. This system backpressure was steadily maintained at approximately 23 MPa.

3.3. Internal standard and sample processing

The I.S. used in one of the previously developed in-house methods was *p*-chlorowarfarin (coumachlor). *p*-Chlorowarfarin, being similar in chemical characteristics to warfarin, is an appropriate I.S. to correct for processing/extraction and chromatographic variability. However, it may not be a suitable I.S. for a pSFC-MS/MS method. During early method development, with ESI used as the ionization source, the signal intensities of the *p*-chlorowarfarin and warfarin enantiomers drifted in different directions within an analytical run. This indicated that the ionization behavior of the *p*-chlorowarfarin and warfarin enantiomers in the MS were different. An I.S. not being able to track ionization is problematic due to the unpredictable effect of matrix components. Although preliminary results with *p*-chlorowarfarin as I.S. were deemed acceptable, it was decided that the potential problem must be addressed to produce a robust method that would allow long bioanalytical sample runs. Therefore, d_8-warfarin was synthesized in-house to be used as the I.S.

The extraction procedure used was based on our previous HPLC method [11], which has been applied to multiple clinical studies and shown to be robust. Because of the higher sensitivity of the SFC-MS/MS compared to that of HPLC–UV, the sample volume was reduced from 1.0 to 0.2 ml. To increase sample throughput, the test tube extraction format of the HPLC method was scaled down to a 96-well plate format for the application of the Tomtec Quadra-96® semi-automated workstation. The recovery of the semi-automated method was less than that of the previous manual extraction method, since only about half of the organic extract layer could be removed for the subsequent dry down step. If the Tomtec Quadra-96® tips were too close to the centrifuged aqueous layer, extraneous amount of matrix components might be transferred into the sample extract, resulting in dramatically increased matrix suppression. As shown in Table 1, the overall extraction recovery was 39%, which was consistent over the concentration range of the LLOQ of 13.6 ng/ml to the ULOQ of 2500 ng/ml. Matrix suppression was evaluated from the plasma extracts of the LLOQ, middle, and ULOQ concentration samples against that of the non-matrix solvent solutions. An overall 29% matrix suppression was calculated. The amount of matrix suppression was consistent over the concentration range tested as well as among individual matrix lots (see Section 3.5 selectivity below). In addition, a filtration step was used to produce cleaner extracts for injection. The pipetting station transferred the extracts into a Millipore Multiscreen-BV® 96-well plate, placed onto a clean collection plate. The assembly was centrifuged at 5600 rpm for 2 min. The entire sample processing for one 96-well plate took only 20 min.

Table 1
Extraction recovery of plasma sample and matrix suppression by the plasma extracts[a]

	13.6 ng/ml			250 ng/ml			2500 ng/ml		
	Spiked before extraction	Spiked after extraction	Spiked to solvent	Spiked before extraction	Spiked after extraction	Spiked to solvent	Spiked before extraction	Spiked after extraction	Spited to solvent
Mean peak area	4940	13269	17538	105303	244194	362233	974366	2575812	3628804
C.V. (%)	13.1	20.3	6.8	8.3	15.8	8.6	9.9	17.8	8.4
N		6	6	6	6	6	4	5	6
Extraction recovery (%)[b]			37			43			38
Matrix effect (%)[c]			−24			−33			−29
Overall mean recovery (%)	39								
Overall mean matrix suppression (%)	29								

[a] Experiment was performed on a single plasma lot over three concentrations.
[b] Extraction recovery (%) = mean peak area spiked after extraction/mean peak area spiked before extraction × 100%.
[c] Matrix effect (%) = mean peak area spiked before extraction/mean peak area spiked to solvent × 100%.

Table 2
Precision and accuracy of standard calibrator[a]

	STD B 13.6 ng/ml	STD C 271 ng/ml	STD D 50 ng/ml	STD E 100 ng/ml	STTD F 250 ng/ml	STD G 542 ng/ml	STD H 1000 ng/ml	STD I 2000 ng/ml	STD J 2500 ng/ml
R-Warfarin									
Mean	13.7	26.7	49.7	100	249	558	992	1990	2510
S.D.	0.263	0.499	1.77	3.89	2.38	6.22	14.2	48	66
C.V. (%)	1.9	1.9	3.6	3.9	1.0	1.1	1.4	2.4	2.6
R.E. (%)	0.7	−1.5	0.6	0.0	−0.4	3.0	−0.8	−0.5	0.4
S-Warfarin									
Mean	13.7	27.0	48.8	99.3	249	561	972	2030	2530
S.D.	0.275	0.753	1.82	1.95	2.5	6.35	7.59	48.3	70.4
C.V. (%)	2.0	2.8	3.7	2.0	1.0	1.1	0.8	2.4	2.8
R.E. (%)	0.7	−0.4	−2.4	−0.7	−0.4	3.5	−2.8	1.5	1.2

[a] Data from four validation batches, with single standard per analytical batch.

3.4. Precision, accuracy and linearity

Table 2 shows the accuracy and precision data of calibrator standards from four validation batches with an analytical range of 13.6–2500 ng/ml for *R*- and *S*-warfarin. The absolute bias of the back-calculated standards was ≤3.5% R.E. and the imprecision was ≤3.9% C.V. The correlation coefficients of the four validation curves were all ≥0.9982. The low C.V.% (≤6.7%) for the slope of each analyte from two different MS/MS instruments indicated reproducible LC–MS/MS instrument response and linearity. The choice of the regression methods was determined. Both *R*- and *S*-warfarin data fit well with a linear regression model, and weighting of 1/concentration^2 had lower variability than that of 1/concentration or unweighted.

Tables 3 and 4 show the interday accuracy and precision of QCs for *R*- and *S*-warfarin, respectively. The sensitivity of the assay at an LLOQ is established by the accuracy of ≤3.7% R.E. and precision of ≤7.0% C.V. For the low, mid and high QCs, the accuracy was ≤3.7% R.E. and precision was ≤3.5% C.V. The interday data show that this method is consistent and reliable with very low variability and bias. The method is also robust because the validation batches were processed by four analysts using two analytical column lots and two MS instruments.

3.5. Selectivity

Selectivity against matrix components was demonstrated by the lack of interfering peaks at the retention times of the analytes and the corresponding I.S. and the consistency of spike-recovery.

Table 3
Interday precision and accuracy of QC for *R*-warfarin[a]

QCs	LLOQ	Low	Mid	High
Nominal (ng/ml)	13.6	38.7	146	1880
Mean (ng/ml)	14.1	38.9	147	1870
S.D.	0.99	1.18	4.49	44.8
C.V. (%)	7.0	3.0	3.1	2.4
R.E. (%)	3.7	3.7	−2	−0.5
n	24	24	24	24

[a] Data from four validation batches, with six replicates of each QC level per analytical batch.

Human plasma from 10 different individuals were screened for interfering peaks and matrix effect by extracting the unspiked control samples as well as samples from each lot spiked with the LLOQ and ULOQ concentrations. No significant interference at the analyte or I.S. retention times was observed in any of the lots screened as shown by the representative chromatograms of the extracted blank plasma sample in Fig. 2. The representative chromatograms of extracted samples at the LLOQ concentration are shown in Fig. 3. During method development, 29% matrix suppression was observed from the plasma extract versus that of the non-matrix solvent solution (see Table 1). Since the standard calibrator was prepared in blank control plasma (not in a non-matrix solvent solution), it was more important to establish that the matrix effect was consistent than the total absence of it. Table 1 shows the consistency of matrix effect over the entire calibrator range. In addition, the matrix consistency among the 10 human plasma lots was further demonstrated by the low variability (≤5.1% C.V.) and high accuracy (≤5.1% R.E.) at both the LLOQ and near the upper limit of the analytical range for *R*-warfarin (Table 5) and *S*-warfarin (Table 6).

A stress test was performed to check for cross-well contamination during sample processing and there was no evidence of contamination in the blank control samples or standard zero samples that were arranged with adjacent ULOQ samples.

3.6. Selectivity against a co-administered compound

One of the intended purposes of the method validation was for the application to drug interaction studies. Selectivity against the co-administered compound must be evaluated during the method validation. The following is an example of such evalua-

Table 4
Interday precision and accuracy of QC for *S*-warfarin

QCs	LLOQ	Low	Mid	High
Nominal (ng/ml)	13.6	37.5	146	1880
Mean (ng/ml)	14.0	38.7	146	1900
S.D.	0.84	1.04	5.14	57.1
C.V. (%)	6.0	2.7	3.5	3.0
R.E. (%)	2.9	3.2	−2.7	1.1
n	24	24	24	24

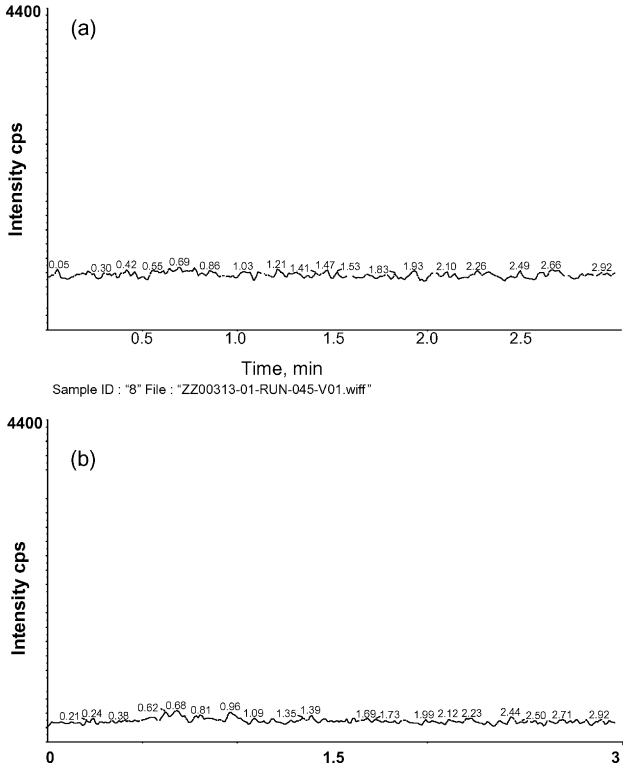

Fig. 2. Representative chromatogram of extracted blank control plasma: (a) warfarin channel and (b) I.S. channel.

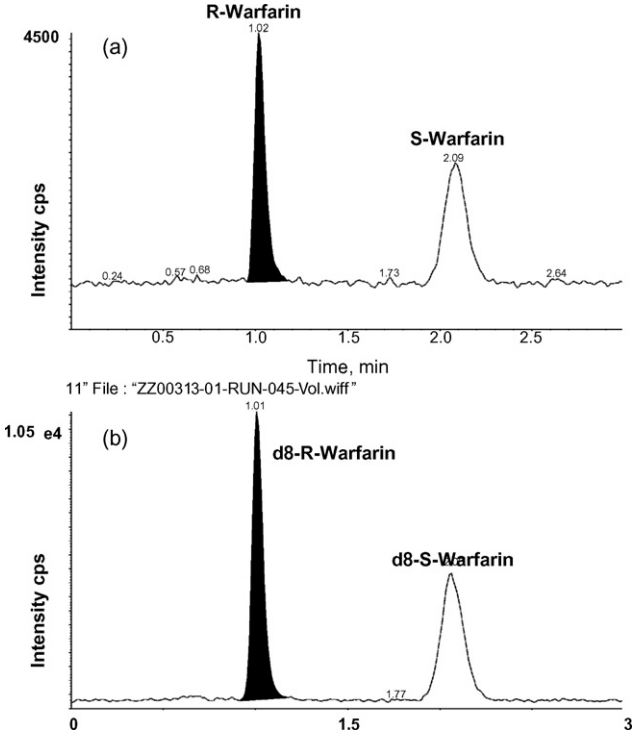

Fig. 3. Representative chromatogram of an extracted LLOQ (13.6 ng/ml warfarin) plasma sample: (a) warfarin channel and (b) I.S. channel.

Table 5
Matrix effect for *R*-warfarin in human plasma[a]

Lot no.	LLOQ		High	
	13.6 ng/ml	% Dev.	1880 ng/ml	% Dev.
1	14.7	+8.1	1830	−2.7
2	14.7	+8.1	1880	+0.0
3	14.4	+5.9	1870	−0.5
4	15.5	+14.0	2010	+6.9
5	13.3	−2.2	1910	+1.6
6	13.9	+2.2	1840	−2.1
7	14.6	+7.4	1780	−5.3
8	14.0	+2.9	1900	+1.1
9	14.0	+2.9	1830	−2.7
10	13.7	+0.7	1870	−0.5
Mean	14.3		1870	
C.V. (%)	4.4		3.3	
Theoretical (%)	105.1		99.5	

[a] Spike-recovery experiment performed on 10 lots of individual plasma.

tion: the experiment was performed by spiking the low and high QC samples with the co-administered drug, thalidomide, at a concentration of 2000 ng/ml that exceeded the expected *in vivo* level. The samples were analyzed together with the regular QC samples at low and high concentrations and standard calibrators. Results of the thalidomide-spiked samples were compared with those of the regular QCs. Furthermore, blank control plasma and zero standard samples were also processed with the addition of the same pure solution of thalidomide. No quantitative interference was observed in samples spiked with thalidomide only or thalidomide and I.S. only.

3.7. Stability of the analytes

Stability tests were designed to investigate the effects on assay performance of the method mimicking anticipated sample handling conditions. Stabilities of sample storage (long-term storage), processing (freeze-thaw, short-term, and post-

Table 6
Matrix effect for *S*-warfarin in human plasma[a]

Lot#	LLOQ		High	
	13.6 ng/ml	% Dev.	1880 ng/ml	% Dev.
1	13.2	−2.9	1800	−4.3
2	14.4	+5.9	1980	+5.3
3	13.0	−4.4	1910	+1.6
4	14.5	+6.6	2090	+11.2
5	12.8	−5.9	1930	+2.7
6	13.5	−0.7	1840	−2.1
7	13.9	+2.2	1790	−4.8
8	12.4	−8.8	1920	+2.1
9	13.0	−4.4	1900	+1.1
10	13.6	0.0	1850	−1.6
Mean	13.4		1900	
C.V. (%)	5.1		4.7	
Theoretical (%)	98.5		101.1	

[a] Spike-recovery experiment performed on 10 lots of individual plasma.

extraction) and chromatography (re-injection) were tested and shown to be of insignificant effect. The results showed that QC samples that went through six freeze/thaw cycles remained within 10% of nominal concentration. QCs remained within 4% of nominal concentration when exposed to room temperature for 65 h under UV shielded lights. QCs were stable for 281 days when stored at −20 °C, with <4% difference from theoretical concentration. Extracted samples were stable in the polypropylene plates for at least 7 days when stored at 5 °C, staying within 4% of nominal. Stability of the analytes in stock solutions was also established against a fresh preparation. They proved to be stable at 5 °C for 112 days in polypropylene containers when compared to freshly weighed and prepared stock solutions.

3.8. Stability of conversion of single enantiomers

The individual enantiomers of warfarin, *R*-(+)-warfarin and *S*-(−)-warfarin, were spiked into control human plasma and stored at −20 °C to test the appearance of their corresponding counter enantiomer and extracted after four freeze and thaw cycles. No appearance of the other enantiomer was detectable, thus there was insignificant enantiomeric conversion through the normal processes of storage and sample processing.

4. Application

The validated pSFC-MS/MS method was applied for bioanalysis in a drug-interaction clinical study of approximately 460 samples. Fourteen sample batches were run (14 plates). The interday C.V.% of QCs was ≤4.5%, and R.E.% was ≤7.3%. Since the extraction took one analyst only 20 min per batch on the Tomtec Quadra-96® workstation, the samples for the entire study were processed in only 2 days. To further increase the efficiency of the method, clinical samples, standards, and QCs were pre-aliquotted by a Tecan Genesis® RSP pipetting station and stored at −20 °C until processed.

5. Conclusion

A pSFC-MS/MS method for the analysis of chiral warfarin plasma samples was developed and validated. The standard curve range was 13.6–2500 ng/ml. Precision of QCs from four validation runs was ≤7.0% C.V. for *R*-warfarin, and ≤6.0% for *S*-warfarin; the bias was ≤3.7% and 3.2% R.E., respectively. The validated method was applied successfully to assay samples from a drug-interaction clinical study. A rapid turnaround time was achieved within 2 days for approximately 460 samples.

The method was robust and reduced the cycle time of our previous LC–MS/MS method for *R*/*S*-warfarin by one-half. In spite of the faster cycle time, the quality of chromatographic separation was improved, with two-fold increased resolution of the enantiomers. Moreover, the sensitivity was also improved due to the low background noise of the methanol–CO_2 mobile phase and the sharpened peaks. Sample processing was made efficient and fast using an automated off-line procedure. The resulting pSFC-MS/MS method is simple, efficient, with no mobile phase to be prepared, and thus provides a cost-savings of the labor time and solvent costs.

Acknowledgements

We thank Dr. Lee Zhu for the synthesis of the stable-labeled internal standard and Paul Brown, Alan Dzerk, Tom Johnson, and Chad Briscoe for their discussions and review of the manuscript.

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