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The application of fully automated on-line solid phase extraction in bioanalysis

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ABSTRACT

This paper describes the application of fully automated on-line solid phase extraction to the bioanalysis of three example compounds using the Symbiosis platform. The on-line assay performance is compared to off-line methodologies for the same compounds. The three example compounds possess a variety of physicochemical properties and different extraction modes were applied in off-line methods. These methods were developed through optimisation of solid phase or liquid–liquid extraction and chromatographic separation conditions for each of the analytes. Both on-line and off-line methods were evaluated for linearity, carryover, imprecision and inaccuracy. Experiments were also performed investigating modification of ionisation and selectivity against different batches of plasma. On-line and off-line methods were found to be comparable in performance. In conclusion, on-line methodology has distinct advantages for the analysis of large numbers of samples with a marked reduction in manual operation.

1. Introduction

In bioanalysis, the removal of protein and other components of matrices has always necessitated the development of multi-step methods. Whilst this has led to highly selective and sensitive assays, the extraction steps of these methods are lengthy and often involve a number of manual operations. The direct analysis of plasma samples has always been a goal to speed up sample analysis. The challenge has been to clean up these samples sufficiently to allow highly sensitive and robust assays with the minimum of analyst time spent on sample preparation. Previous solutions have involved the use of turbulent flow chromatography [1] or column switching techniques [2,3]. These techniques often suffer from limited sensitivity or analyte carryover, respectively.

The Symbiosis system developed by Spark Holland is an automated on-line sample preparation system that uses separate solid phase extraction (SPE) cartridges to extract drugs from plasma. There have been several examples of bioanalysis of pharmaceutical compounds from direct injection of plasma using this platform [4,5]. The principles of extraction are essentially the same as off-line SPE. However, the system design allows the SPE eluate to be directly injected onto the chromatography system, thus removing the need for an evaporation step. Fig. 1 compares the extraction processes between off-line and on-line SPE methodology.

The Symbiosis pharma system has been specifically developed for the pharmaceutical industry. This system is not restricted to small volumes of plasma with a capacity up to 500 μL. Also, the system design uses two separate liquid flows for the extraction and chromatography steps, thus much reducing the potential for carryover. The SPE cartridge used by the system is available in a variety of phases to allow selective methods to be developed for a wide range of drugs with varying physicochemical properties.

This paper presents on-line (Symbiosis) methods and off-line methods for three separate pharmaceutical compounds with varying physicochemical properties. This is highlighted by the range of log *D*'s in Fig. 1 and the differing methods developed. A comparison is made of the method performance of these methods together with the respective advantages and disadvantages of the different approaches.

2. Materials and methods

2.1. Reagents and apparatus

Solid analytical standards of CJ-040714, PF-00821385, fluconazole and candoxatril (Fig. 2) were obtained from Pfizer Global Research and Development, Sandwich. Solid CJ-044263, PF-04160891 and UK-051453 were also obtained from Pfizer Global Research and Development, Sandwich as internal standards.

Methanol, pre-prepared methanol–ammonium acetate (2 mM)–formic acid (90:10:0.027, v/v/v) (MF4) and pre-prepared methanol–ammonium acetate (2 mM)–formic acid (10:90:0.027, v/v/v) (MF5) were purchased from Romil Chemicals (Cambridge, UK). Acetonitrile, formic acid, isopropanol and tertiary butyl methyl ether (TBME) were purchased from Riedel-de-Haen (now Sigma–Aldrich (Dorset, UK). Glacial acetic acid, ammonium acetate,

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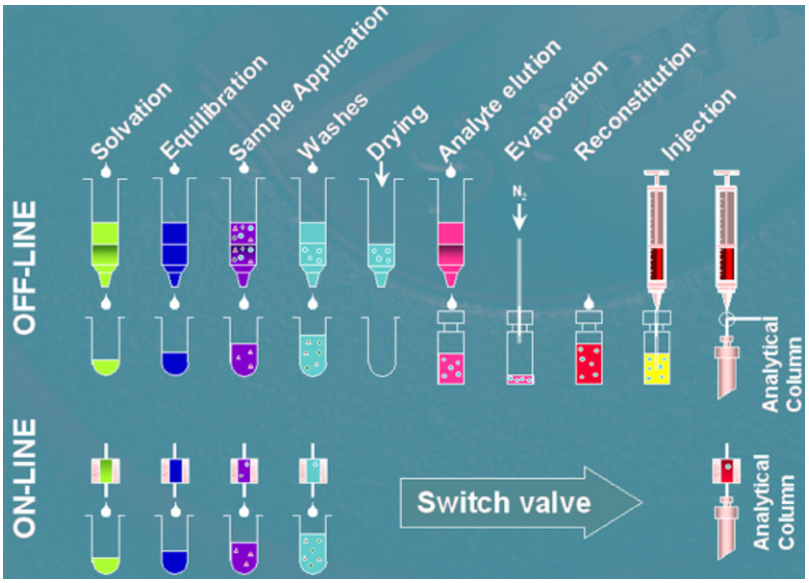
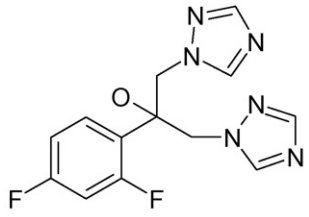
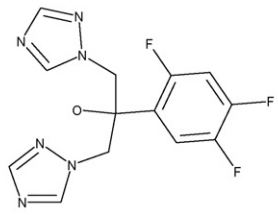


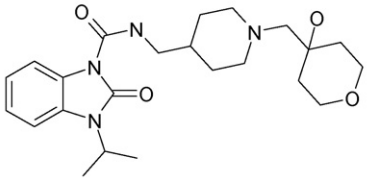
Fig. 1. Comparison between on-line and off-line solid phase extraction methodology.



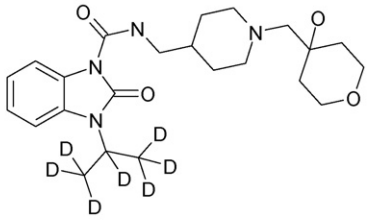
Fluconazole



UK-051453



CJ-040714



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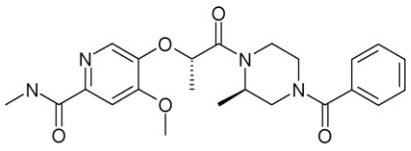

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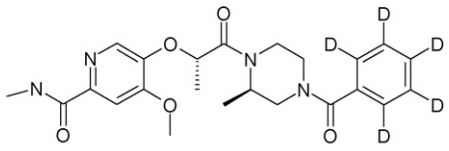

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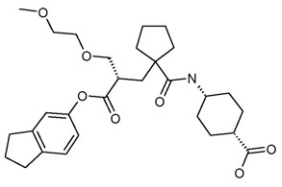

Candoxatril

Fig. 2. The structures of analytes with the corresponding internal standards.

pH 10 buffer and ammonia were purchased from Fisher Scientific (Loughborough, UK).

Ammonium hydrogen carbonate (10 μM, w/v) (pH 7.5) (mobile phase A).

Hysphere CN, C2, C8, C8 end-capped, C18, C18 High density, GP & SH Symbiosis™ cartridges were purchased from Spark Holland (Emmen, The Netherlands). Oasis MCX Symbiosis™ cartridges were supplied by Waters (Elstree, UK). SPEC MP1 and Bond Elut C8 50 mg 96-well SPE plates were purchased from Varian (Oxford, UK).

2.2. Plasma

Drug free heparinised human plasma was purchased from Richmond Pharmacology (London, UK). Drug free dog plasma was obtained from Pfizer Global Research and Development (Sandwich, UK).

2.3. Preparation of standard solutions

2.3.1. CJ-040714

Stock solutions of CJ-040714 and CJ-044263 were prepared in methanol–water (50:50, v/v) at a concentration of approx. 200 μg/mL. To prepare working solutions, serial dilutions of CJ-040714 were prepared from the stock solution at concentrations of 3 μg/mL, 150, 120, 75, 45, 15, 7.5, 4.5, 3.0 and 1.5 ng/mL in methanol–water (50:50, v/v). The CJ-044263 stock solution was diluted in methanol–water (50:50, v/v) to give a concentration of 1 μg/mL. This was then further diluted in pH 10 buffer to prepare internal standard working solution (ISWS) at a concentration of 5 ng/mL.

2.3.2. PF-00821385

Stock solutions of PF-00821385 and PF-04160891 were prepared in methanol–water (50:50, v/v) at a concentration of approximately 200 μg/mL for PF-00821385 and approximately 500 μg/mL for PF-04160891. To prepare working solutions, serial dilutions of PF-00821385 were prepared from the stock solution at concentrations of 2.5, 0.25 and 0.025 μg/mL in methanol–water (50:50, v/v). The PF-04160891 stock was diluted in 1% acetic acid to give a concentration of 0.035 μg/mL.

2.3.3. Fluconazole

Stock solutions of fluconazole and UK-051453 were prepared in methanol–water (50:50, v/v) at a concentration of approximately 200 μg/mL. To prepare working solutions, serial dilutions of fluconazole were prepared from the stock solution at concentrations of 1 μg/mL, 100 ng/mL and 20 ng/mL in methanol–water (50:50, v/v)—working solutions. The UK-051453 stock solution was diluted in methanol–water (50:50, v/v) to give a concentration of 10 μg/mL. To prepare the ISWS, this was then further diluted in water to a concentration of 50 ng/mL.

2.3.4. Candoxatril

A stock solution of candoxatril was prepared in water–formic acid (98:2, v/v) at a concentration of 10 μg/mL.

2.4. SPE–LC–MS/MS

2.4.1. CJ-040714: on-line SPE (XLC)

The calibration range of the method was 0.05–5 ng/mL with 250 μL plasma using a Sciex API 3000 mass spectrometer. All injections used the partial loopfill injection mode. Hysphere C18HD cartridges were used for extraction. After conditioning with 1 mL acetonitrile at a flow rate of 5 mL/min, the cartridge was equilibrated with 1 mL water–acetonitrile–ammonia (94/5/1, v/v/v) again at a flow rate of 5 mL/min. The plasma sample was then applied to the cartridge in water–acetonitrile–ammonia (94/5/1, v/v/v) at a flow rate of 2 mL/min. The cartridge was then washed with 1 mL water–acetonitrile-ammonia (40/59/1, v/v/v) at a flow rate of 5 mL/min. The cartridge was then moved to the right clamp for elution with an MF4/MF5 gradient and an elution time of 1 min. A clamp flush was performed with 0.5 mL acetonitrile–water–formic acid (5/94.8/0.2) at a flow rate of 5 mL/min. This reduces carryover by washing the clamp apparatus (that holds the cartridge during the extraction procedure) to remove residual extract. Chromatography was performed on a Chromolith SpeedROD C18 50 mm × 4.6 mm reverse phase column using a gradient of MF4/MF5 as follows:

Total time (min)	Flow (mL/min)	% MF5	% MF4
0.00	2	100	0
0.50	2	70	30
0.67	2	40	60
1.33	2	20	80
1.67	2	20	80
2.33	2	100	0
3.17	2	100	0

The LC flow was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.4 mL/min.

2.4.2. CJ-040714: off-line

An off-line method for CJ-040714 was developed independently from the on-line method. The calibration range for this method was 0.1–10 ng/mL on a Sciex API 4000 mass spectrometer.

200 μL pH 10 buffer containing internal standard working solution (ISWS) (blank pH 10 buffer for double blanks) was added to 200 μL human plasma. 910 μL TBME was then added and mixed using a liquid handling robot. Samples were centrifuged for 5 min at 3000 rpm. 850 μL of the TBME layer was then transferred to a 96-well block using a liquid handling robot and evaporated to dryness under nitrogen at 40 °C, then reconstituted with 100 μL MF5. 70 μL was injected onto the LC–MS system.

Chromatography was performed on a Chromolith SpeedROD C18 50 mm × 4.6 mm reverse phase column using a gradient of MF4/MF5 as follows:

Total time (min)	Flow (mL/min)	% MF5	% MF4
0.00	2	100	0
0.10	2	70	30
0.50	2	70	30
0.60	2	40	60
0.80	2	40	60
1.40	2	20	80
1.80	2	20	80
3.30	2	100	0

The LC flow was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.4 mL/min.

2.4.3. PF-00821385: on-line SPE (XLC)

An on-line method using mixed mode SPE was developed for PF-00821385 with a range of 5–500 ng/mL on a Sciex API 3000 mass spectrometer. Oasis MCX cartridges were conditioned with 1 mL methanol–water (90:10, v/v) at a flow rate of 5 mL/min and then equilibrated with 1 mL water–acetic acid (99:1, v/v) again at a flow rate of 5 mL/min. 100 μL dog plasma was injected onto the system in microlitre pickup mode with 1 mL water–acetic acid (99:1, v/v) at 2 mL/min. The cartridge was then washed with 1 mL methanol–water (90:10, v/v) at 3 mL/min. The cartridge was then moved to the right clamp for HPD focusing elution with 0.2 mL

methanol–water–ammonia (50:49:1, v/v/v) at 0.1 mL/min. A clamp flush was performed with 0.5 mL methanol–water (10:90, v/v) at 1 mL/min.

Chromatography was performed on a Chromolith SpeedROD C18 50 mm × 4.6 mm reverse phase column using a gradient of MF4/MF5 as follows:

Total time (min)	Flow (mL/min)	% MF5	% MF4
0.00	3	100	0
2.00	3	100	0
2.50	3	0	100
3.50	3	0	100
4.00	3	100	0
4.50	3	100	0

The LC flow was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.6 mL/min.

2.4.4. PF-00821385: off-line

The mixed mode cation exchange method of extraction was also utilised in an independently developed off-line method with a range of 1–100 ng/mL on a Sciex API 4000 mass spectrometer.

250 μL of ISWS was added to 250 μL human plasma. A Varian SPEC MP1 96-well plate was then conditioned with 250 μL methanol and equilibrated with 250 μL water–acetic acid (99/1, v/v), A light vacuum was applied between each step of the sample extraction procedure. Buffered plasma samples were then loaded onto the extraction plate. The plate was then washed with 250 μL H_2O, 250 μL water–acetic acid (99/1, v/v) and 250 μL methanol sequentially. Elution from the extraction plate into a collection block was achieved with 2× 125 μL methanol–ammonia (99:1, v/v). The collection block was then evaporated to dryness under a stream of nitrogen gas at 40 °C and reconstituted with 150 μL MF5. 50 μL was injected onto the LC–MS system.

Chromatography was performed on a Chromolith SpeedROD C18 50 mm × 4.6 mm reverse phase column using a gradient of MF4/MF5 as follows:

Total time (min)	Flow (mL/min)	% MF5	% MF4
0.00	3	100	0
0.50	3	100	0
1.00	3	0	100
2.00	3	0	100
2.50	3	100	0
3.00	3	100	0

The LC flow was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.6 mL/min.

2.4.5. Fluconazole: on-line SPE (XLC)

An on-line method for fluconazole utilising reverse phase SPE was developed with a calibration range of 1–100 ng/mL on a Sciex API 4000 mass spectrometer.

Hysphere C8 cartridges were conditioned with 1 mL acetonitrile at a flow rate of 5 mL/min and then equilibrated with 1 mL water at a flow rate of 5 mL/min. 160 μL of human plasma which had been diluted 1:1 with H_2O containing internal standard (ISWS) (blank water for double blanks) was then injected onto the system at 2 mL/min using the partial loopfill injection mode. The cartridge was then washed with 1 mL acetonitrile–water (5:95, v/v) at a flow rate of 5 mL/min. The cartridge was then moved into the right clamp for gradient elution with MF4/MF5 (elution time was 1 min). A clamp flush was performed with 0.5 mL acetonitrile–water–formic acid (5:94.8:0.2, v/v/v) at 5 mL/min.

Chromatography was performed on a Phenomenex Onyx C18 100 mm × 4.6 mm 5 μm reverse phase column using a gradient of MF4/MF5 as described in Section 2.4.4.

The LC eluent was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.6 mL/min.

2.4.6. Fluconazole: off-line

An off-line method using reverse phase SPE was also developed for fluconazole with a calibration range of 1–100 ng/mL on a Sciex API 4000 mass spectrometer.

200 μL human plasma was diluted with 200 μL H_2O containing internal standard (ISWS) (blank water was added to double blanks). A Varian Bond Elut C8 96-well plate was conditioned with 2× 500 μL acetonitrile. A weak vacuum was applied between each step of the extraction procedure. The plate was then equilibrated with 2× 500 μL water and the diluted plasma was then loaded into the wells of the extraction plate. The plate was then washed with 2× 500 μL water followed by 2× 500 μL acetonitrile–water (5:95, v/v). Elution into a collection block was achieved with 2× 500 μL acetonitrile. The collection block was then evaporated to dryness under a stream of nitrogen at 40 °C and then reconstituted with 100 μL MF5. 40 μL was injected onto the LC–MS system.

Chromatography was performed on a Phenomenex Onyx C18 100 mm × 4.6 mm reverse phase column using the gradient of MF4/MF5 as described in Section 2.4.4.

The LC eluent was split 1:5 post-column, so that the flow rate into the mass spectrometer was 0.6 mL/min.

2.4.7. Candoxatril: post-column infusion experiments with injections of plasma extracts

Blank plasma and water were extracted using the on-line extraction system and injected onto the chromatography system below. Candoxatril was added as a post-column infusion and the specific transition monitored to establish changes in ion suppression. The thermally assisted solid phase extraction (TASPE) module was then used and changes in ion suppression monitored. This module can heat wash solutions up to 80 °C increasing the cleansing ability without affecting the retention of the analyte and is particularly useful when the organic content of such washes is limited by weak retention of the analyte. Chromatography was performed on a Phenomenex Onyx C18 50 mm × 4.6 mm reverse phase column and a Chromolith guard column RP-18e (5 mm × 4.6 mm, USA) using a gradient as described below:

Total (min)	Flow (mL/min)	% mobile phase A	% methanol
00.01	1.5	65	35
02.00	1.5	65	35
03.00	1.5	15	85
05.06	1.5	15	85
05.12	1.5	65	35
06.00	1.5	65	35

Hysphere C18HD cartridges were used for extraction. After conditioning with 1 mL methanol at a flow rate of 5 mL/min, the cartridge was equilibrated with 1 mL water–formic acid (98:2, v/v) again at a flow rate of 5 mL/min. The plasma sample was then applied to the cartridge in 1 mL water–formic acid (98/2, v/v) at 2 mL/min. The cartridge was then washed with 2 mL water–methanol (90/10, v/v) at a flow rate of 2 mL/min. The cartridge was then moved to the right clamp for elution in focussing mode and eluted with 0.2 mL methanol–formic acid (98/2, v/v) at 0.1 mL/min. A clamp flush was performed with 0.5 mL water at a flow rate of 1 mL/min.

2.4.8. Mass spectrometry

Sciex API 4000, 3000 and 2000 mass spectrometers were used for analyte detection. Ionisation was achieved using positive mode TurboIonSpray®. Infusion experiments were performed to establish the optimum MRM transitions and mass spectrometer parameters for each compound. All aspects of data acquisition were controlled using Analyst 1.4 software by Applied Biosystems (Warrington, UK) with the Symbiosis™ Pharma software from Spark Holland (Emmen, The Netherlands) for on-line extraction methods.

API 4000 parameters:

Analyte	Precursor ion (m/z)	Product ion (m/z)	Source temp. (°C)	Ionspray voltage
CJ-040714 (off-line)	431	237	400	4500
CJ-044263 (off-line)	438	255	400	4500
PF-00821385 (off-line)	441	183	500	5000
PF-04160891 (off-line)	446	183	500	5000
Fluconazole	307	220	500	5500
UK-51453	325	256	500	5500

API 3000 parameters:

Analyte	Precursor ion (m/z)	Product ion (m/z)	Source temp. (°C)	Ionspray voltage
CJ-040714 (on-line)	431	255	500	4400
CJ-044263 (on-line)	438	255	500	4400
PF-00821385 (on-line)	441	183	500	5000
PF-04160891 (on-line)	446	183	500	5000

API 2000 parameters:

Analyte	Precursor ion (m/z)	Product ion (m/z)	Source temp. (°C)	Ionspray voltage
Candoxatril	516	306	500	4000

2.5. Assay validation

Analytical validation of linearity, limit of quantification, precision, accuracy, selectivity and modification of ionisation was performed according to the recommendations presented in the document "Guidance for Industry, Bioanalytical Method Validation" [6]. It also reflects industry recommendations defined in two conferences [7,8].

2.5.1. Linearity, precision and accuracy

Quantification was performed by integration of the area under the specific multiple reaction monitoring MRM chromatograms. Calibration lines were constructed by plotting the peak area ratio of analyte to internal standard in calibration standards against analyte concentration using least squares regression with a weighting factor of $1/y^2$. The calibration line must contain a minimum of six concentration levels excluding blanks, with at least 75% of the total number of calibration standards included in the final curve. At least one blank matrix sample was included in the run after the top calibration standard, to assess carryover.

Imprecision and inaccuracy were evaluated using validation (QC) samples prepared from a separate stock weighing to the standard analyte stock. A minimum of five replicates are prepared at four concentrations: lower limit of quantification (LOQ), low, mid and high. Imprecision was expressed as percent relative standard deviation (% CV) and accuracy as percent bias (%Bias). Acceptable imprecision was ≤15% (≤20% at the LOQ). Inaccuracy was acceptable where %Bias was ±15% (±20% at the LOQ).

The method was said to be validated where at least three validation batches were acceptable, and overall batch imprecision was ≤15% at all levels except the LOQ (≤20%).

2.5.2. Selectivity and ionisation modification experiments

Selectivity was assessed by the inclusion of three independent sources of biological matrix double blanks ($n = 3$ samples per plasma batch). Any response with a similar retention to the analyte was considered acceptable only if it was ≤20% of the analyte response for the LOQ standard. The method was acceptable if ≤10% of sources failed.

Modification of ionisation was assessed in three independent sources of biological matrix which were spiked at the level of the low QC ($n = 3$ samples per plasma batch). Accuracy of the response compared to nominal was calculated and accepted if %Bias was ≤15%. The method was acceptable if ≤10% of sources failed.

2.5.3. Calculation of imprecision (%CV) and inaccuracy (%Bias)

Within-batch (intra-batch) and between-batch (inter-batch) imprecision and inaccuracy are calculated as for validation samples using the statistical procedures shown below.

Imprecision is expressed as percent relative standard deviation (%CV):

$$\%\mathrm{CV} = \frac{\sqrt{\sum_{i=1}^{n}(x_i - \overline{x_i})^2/(n-1)}}{\overline{x_i}} \times 100$$

where x_i is the ith observed value of n observations; n is the total number of observations; a minimum of 5 (within-batch) and 15 (between-batch); x_i is the within-batch mean (e.g., $n \geq 5$) or overall mean (e.g., $n \geq 15$).

Inaccuracy is expressed as percent relative error (%Bias):

$$\%\mathrm{Bias} = \frac{E - T}{T} \times 100$$

where E is the mean experimental concentration and T is the theoretical concentration (nominal value).

3. Results

3.1. Validation data for fluconazole methods

Both on-line and off-line methods for fluconazole were successfully validated, producing almost identical imprecision and inaccuracy data. At the LOQ (1 ng/mL), the imprecision (%CV) and inaccuracy (%Bias) were 3.3% and 9.2% for on-line and 5.1% and 10.1% for off-line (Table 1). Carryover for on-line and off-line methods were both acceptable at 0.03% and 0.06%, respectively.

Table 1
Imprecision and inaccuracy data for on-line and off-line methods for fluconazole, CJ-040714 and PF-00821385.

Compound	On-line				Off-line			
	QC conc. (ng/mL)	Mean conc. (ng/mL)	% CV	%Bias	QC conc. (ng/mL)	Mean conc. (ng/mL)	% CV	%Bias
Fluconazole	1	1.10	3.3	9.2	1	1.10	5.1	10.1
	2.5	2.64	2.4	5.7	2.5	2.65	3.7	2.2
	50	52.5	1.0	5.0	50	52.7	2.2	5.3
	80	83.5	2.0	4.4	80	83.4	2.0	4.3
CJ-040714	0.05	0.0536	6.1	7.2	0.1	0.107	7.0	7.0
	0.15	0.151	7.0	1.2	0.3	0.308	3.2	2.7
	2.5	2.52	5.5	0.9	5.0	5.29	3.6	5.8
	3.5	3.39	7.1	−4.0	7.5	7.76	1.4	3.5
PF-0821385	5	4.81	8.6	−3.8	1.0	1.04	5.9	4.0
	10	10.5	4.6	5.4	2.0	2.11	4.5	5.5
	200	203	1.4	1.5	50	50.4	2.3	0.8
	400	381	4.7	−4.7	80	81.8	1.8	2.3

Table 2
Concentration of fluconazole in ionisation effects experiments with different batches of human plasma.

Mode	Replicate	Concentration of fluconazole (ng/mL)		
		Batch 1	Batch 2	Batch 3
On-line	1	2.90[a]	2.72	2.75
	2	3.03[a]	2.65	2.78
	3	3.00[a]	2.85	3.15[a]
	Mean	2.97	2.74	2.89
Off-line	1	3.49[a]	2.75	2.77
	2	3.16[a]	2.69	2.76
	3	3.18[a]	2.73	2.59
	Mean	3.27	2.72	2.71

[a] Replicate that was outside the acceptance criteria.

Table 3
Peak areas at the ion transition for fluconazole in selectivity experiments with different batches of blank human plasma.

Mode	Replicate	Peak area (cps)		
		Batch 1	Batch 2	Batch 3
On-line	1	161.5	56.7	119.7
	2	540.4	60.3	80.5
	3	288.8	60.1	80.3
Off-line	1	80.6	78.7	59.7
	2	93.3	81.5	60.6
	3	223.9	59.8	46.2

In the modification of ionisation experiments where low QC samples were assayed from independent matrix sources, batch 1 failed in both methods. The mean concentration in the on-line method (2.97 ng/mL) and off-line method (3.27 ng/mL) was greater than 15% higher than the nominal concentration (2.5 ng/mL) (Table 2).

In the selectivity experiments, all batches passed the acceptance criteria. Peak areas for batch 1 were slightly higher than for the other batches but were still within the acceptance criteria (≤20% peak area of LOQ).

The variation of internal standard peak areas for both the on-line and off-line fluconazole methods is shown in Figs. 3 and 4. For the on-line method, injections with batch 1 plasma show a significantly higher internal standard response than other batches. This is not observed with the off-line method (Table 3).

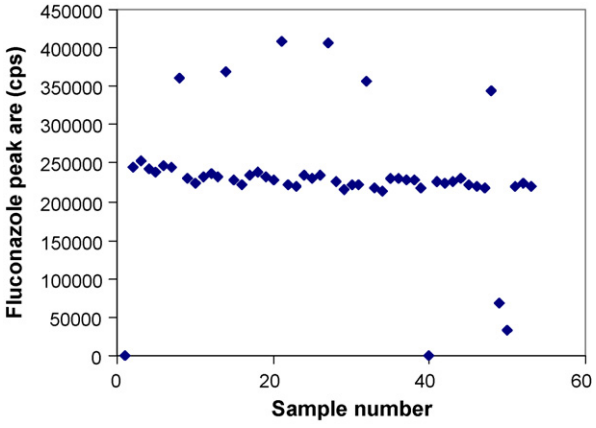

Fig. 3. A plot of fluconazole internal standard area against sample number for the on-line method.

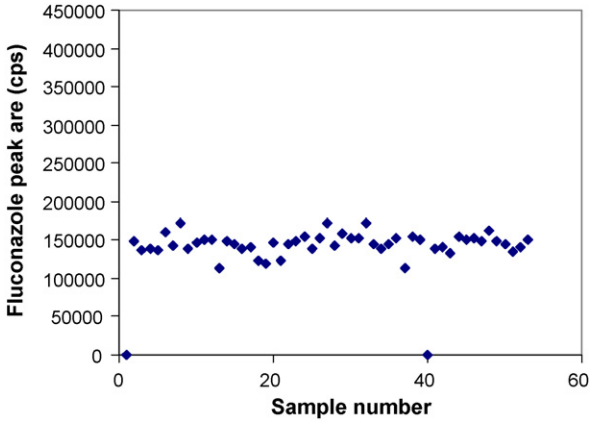

Fig. 4. A plot of fluconazole internal standard area against sample number for the off-line method.

3.2. Validation data for CJ-040714 methods

Despite the differences in extraction modality of the on-line (SPE) and off-line (LLE) methods, both assays behaved similarly and were successfully validated using similar ranges. The LOQ established for the on-line method was 0.05 ng/mL versus 0.1 ng/mL for the off-line method despite utilising the less sensitive mass spectrometer. These LOQ values were appropriate for the target assay range and do not necessarily reflect the maximum sensitivity that could be achieved. As shown in Table 1, imprecision (%CV) and inaccuracy (%Bias) at the LOQ were almost identical (6.1% and 7.2% for on-line and 7.0% and 7.0% for off-line). Similar %CV and %Bias values between off-line and on-line methods were observed throughout the range. Carryover for on-line and off-line methods was both acceptable at 0.12% and 0.04%, respectively. Mean correlation coefficients for on-line and off-line were 0.9978 and 0.9971, respectively. When taking into account the less sensitive mass spectrometer used for the on-line Symbiosis method, the on-line method was considered significantly more sensitive.

3.3. Validation data for PF-00821385 methods

The LOQ established for the on-line method was 5 ng/mL versus 1 ng/mL for the off-line method. This was due to utilising a less sensitive mass spectrometer and a smaller volume of samples. As shown in Table 1, imprecision (%CV) and inaccuracy (%Bias) were acceptable at the LOQ (8.6% and −3.8% for on-line and 5.9% and 4.0% for off-line) and throughout the range. Carryover for on-line and off-line methods was both acceptable at 0.12% and 0.02%, respectively. Mean correlation coefficients for on-line and off-line were 0.9988 and 0.9971, respectively. The sensitivity differences seen between the on-line and off-line methods are largely due to the differences in sample volume and mass spectrometer used for detection.

3.4. Thermally assisted solid phase extraction (TASPE)

Chromatograms following injections of water, plasma and plasma with TASPE with post-column infusion of candoxatril were obtained (Fig. 5). Significant areas of ion suppression were observed at 0.8, 2.1 and 2.5 min and mass spectrometric response at the transition of candoxatril was reduced by approximately 95%, 25% and 60%, respectively. The retention time of candoxatril was 2.7 min. No ion suppression was observed for the water injection. When the TASPE module was used to heat the wash to 80 °C, the areas of ion suppression at 2.1 and 2.5 min were removed completely and the area at 0.8 min was reduced to a reduction of response of 25%.

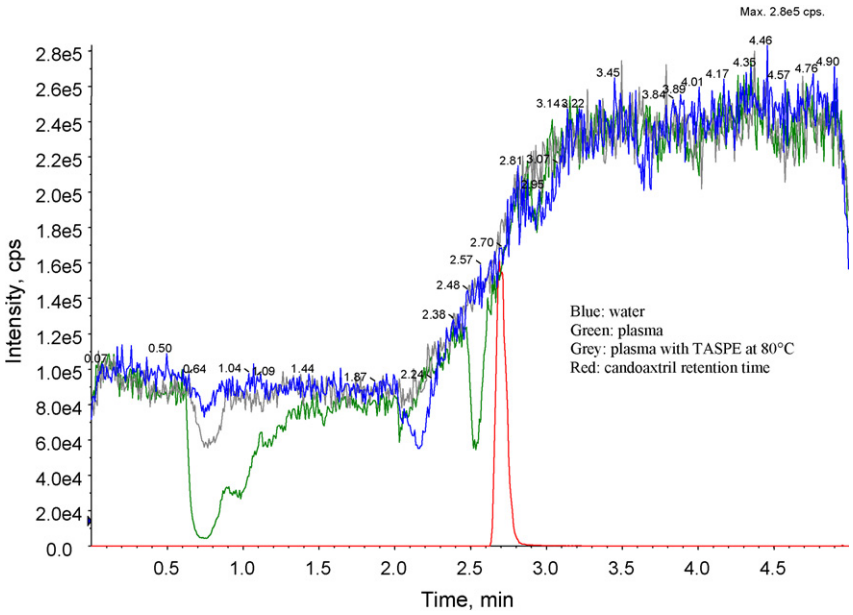

Fig. 5. Chromatogram at the ion transition following post-column infusion of candoxatril and injections of water, plasma with and without TASPE washes.

The results from the TASPE experiment showed that significant reductions in ion suppression could be achieved by washing with water heated to 80 °C compared to unheated water. This is of particular importance when the use of washes to remove interferences is limited by loss of compound from the solid phase. In this particular example the SPE method for candoxatril was not limited to low organic washes. Other experiments have shown that using a 45% organic wash instead of the 10% organic wash in this experiment removed all interferences adequately.

4. Discussion

The three on-line methods developed for fluconazole, CJ-040714 and PF-00821385 were all successfully validated for carryover, linearity, imprecision and inaccuracy. These data compare well with those obtained for the off-line methods. The on-line and off-line methods for CJ-040714 differ in their extraction mechanism. An increased internal standard response for batch 1 plasma was observed with the PF-00821385 assays presumably due to the presence of endogenous material in extracts from the on-line method but not in extracts from the off-line method.

Developing analytical methods on the Symbiosis system is potentially more complex given the large number of parameters (e.g., elution time, elution flow rate) that can be varied. These parameters are not modified in off-line methods. Thus for studies involving only a small number of samples, developing an on-line method may not be the most efficient approach. The TASPE module of the Symbiosis system allows heating of the washes which can be useful when the use of highly organic washes is limited by analyte retention. The addition of heated washes to 96-well SPE plates in off-line methods would be difficult to control.

Whilst there is no improved performance of the assay when analysed using the Symbiosis system compared to off-line method, there is the potential to increase sensitivity by minimising the losses made during liquid transfer and reconstitution steps. The off-line methods all provided appropriate levels of sensitivity for the intended purpose. This reflects the improvement in mass spectrometer sensitivity through technological advances in recent years which has meant that in general, pharmacokinetic analysis in drug discovery and development can be readily achieved using available technologies [9].

In conclusion, the benefits of on-line SPE are largely due to the reduced analyst and extraction time in the analysis of samples. By reducing manual involvement, the potential for human error is reduced. However there are several advantages to using off-line methods in terms of their simplicity and the use of automation can reduce manual involvement. Overall this comparison of on-line and off-line methods has shown that they are comparable in terms of general bioanalytical assay performance characteristics.

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