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Development of a μ-turbulent flow chromatography focus mode method for drug quantitation in discovery bioanalysis

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Abstract

An online turbulent flow chromatography method coupled to tandem mass spectrometry (TFC-MS/MS) has been developed within our bioanalytical group, suited to the analysis of mid to late stage discovery compounds. A dual column configuration utilising isocratic focusing of the analyte upon the analytical column maintained an excellent peak shape for a large proportion of compounds encountered and enabled consistent quantitation to sub-nanogram concentrations (<15 pg on column). Furthermore, the low sample injection volume coupled with rapid column washing using basic and acidic mobile phases, has proved advantageous in removing sample carryover and also the overall exposure to biological material; favourable for good system robustness. All the data discussed were generated with a method cycle time of 5 min providing accurate quantitation (acceptance criteria based upon FDA method validation guidelines) with multiple analytes and biological matrices.

Keywords: Focus mode; Tandem mass spectrometry; Turbulent flow chromatography (TFC); Online extraction; Low volume samples; Biological matrices; Analytical column

1. Introduction

A significant challenge within the pharmaceutical industry bioanalytical field, is maintaining an optimal balance of assay sensitivity, selectivity and applicability using multiple sample matrices and volumes; whilst reducing the cycle time for delivery of quantitative data. Such 'fit for purpose' assays in a discovery environment impact the timescale with which drug candidate selection decisions can be made. Consequently, both the sample throughput and data quality are of prime concern during analytical method development.

Turbulent flow chromatography (TFC) is an online application that enables quantitative determination without prior sample cleanup and the technology is well described within the literature using a variety of biofluids [1–7], predominantly coupled to a tandem mass spectrometric endpoint (TFC-MS/MS). The ability to dilute and inject samples online with minimal intervention reduces the preparation time necessary and consequently offers a time saving advantage over more labour intensive liquid–liquid extraction or solid phase extraction techniques. Several authors over the years have demonstrated successfully how such a reduced sample preparation time, coupled to a short turbulent flow chromatographic gradient can reduce the bottleneck of discovery sample throughput, particularly during quantification of in vitro [8,9] and in vivo samples [10–12]. Recently, TFC operating in a dual column (sample extraction and isocratic focusing) mode has utilised a monolithic column compatible with high flow rate and low backpressures [13], to reduce cycle times to shorter than 1.5 min.

Further useful applications of TFC have been the setup of high throughput GLP validated assays to analyse pharmaceutical compounds entering the development stage [14–16] and clinical monitoring of drug exposure levels in hospital patients [17,18]. However, in each of these cases method development time is significantly extended to optimise the quantitation to the analyte/s of interest. As such, the successful application of any TFC system therefore needs to consider specific requirements relative to intended use.

At Pfizer within our discovery bioanalytical group, due to the diverse array of compound libraries and a high throughput

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in vitro ADME screening platform [19,20] that quickly identifies candidate molecules; a significant variety of compounds require a high quality analytical method suitable for further in vivo investigations, lead optimisation and ultimately candidate nomination. Therefore, it is necessary to achieve robust analysis that incorporates analytical sensitivity and accuracy over a wide range of compound physicochemistries. In particular, achieving good chromatography with analytically challenging molecules such as strongly lipophilic neutrals and charged species whilst minimising the carryover often associated with such analytes is key to delivering quality data. Equally, simultaneous retention of polar analytes to monitor compound metabolites would allow a platform to be utilised in a more generic manner for a variety of sample types in discovery bioanalysis. This paper describes a sensitive μ-TFC focus mode method developed in our discovery research group that could be applied to a wide range of compounds. The method uses a small biological sample volume (15 μl) and broad organic gradient (3–97%) coupled with several aggressive wash steps to minimise carryover problems.

2. Experimental

2.1. Materials and reagents

The analyte test mixture used during method development was obtained from Pfizer Global R&D, Sandwich Laboratories, Kent, UK. All other compounds analysed using the developed method were obtained from the same source. The chemical structures within the test mixture (Fig. 1) were used to optimise μ-TFC conditions and are representative of a wide physicochemical range within pharmaceutical discovery (Table 1). Other mobile phase and analytical reagents were HPLC grade or better (Sigma–Aldrich Ltd., Dorset, UK) and were used without further purification. Control plasma matrices were obtained in-house and from UK suppliers (Charles River, Harlan Sera-Labs & Richmond Pharmacology).

Table 1
Physicochemical properties of the test mix

Compound	Molecular weight	$c \log P$	pK_a	Notes
Fluconazole (A)	306	−0.11	4.5/5.3	Polar, neutral
Compound B	365	−0.05	9.1	Polar, weak base
Dofetilide (C)	441	1.56	7/9.2	Weak base
Candoxatril (D)	515	2.94	4.5	Weak acid
Compound E	683	4.2	NR_4^+	Lipophilic
Compound F	730	3.94	6.1	Lipophilic, neutral

2.2. HPLC

The LC system used for all analyses was a parallel ARIA TX2 platform (Cohesive Technologies, Franklin, MA, USA). Predominantly, only one system was operational at a time but sample throughput could be potentially doubled using two equivalent μ-TFC systems. The overall platform comprised four HPLC pumps, two binary and two quaternary, one autosampler with two injection valves and a switching valve module. For each system operating in the μ-TFC focus mode configuration, a Hewlett-Packard 1100 series quaternary pump (Cohesive Technologies) delivered the mobile phase for extraction on a 50 mm × 0.5 mm i.d. μCyclone polymeric column (60 μM particle diameter, 100 Å pore size; Cohesive Technologies). The flow rate was maintained at 2 ml/min using an aqueous solution of formic acid for extraction (solvent A, 1/999, w/v), and wash solutions comprised NH_3 in methanol (solvent B, 1/99, v/v) and glacial acetic acid (solvent D, 15/85, w/v). Additionally, formic acid in acetonitrile (solvent C, 1/999, w/v) acted as the organic loop reagent and a further wash solvent. A biocompatible PAT online frit (5 μm, 0.25 in. × 0.067 in., VICI, AG International) was fitted before the extraction column. A CTC HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) injected 30 μl aliquots of 1:1 diluted plasma fortified with drug and internal standard onto the μCyclone extraction col-

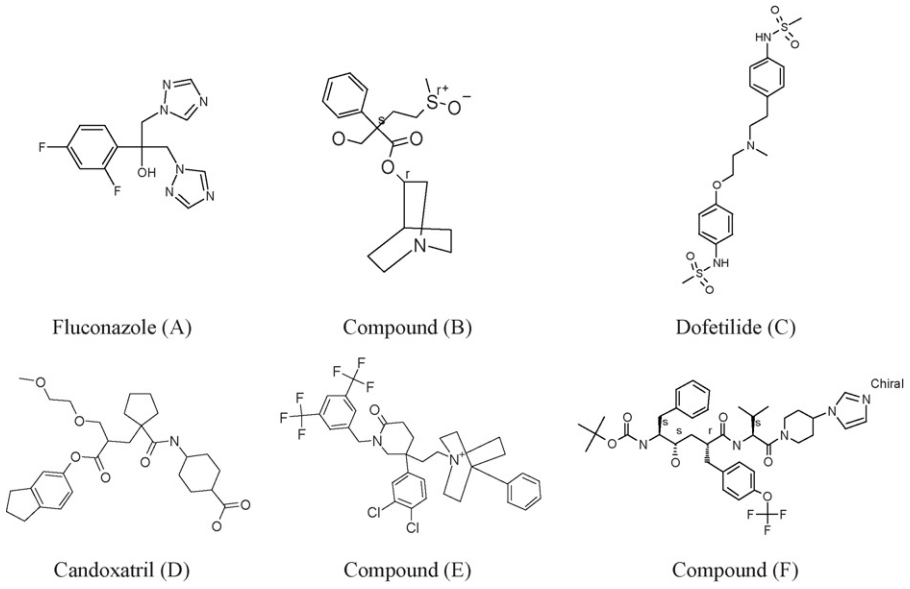

Fig. 1. Test mixture used to optimise the μ-TFC method (Ref. [14]).

umn. The autosampler was equipped with two injection ports as well as a Peltier-cooled stack set to maintain the temperature at 10 °C. Acetic acid solution (1/99, w/v) in water (wash 1) and acetic acid/acetone/isopropanol/methanol (1/30/30/39, w/v, wash 2) were used as aggressive wash solvents for the CTC autosampler injectors and syringe. After each injection, wash 1 was used first to wash out the plasma residue. An aqueous wash was used in order to avoid blockage from potential protein precipitation that may occur with a methanol wash.

A Hewlett-Packard 1100 series binary pump (Cohesive Technologies) delivered mobile phase concurrently to the analytical column running through a 2 μM guard filter. The standard analytical column used was a 50 mm × 4.6 mm i.d. C18 Zorbax Extend column (5 μM particle, 80 Å pore size, Agilent Technologies, Palo Alto, CA, USA) but alternative columns were assessed during the investigation selected using a requirement for pH stability between the range of 2 and 12. The flow rate was set at a rate of 1.2 ml/min using a mixture of aqueous solvent A (formic acid/water, 1/999, w/v) and organic solvent B (acetic acid/heptafluorobutyric acid/MeOH, 5/0.1/994.9, w/v) delivered without splitting into the mass spectrometer. The total cycle time for each injection, including wash steps was approximately 5 min and this could be reduced by half to 2.5 min when the two systems were used in parallel.

2.3. Column switching setup

The Rheodyne six-port switching valve interface module (VIM) as well as the LC pumps was controlled by ARIA software v1.5 (Cohesive Technologies). The switching valve configuration was set up in focus mode as shown in Fig. 2a and b, to focus analytes in a narrow band upon the head of the analytical column using the following steps, listed in detail in Table 2. The plasma was loaded onto the extraction column with solvent A (100%), whilst the analytical column was equilibrated with a mixture of solvents A and B (Step 1). Both valves A and B were switched, opening flow of the 100 μl plug of organic solvent through the extraction column and enabling transfer to the analytical column where flows were connected with a specially adapted T-valve. Transfer eluent was diluted sufficiently in aqueous mobile phase (1 plus 11) to enable focusing on the analytical column head (Step 2). Analytes were eluted to MS using a rapid gradient mixture of solvents A and B (Step 3). The valves, analytical and extraction columns were washed sequentially with 100% of solvents A–D (Table 2) and the loop at valve A refilled (Step 4). The system was then re-equilibrated (Step 5).

2.4. Solutions and standards

All stock solutions of analytes (A–F) were made in methanol at 1 mg/ml and stored frozen (−20 °C). Working solutions con-

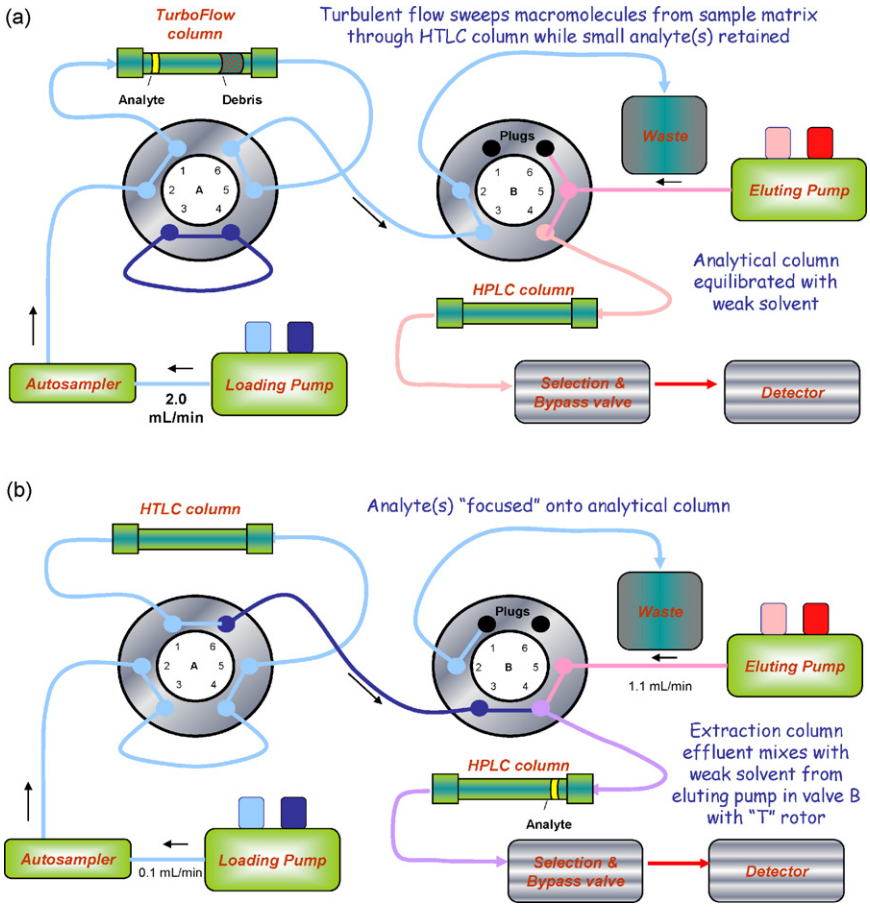

Fig. 2. (a and b) Schematic diagram of the valve switching units.

Table 2
Valve switching method for μ-TFC

Step	Duration		Quaternary pump					Binary pump				Valve position	
	Start	Sec	Flow (ml/min)	%A	%B	%C	%D	Flow (ml/min)	Grad	%A	%B	A (loop)	B (tee)
1	0	30	2	100	–	–	–	1.2	Step	97	3	Out	
2	0.5	70	0.1	100	–	–	–	1.1	Step	97	3	In	T
3	1.67	35	2	100	–	–	–	1.2	Ramp	3	97	In	
4	2.25	5	2	–	–	100	–	1.2	Step	3	97	Out	
5	2.33	5	2	100	–	–	–	1.2	Step	3	97	In	
6	2.42	5	2	–	–	100	–	1.2	Step	3	97	In	
7	2.5	5	2	–	–	100	–	1.2	Step	3	97	Out	
8	2.58	15	2	–	100	–	–	1.2	Step	3	97	Out	
9	2.83	15	2	–	100	–	–	1.2	Step	3	97	In	
10	3.08	5	1.25	–	–	100	–	0	n/a	3	97	Out	T
11	3.17	5	1.25	–	–	100	–	0	n/a	3	97	In	T
12	3.25	10	1.25	–	–	–	100	0	n/a	3	97	In	T
13	3.42	10	1.25	–	–	–	100	0	n/a	3	97	Out	T
14	3.55	45	2	–	–	100	–	1.2	Step	97	3	In	
15	4.33	45	2	100	–	–	–	1.2	Step	97	3	Out	

Solvent consumption per 100 samples: (A)—800 ml (B)—100 ml (C)—200 ml (D)—40 ml (B, eluting)—165 ml.

taining all analytes at 5, 0.5, 0.05 and 0.005 μg/ml in 50/50 (v/v) water/methanol were obtained by serial dilution. Calibration standards and quality control (QC) samples were prepared in plasma (50 μl) The final volume was made up to 100 μl using a maximum of 10 μl working solution (≤5% methanol concentration) and dilution media containing internal standard (100 ng/ml). The internal standard used was a structural analogue of the analyte of interest. Calibration lines consisted of eleven plasma standards at 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml and QC samples were spiked independently to plasma concentrations of 1, 10 and 100 ng/ml ($n = 3$, analytes A–F) and 0.1 and 200 ng/ml ($n = 6$, analyte C) to measure imprecision and inaccuracy. All other bioanalytical studies carried out encompassed a similar dynamic range. Data were obtained using Analyst 1.4 (Applied Biosystems/MDS Sciex, Canada). The peak area ratio (drug/IS) was plotted versus the concentration in ng/ml, using linear weighted least squares regression. The linear regression calibration was modelled against weighting factors of $1/y^2$.

2.5. Bioanalytical acceptance criteria

All discovery data deemed to exhibit acceptable quantitation accuracy and precision using the μ-TFC method, exhibited the following features: the lower limit of quantitation (LLOQ) peak intensity three times greater than background noise and the peak area ratio four times greater than the calibration y-intercept. The coefficient of determination (r^2) greater than 0.99 containing more than 75% points with less than 20% inaccuracy; low, medium, high and dilution QC's greater than 66% points with less than 20% inaccuracy. Carryover immediately following the highest calibration standard at a level that does not compromise the quantitation of the LLOQ. A consistent internal standard response and all study sample concentrations within ±20% the upper limit of quantitation/LLOQ, respectively. The described acceptance criteria were defined considering the FDA guidelines for GLP bioanalytical work and acknowledging the fact that non-GLP regulated studies were performed requiring a rapid turnaround from sample receipt to data delivery.

2.6. Sample dilution procedure

Plasma samples were thawed at ambient temperature. Aliquots (50 μl) were transferred into a 96-well block and mixed with an equal volume of dilution media (1 M monochloroacetic acid/acetonitrile, 90/10, v/v). Other sample dilution media combinations (formic acid/water/acetonitrile, 1/899/100, 5/895/100, 1/949/50 and 5/945/50, w/v) were also investigated. The block was then centrifuged at 3000 rpm ($2000 \times g$) for 10 min at approximately 4 °C and transferred into the autosampler-cooled tray for analysis. The dilution step did not result in any visible protein precipitation and organic solvent was present to help keep the analytes in solution.

2.7. Mass spectrometry: detection conditions

An API 4000™ Triple Quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Canada) operating in the positive ion mode with a TurboIonSpray interface at 650 °C was employed for the detection of the analytes. Nitrogen was used as both nebuliser gas with an applied voltage of 5000 V and collision gas at a setting of 4. Detection was performed using multiple reaction monitoring (MRM) and a dwell time of 50 ms per transition for the analytes and the internal standards. The mass spec parameters, such as MRM transitions were quickly determined by injecting a 200 ng/ml solution (90/10, v/v, water/methanol) and running an appropriate Q1 and a product ion scan at three collision energies (25, 40 and 55 eV) to identify the unit mass values for each analyte. Declustering potential and exit potential values defaulted to 60 and 10, respectively.

3. Results and discussion

3.1. Sample preparation

The preparation steps prior to sample injection were continued in the format previously described by our group [14] where dilution 1:1 (v/v) in 1 M monochloroacetic acid buffer containing 10% organic helps to release protein bound drug material and keep it in solution. This type of acidification weakly denatures the protein but results in no visible protein precipitation within the sample, enabling direct injection. Our observation is that this type of sample preparation precludes an extra step of prolonged centrifugation, supernatant transfer to a separate vessel (necessary when using protein precipitation) and sample concentration, offering an extra time saving advantage. Furthermore, this approach can be beneficial in the analysis of some compounds liable to bind to plastic 96-well plates because the presence of protein in each well, aids compound solubilisation when compared to conventional reconstitution media of lower solvating strength. Further investigation of the strength of the acid buffer as well as the organic composition (5–10%) was conducted to assess the affects upon analytical sensitivity and was shown to be variable for different analytes; therefore, the aforementioned buffer was used with all the data described in this method. In using acidic media an important caveat to consider was the chemical stability of the analytes of interest. On one hand, the degradation of enzymatic activity in neat biological sample once the sample is thawed can be an advantage [4] but equally acid labile compounds can be degraded over time affecting the accuracy and reproducibility of bioanalytical data. However, so far using the technique in discovery pharmaceutical analysis we have yet to noticeably encounter this problem with μ-TFC.

An injection volume of 30 μl (15 μl biological matrix) was considered most appropriate because of its applicability with multiple discovery study types where in vivo sampling volumes can sometimes be low and also to maintain robustness of the μ-TFC system. The biocompatible online frit prior to the turbulent flow column was frequently the point where pressure build-up could occur but this was easily solved by replacement, necessary after approximately every 300–400 samples. In our experience larger injection volumes can be made (50–100 μl) but it is necessary to replace the frit more regularly to avoid rapid pressure build-up or alternatively use a larger pore size, exposing the column and valve switching unit to larger size biological debris material. The overall advantage gained through having a con-

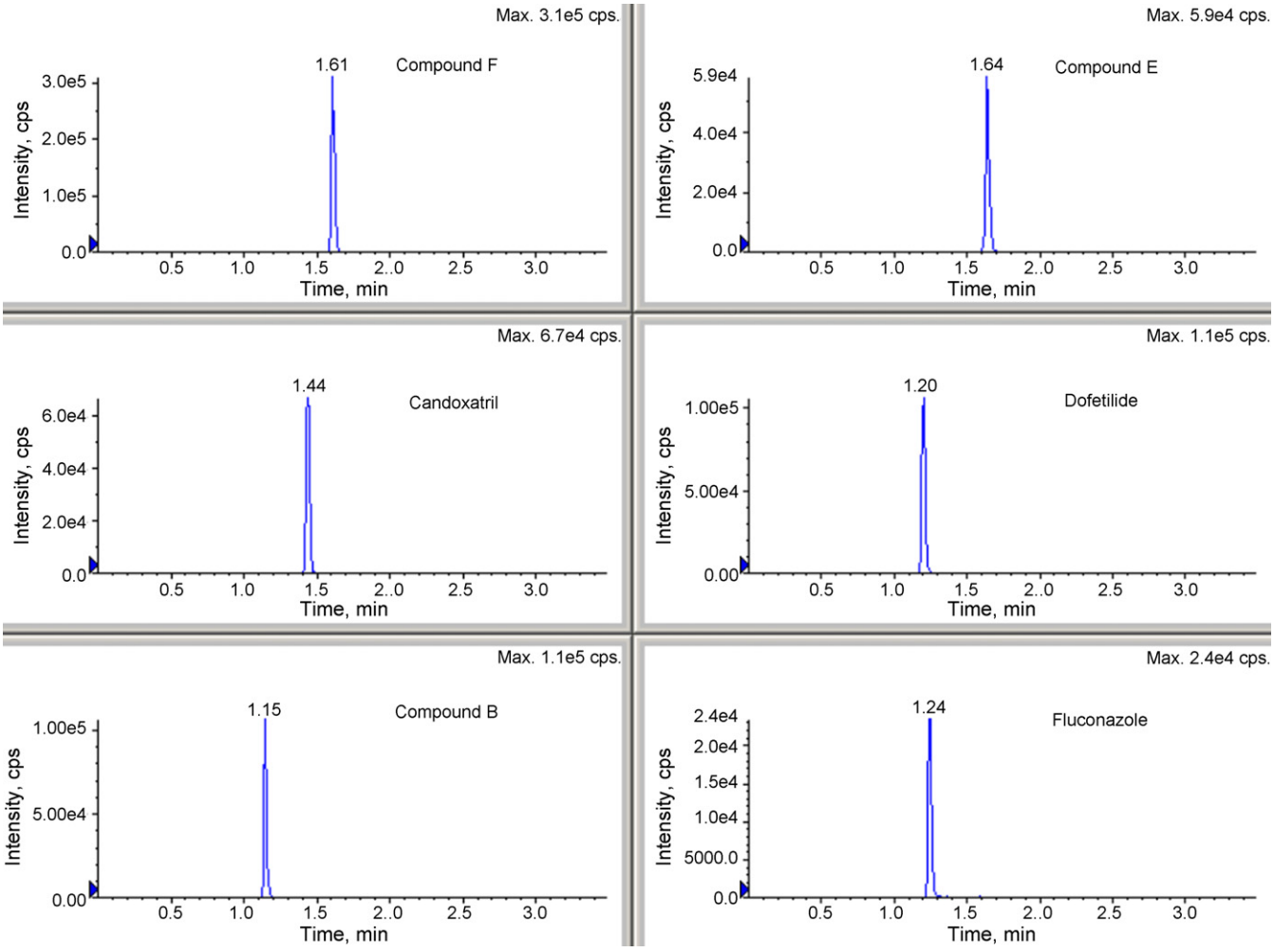

Fig. 3. Representative chromatograms for the analytical test mixture (50 ng/ml rat plasma injection).

tinuously operational system outweighs any potential gains in analytical sensitivity with a single analyte of interest or specific study requirement.

3.2. μ-TFC method development

Using the test mixture described, alterations in the chromatographic method were made until a satisfactory peak shape was achieved for all the analytes of interest; this followed injection of a 50 ng/ml rat plasma standard solution (0.75 ng on column). The peaks (Fig. 3) obtained in the final method were of a consistent high quality demonstrated by a low peak width and peak asymmetry and high recovery (>90%). Furthermore, despite using a rapid 30 s gradient chromatographic separation of four of the six analytes was achieved.

The primary improvement was made through optimising the isocratic focusing upon the analytical column during sample transfer, where loop material (100 μl acetonitrile) was diluted approximately 12-fold by the high aqueous mobile phase composition (97%, formic acid/water, 1/999, w/v) and flow (1.1 ml/min). The resulting flow reaching the column had a low organic content (8%) which was sufficient to retain all analytes in the mixture. By contrast, the acetonitrile present in the loop was a strong enough organic eluent to wash even the most lipophilic analytes off the turbulent flow column in a tight focused band. Furthermore, by easily adjusting this loop content (e.g. 50% acetonitrile giving 4% organic content at the analytical column) it has also been possible to retain polar analytes. Secondly, the flow rate of the eluting pump (1.2 ml/min; Fig. 4) was found to be optimal for the API4000 detection system because flow splitting was not required and the majority of the drug material could be ionised to maximise the response. The mass spectrometer source was run at a high temperature of 650 °C to aid this process and the discharge needle positioned with minimal exposure (<1 mm) from the TurboIonSpray probe to allow fluid dispersion over the dual ceramic heater rods.

The analytical method was characterised using the calibration standards and imprecision and inaccuracy samples previously described. Data shown in Table 3 express QC inaccuracy and imprecision values obtained over the concentration range 0.1–200 ng/ml, calculated as follows:

$$\text{Inaccuracy (\%error)} = \frac{\text{measured value} - \text{spiked value}}{\text{spike value}} \times 100$$

$$\text{Imprecision (\%RSD)} = \frac{\text{standard deviation}}{\text{mean measured value}} \times 100$$

A linear fit ($r^2 > 0.99$) for the calibration curve and low inaccuracy and imprecision (<10%) values were obtained for the majority of assay samples included in this run, indicating a robust method capable of quantifying discovery samples well within the discovery analytical criteria previously described. The extremely wide dynamic range of this assay (2000-fold) was performed to test the upper and lower limits of quantitation for each analyte in the test mixture (Table 4). Normally a 500- or 1000-fold range is considered fit for purpose within the discovery analytical group when using mass spectrometric detection. The

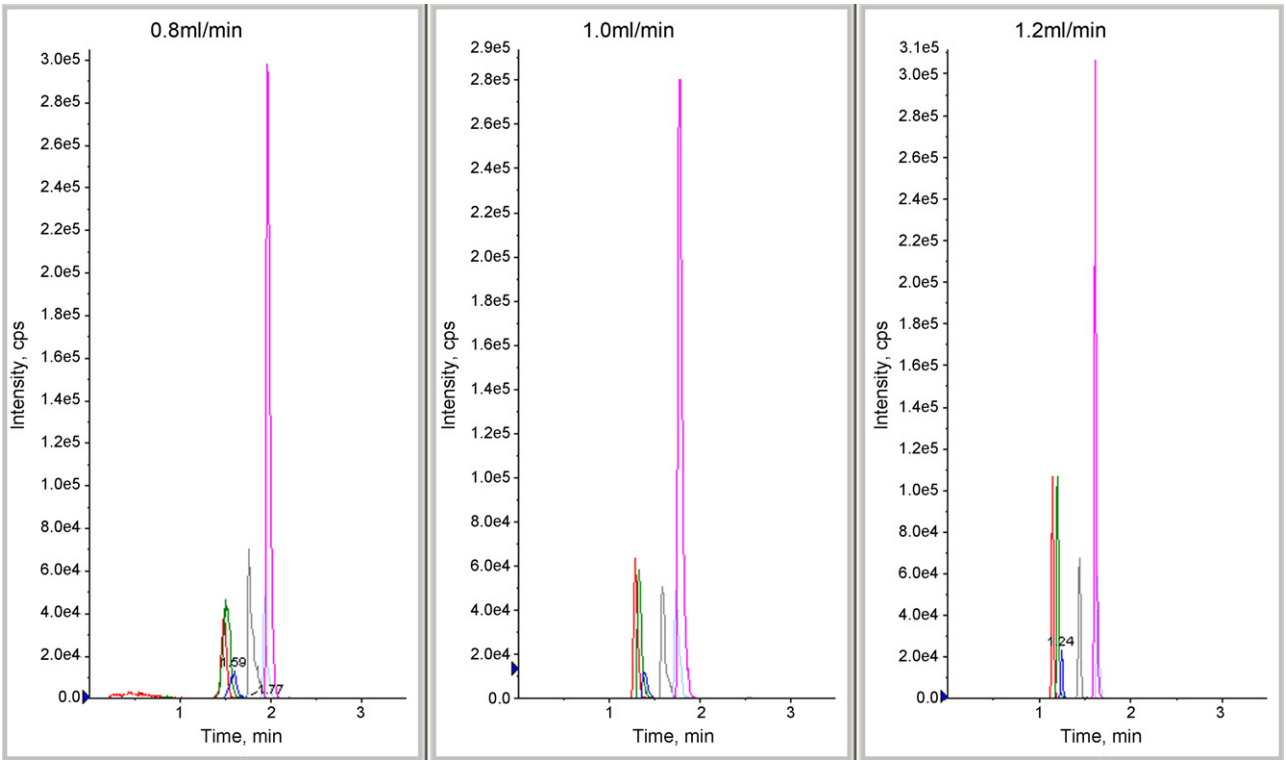

Fig. 4. Chromatograms captured during LC-method development to determine the optimal flow rate to the analytical column (50 ng/ml, rat plasma).

Table 3
Test mixture characterisation; imprecision and inaccuracy data

Compound	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Standard deviation	Imprecision (%RSD)	Inaccuracy (%error)	*n*
UK-112166	1	0.98	0.03	3.1	−2.0	3
	10	9.75	0.3	3.1	−2.6	3
	100	102	1	1.0	2.0	3
Fluconazole	1	1.09	0.1	9.2	8.3	3
	10	9.49	0.35	3.7	−5.4	3
	100	107	4.16	3.9	6.5	3
Candoxatril	1	0.89	0.14	15.7	−12.4	3
	10	9.13	0.54	5.9	−9.5	3
	100	116	6.6	5.7	13.8	3
UK-258300	1	1.07	0.03	2.8	6.5	3
	10	11.4	0.68	6.0	12.3	3
	100	113	2.52	2.2	11.5	3
UK-141495	1	1	0.03	3.0	0.0	3
	10	9.9	0.3	3.0	−1.0	3
	100	102	2.52	2.5	2.0	3
Dofetilide	0.1	0.102	0.02	19.6	2.0	6
	1	0.97	0.08	8.2	−3.1	6
	10	10.4	0.6	5.8	3.8	6
	100	96.7	3.19	3.3	−3.4	6
	200	162.2	3.34	2.1	−23.3	6
Overall	1	1.00	0.07	7.00	−0.45	18
	10	10.01	0.46	4.57	−0.39	18
	100	106	3.33	3.09	5.39	18

most sensitive of the compounds (C, Dofetilide) gave a large response at 0.1 ng/ml and therefore at 200 ng/ml there was a reduced response due to detector saturation, explaining why the inaccuracy reached −23% at this concentration. With the other compounds, all five had a lower limit of quantitation between 0.1 and 0.5 ng/ml (1.5–7.5 pg on column) and displayed a linear fitted calibration up to higher concentrations (mean inaccuracy 100 ng/ml [$n = 18$] = 5.4%).

Typically, the sensitivity range given by the developed method has proved to be sufficient for a large proportion of the in vivo studies conducted The significant variety of doses and dose administration routes, as well as sample collection times associated with biological efficacy investigations (where drug quantitation is required for PK/PD modelling) meant a wide dynamic range was useful when predicted concentrations were not certain.

Table 4
Dynamic ranges and %carryover immediately following the top calibration standard (measured peak heights)

Compound	LLOQ (ng/ml)	ULOQ (ng/ml)	Carryover (%)
UK-112116	0.1	200	0.015
Fluconazole	0.5	200	0.024
Dofetilide	0.1	100	0.045
Candoxatril	0.5	200	0.025
UK-258300	0.2	200	0.046
UK-141495	0.1	200	0.028
Mean	0.25[a]	183	0.031

[a] Equivalent to 3.75 pg on column.

3.3. Applicability as a routine method

The μ-turbulent flow focus mode method was adapted from the fast elution technique [1,21] we had available within our bioanalytical group where the turbulent flow and analytical columns were back-flushed with elution solvent and directed to the detector. The advantages gained using the isocratic focusing mode with respect to peak sensitivity, shape and reproducibility are significant [4] and this has been demonstrated by its successful use over the past 6 months in an LC–MS open access analytical environment. Of the studies performed using this method, a random selection are listed in Table 5 highlighting the wide diversity of compound physicochemical properties ($c \log P$ range: −1.7 to 5.7, variety of ionisation states) amenable to μ-TFC analysis. All studies adhered to the discovery analytical acceptance criteria defined in the methods. Furthermore, several of these analytes display a negative calculated CLogD (ACD Labs v8, Toronto, Canada) at the pH of the loading mobile phase; therefore, retention on the copolymer column chemistry (styrene–divinylbenzene) is perhaps not exclusively limited to hydrophobic interactions. This is also notable with the observation that two polar analytes with permanent quaternary ammonium charges (tiotropium and compound B) are also retained under these mobile phase conditions.

Different biological matrices that have been analysed are also contained within Table 5. Simple dilution and injection of all matrices except lung homogenate has been performed. However, with other homogenised tissues containing a large proportion of lipophilic material (brain homogenate) it has been preferable to

Table 5
Discovery bioanalytical data for recently run compounds, using the μ-TFC focus mode method

Compound	Molecular weight	*c* log *P*	Ionisable groups	LLOQ	Carryover (%)	Sample matrix
1	374	2.3	Neutral	1	0.1	Plasma/brain
2	399	3.71	Neutral	1	0.09	Plasma
3	422	3.42	Neutral	1	0.01	Plasma/urine
4	373	3.29	Neutral	0.5	0.06	Plasma
5	335	3.09	Neutral	0.5	0	Plasma
6	333	3.52	Neutral	0.5	0.04	Plasma
Morphine[a]	285	0.572	Neutral	1	0.3	Plasma
Oxycodone[a]	315	−0.039	Neutral	1	0.04	Plasma
Tiotropium	393	−1.7	Quaternary ammonium	0.5	0.19	Lung tissue
10	419	2.41	Strong acid	2	0	Plasma
11	404	5.34	Strong acid	2	0.05	Plasma
12	388	5.42	Strong acid	2	0.5	Plasma
13	346	1.83	Strong base	1	0	Plasma
14	410	2.54	Strong base	0.5	0.04	Plasma/brain/CSF
15	343	4.34	Strong base	0.5	0.2	Plasma
16	368	4.18	Strong base	0.5	0.07	Plasma
17	472	3.6	Weak acid	0.2	0.04	Plasma/urine/brain/CSF
18	386	5.72	Weak acid	2	0.07	Plasma
19	458	3.08	Weak acid	0.5	0.02	Plasma
20	326	2.98	Weak base	0.5	0.06	Plasma/urine
21	363	2.75	Weak base	0.5	0.06	Plasma/urine
22	450	3.97	Weak base	0.5	0	Plasma
23	378	3.82	Weak base	2	0.07	Plasma
24	322	3.86	Weak base	0.5	0.04	Plasma/CSF
Sibutramine	280	2.9	Weak base	0.5	0.08	Plasma/urine
26	330	2.8	Weak base	0.5	0.05	Plasma/urine
Propranolol	259	2.75	Weak base	0.2	0.19	Plasma
28	438	4.82	Weak base	0.5	0.09	Plasma
29	440	0.961	Weak base	0.2	0.09	Bile/urine/plasma/CSF
30	381	3.58	Weak base	1	0.12	Plasma
Rolipram	275	2.9	Weak base	0.5	0	Plasma/brain homogenate
32	338	3	Weak base	0.5	0.18	Plasma
33	386	3.37	Weak base	1	0.02	Plasma
34	468	1.57	Zwitterionic	0.2	0.05	Plasma/CSF
35	786	5.15	Zwitterionic	0.5	0.13	Plasma
36	703	5.43	Zwitterionic	0.5	0.07	Plasma
Mean				0.79	0.09	

[a] Utilised alternative columns—Cyclone-P (0.5 mm × 50 mm) and Curosil PFP (4.6 mm × 50 mm).

protein precipitate samples prior to injection to reduce system downtime. Many of the listed compounds have been analysed in multiple assay matrices and the analytical data generated have demonstrated excellent intra-assay variability.

In summary, the focus mode method described, works successfully for the large majority of analytes encountered in our laboratory. However, certain physicochemistries are not ideally suited to the column chemistry employed and will not be detectable on the system. In this situation, the first alteration we consider is the selection of an appropriate column set, rather than changing the chromatographic method. For example, morphine and oxycodone (both very hydrophilic analytes not initially detectable) were quantifiable down to 1 ng/ml by changing to the Cyclone-P turbulent flow column and Curosil-PFP analytical column (4.6 mm × 50 mm, 5 μM, Phenomenex, CA, USA), with no LC-method changes. Similarly, our experience with extremely hydrophobic analytes (*c* log *P* > 5) is that peaks become detectable or sometimes are enhanced when the generic Zorbax C18 extended column is changed to a less retentive C8 column (X-Bridge, 3 mm × 50 mm, 5 μM, Waters, Milford, USA; has been our preference). Due to the pH extremes of the mobile phases (see section below) it was necessary to use columns stable between pH 1 and 11; therefore, this precludes the use of the less retentive silica C2 turbulent flow column. However, a variety of high quality analytical columns are now available on the market, stable up to pH 10–11 suitable for μ-TFC methods. This includes both spherical particle silica based columns [3] and monolithic columns [11,13].

3.4. Carryover

A quaternary pump was utilised to enable, alongside the loading mobile phase—three short washing steps of both turbulent flow and analytical columns [22]. This included one organic basic wash (pH 10) and two acidic washes (pH 2.6 organic and pH 1.9 aqueous) to effectively remove drug and proteinacious material over two pH extremes and as discussed above still be compatible with HPLC columns on the market. The decision to

include mobile phase D (acetic acid in water, 15/85, w/v) follows the investigations of Zeng et al. [23] where they found this wash to significantly weaken protein interaction with the turbulent flow column stationary phase. All washes were kept short to minimise the cycle time of the method and directed between two valve switch positions to ensure the whole valve switching module was cleaned. An acidified aqueous autosampler wash was used first to remove residual matrix material followed by an organic wash to remove lipophilic material.

Overall, significant carryover has been eliminated for all compounds analysed using this chemically diverse range of washing solvents. The test set validation indicated carryover was in the range of 0.015–0.046% (Table 4) which was well within our target for analytical acceptance. With a larger range of compounds (Table 5, $n = 36$), results also indicate mean carryover remains low at approximately 0.09% (range, 0–0.2%), lower than what can be achieved with a binary pump setup with two weakly acidified mobile phases [4]. This has enabled smaller peaks to be accurately quantified and pushes the average system detection limits into the sub-nanogram range, even without spending extra time tuning more sensitive MS/MS parameters ([20], see Methods section). The low carryover is helped by the low sample injection volume (15 μl) which minimises the total mass of both drug and matrix material entering the system.

3.5. System robustness and solvent consumption

Other turbulent flow focus mode methods described, where neat or diluted sample is injected directly, have quoted injection numbers ranging from 200 to 2070 samples (approximate average ~850 injections), before observing a loss in chromatographic performance [4,14,23,24]. As an open access LC–MS system regularly used in our department with different animal matrices, no formal assessment of column robustness has yet been performed. However, changes of both column types, has consistently occurred at the top end of this range and no significant incidences of chromatographic failure have yet been found to be related to column performance. These observations corroborate data by Zeng et al. [23] which show column lifetimes are extended with strong acidic and organic washing. Further use of the biocompatible online frit discussed above, helps to maintain the system robustness, as well as direction of solvent flow away from the mass spectrometer for 3 min of the 5 min cycle time. Indications we have used in the decision to change both columns are the peaks beginning to split and some evidence of band broadening and peak tailing. To date, this has not occurred before 2000 injected samples.

The volumes of solvent used in sample analysis are kept low when compared to other offline chromatographic systems with faster flow rates but shorter cycle times. The total solvent consumption per 100 samples (Table 2) is 1.31 and 64% of this is acidified aqueous media. Following an assessment of different flow rates to the mass spectrometer, 1.2 ml/min (no split) was found to be the optimal rate for analytical performance using the test mixture (Fig. 4). The improvements in peak shape, reduced tailing and relative peak sensitivity can be seen particularly for the earlier eluting compounds as well as faster chromatography for all the analytes. Organic solvent consumption can also be reduced by utilising an analytical column with a narrower bore (2.1 mm cf. 4.6 mm) but we chose the wider dimension in preference, primarily to enhance the analyte focusing process and shorten the re-equilibration and elution times.

4. Conclusion

Turbulent flow chromatography is a well-established technology within the pharmaceutical and other industries that can dramatically reduce sample preparation times to a fraction of other available techniques. However, achieving best use of the system is highly dependant upon the requirements of analysis, by the associated user. At Pfizer, within the non-GLP regulated bioanalytical group it has been necessary to achieve a fast data turn around using a generic μ-TFC approach method whilst maintaining high standards of analytical integrity when working exclusively with in vivo samples.

The method described here, regularly quantifies a variety of analyte chemistries to sub-nanogram levels and has enabled both method development and sample analysis to be performed within 1 day. All the data generated have fallen well within the sensitivity requirements and acceptance criteria, with inaccuracy and imprecision values less than 15% for the majority of compounds. The focus mode setup using an optimised focusing flow rate and chromatographic gradient has enabled excellent peak shape to be achieved for multiple analytes and with a variety of biofluids over three orders of magnitude. Additionally, both system robustness and carryover issues were significantly improved for all analyte chemistries by combining quick washing at different pH extremes with a pH stable column chemistry and using a minimal sample injection volume. In particular, neutral and charged lipophilic analytes ($c \log P > 3$; Table 5, $n = 20$) susceptible to carryover all exhibit carryover values less than our target of 0.2% helping to ensure the LLOQ and dynamic range of each assay is not compromised.

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References

[1] T. Edge, Bioanalytical separations, in: I.G. Wilson (Ed.), Handbook of Analytical Separations, Elsevier Science, BV, 2003, p. 91 (Chapter 4).
[2] J.L. Herman, Rapid Commun. Mass Spectrom. 19 (2005) 696.
[3] V.S. Ong, K.L. Cook, C.M. Kosara, W.F. Brubaker, Int. J. Mass Spectrom. 238 (2004) 139.
[4] J.L. Herman, Int. J. Mass Spectrom. 238 (2004) 107.
[5] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 383.
[6] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallett, R.S. Plumb, Rapid Commun. Mass Spectrom. 11 (1997) 1953.
[7] M. Jemal, Y.Q. Daisy, B. Whigan, Rapid Commun. Mass Spectrom. 12 (1998) 1389.

[8] J. Smalley, P. Kadiyala, B. Xin, P. Balimane, T. Olah, J. Chromatogr. B 830 (2006) 270.
[9] H.K. Lim, K.W. Chan, S. Sisenwine, J.A. Scatina, Anal. Chem. 73 (2001) 2140.
[10] S. Calderoli, E. Frigerio, C.A. James, Chromatographia 59 (2004) 183.
[11] L. Ynddal, S.H. Hansen, J. Chromatogr. A 1020 (2003) 59.
[12] L. Ramos, N. Brignol, R. Bakhtiar, T. Ray, L.M. McMahon, F.L.S. Tse, Rapid Commun. Mass Spectrom. 14 (2000) 2282.
[13] S. Zhou, H. Zhou, M. Larson, D.L. Miller, D. Mao, X. Jiang, W. Naidong, Rapid Commun. Mass Spectrom. 19 (2005) 2144.
[14] C. Chassaing, H. Stafford, J. Luckwell, A. Wright, A. Edgington, Chromatographia 62 (2005) 17.
[15] S. Hsieh, T. Tobien, K. Koch, J. Dunn, Rapid Commun. Mass Spectrom. 18 (2004) 285.
[16] M. Takino, S. Daishima, K. Yamaguchi, T. Nakahara, Analyst 128 (2003) 46.
[17] F.L. Sauvage, J.M. Gaulier, G. Lachatre, P. Marquet, Ther. Drug Monit. 28 (2005) 123.
[18] U. Ceglarek, J. Lembcke, G.M. Fiedler, M. Werner, H. Witzigmann, J.P. Hauss, J. Thiery, Clin. Chim. Acta 346 (2004) 181.
[19] K.C. Saunders, Drug Discov. Today (Tech.) 1 (2004) 373.
[20] P. Wright, C. Chassaing, N. Cussans, D. Gibson, C. Green, M. Gleave, R. Jones, P. Macrae, K.C. Saunders, Biomed. Chromatogr. 20 (2006) 585.
[21] C. Chassaing, J. Luckwell, P. Macrae, K.C. Saunders, P. Wright, R. Venn, Chromatographia 53 (2001) 122.
[22] R.P. Grant, C. Cameron, S.M. McMurter, Rapid. Commun. Mass Spectrom. 16 (2002) 1785.
[23] W. Zeng, A.L. Fisher, D.G. Musson, A.Q. Wang, J. Chromatogr. B 806 (2004) 177.
[24] D. Zimmer, V. Pickard, W. Czembor, C. Muller, J. Chromatogr. A 854 (1999) 23.