Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 834–841

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Turbulent Flow Chromatography TFC-tandem mass spectrometry supporting *in vitro*/*vivo* studies of NCEs in high throughput fashion

Maria Verdirame[a], Maria Veneziano[a], Anna Alfieri[a], Annalise Di Marco[b], Edith Monteagudo[a], Fabio Bonelli[a,*]

[a] *Department of Drug Metabolism and Pharmacokinetics, Istituto di Ricerche di Biologia Molecolare P.Angeletti (IRBM), Merck Sharp and Dohme Research Laboratories, Via Pontina km 30.600, 00040 Pomezia, Roma, Italy*
[b] *Department of Pharmacology, Istituto di Ricerche di Biologia Molecolare P.Angeletti (IRBM), Merck Sharp and Dohme Research Laboratories, Via Pontina km 30.600, 00040 Pomezia, Roma, Italy*

ARTICLE INFO

Article history:
Received 23 July 2009
Received in revised form
25 September 2009
Accepted 2 October 2009
Available online 12 October 2009

Keywords:
TFC-LC–MS/MS
On-line extraction

ABSTRACT

Turbulent Flow Chromatography (TFC) is a powerful approach for on-line extraction in bioanalytical studies. It improves sensitivity and reduces sample preparation time, two factors that are of primary importance in drug discovery.

In this paper the application of the ARIA system to the analytical support of *in vivo* pharmacokinetics (PK) and *in vitro* drug metabolism studies is described, with an emphasis in high throughput optimization.

For PK studies, a comparison between acetonitrile plasma protein precipitation (APPP) and TFC was carried out. Our optimized TFC methodology gave better S/N ratios and lower limit of quantification (LOQ) than conventional procedures.

A robust and high throughput analytical method to support hepatocyte metabolic stability screening of new chemical entities was developed by hyphenation of TFC with mass spectrometry.

An in-loop dilution injection procedure was implemented to overcome one of the main issues when using TFC, that is the early elution of hydrophilic compounds that renders low recoveries. A comparison between off-line solid phase extraction (SPE) and TFC was also carried out, and recovery, sensitivity (LOQ), matrix effect and robustness were evaluated. The use of two parallel columns in the configuration of the system provided a further increase of the throughput.

1. Introduction

The marriage between high throughput and sensitivity represents one of the main goals of bioanalytical activities in support of drug discovery in the pharmaceutical industry.

In early drug discovery, the number of PK studies of different projects is high and a large number of samples to be analyzed are rapidly generated. Therefore it is very important to reduce the entire bioanalytical procedure in terms of sample preparation and sample analysis, TFC is without a doubt useful tool to achieve the aforementioned goal as described in the literature [1–9].

TFC was introduced in 1997 (Quinn and Takarewski [10]) as a technique for the direct injection of biological fluids onto a column packed with 50 μm spherical porous particles. The separation or extraction of analytes from biological matrices is performed in the turbulent flow regime where plasma proteins are eluted to waste while the drugs of interest are retained [11,12]. Turbulence is defined as an eddy-like state of fluid motion where the inertial vortex forces of the eddies are larger than the viscous forces, which tend to dampen the eddies out. Comparison between the two major forces, inertial and viscous, produces a dimensionless constant referred to as the Reynold's number (Re). The Reynold's number establishes the line between laminar and turbulent regimes. In a packed bed the Re number is well expressed by the following equation: $\mu dp/\eta$ = inertial forces/viscous forces, where μ is the linear velocity, dp (particle diameter) and η the viscosity. When the operative conditions give a Re <1 the regime is laminar, while with Re encompassed between 1 and 10, the regime is turbulent.

Turbulent flow conditions can be achieved in 0.5 mm or 1.0 mm internal diameter (ID) columns packed with particles greater than 50 μm at a flow rate of 1.25 mL/min or 4 mL/min, respectively. The use of larger diameter particles eliminates the problem of backpressure, so that high linear flow rates can be reached.

This paper describes the advantages of Turbulent Flow Chromatography in bioanalytical studies. In particular we will focus our attention on PK studies and hepatocyte stability assays. The elimination of sample preparation steps such as solid phase extraction (SPE) or acetonitrile precipitation (APPP) provides new levels of productivity [13–16].

* Corresponding author. Tel.: +39 6 91093606; fax: +39 6 91093225.
E-mail address: fabio_bonelli@alice.it (F. Bonelli).

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doi:10.1016/j.jpba.2009.10.005

Table 1
On-line HTLC method.

	Loading pump									Eluting pump		
	Time (s)	Flow (mL/min)	Grad	%A	%B	%C	%D	FD	Tee	Loop	Flow (mL/min)	Grad	%B
Loading	2	1.25	Step	100	0	0	0	→		Out	1.5	Step	0
Loading	15	1.25	Step	0	0	100	0	→		Out	1.5	Step	0
Loading	30	1.25	Step	100	0	0	0	→		Out	1.5	Step	0
Transfer	30	0.2	Step	100	0	0	0	←		In	1.3	Step	0
Eluting	30	1.25	Step	0	0	0	100	←	T	In	1.5	Ramp	25
Eluting	30	1.25	Step	0	0	0	100	→		Out	1.5	Ramp	50
Eluting	30	1.25	Step	0	100	0	0	←		In	1.5	Ramp	75
Eluting	30	1.25	Step	0	100	0	0	→		Out	1.5	Ramp	100
Eluting	60	1.25	Step	40	60	0	0	←		In	1.5	Step	100
Equilibrate	43	1.25	Step	100	0	0	0	→		Out	1.5	Step	0

Mobile phase A: 0.1% formic acid (FA) aqueous solution; mobile phase B: 0.1% (FA) acetonitrile (ACN) solution; mobile phase C: 15% acetic acid aqueous solution; mobile phase D: 90% IPA solution (IPA:ACN 90:10, v/v); TFC Column: cyclone 50 mm × 0.5 mm ID, 60 μm; analytical column: ACE C_{18} 50 mm × 4.6 mm ID, 5 μm; FD: flow direction.

2. Experimental

2.1. Materials and reagents

NCEs (Compounds A, B, C, D) were synthesized at IRBM P. Angeletti, Merck Research Laboratories (Pomezia, Rome, Italy). 4-Methylumbelliferone (4-MU), umbelliferone (U), 4-methylumbelliferone-β-D-glucuronide (Gluc-4MU) and 6-βOH-testosterone were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade water was purchased from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Acetonitrile (HPLC-grade), methanol (HPLC-grade), isopropanol (HPLC-grade), acetone (HPLC-grade) and acetic acid (glacial) 100% pro-analysis were obtained from Merck KGaA Darmstadt, Germany. Dimethyl sulfoxide (DMSO) was from Sigma Chemical Co. (St. Louis, MO, USA). Formic acid (98%) from Fluka Chemie GmbH (Buchs, Germany) was used for mobile phase preparation. Drug-free rat plasma with heparin lithium was obtained from Charles River Laboratories, Inc. (Les Oncins, France). 96-Well extraction plate packed with Oasis HLB sorbent was obtained from Waters (Milford, MA, USA).

2.2. Instrumentation

A Cohesive 2300 HTLC turboflow system, which included a quaternary pump (used as a loading pump) and a binary pump (used as a eluting pump) (Cohesive Technologies, Inc., Franklin, MA; USA), was used for on-line extraction. A CTC HTS PAL autosampler from LEAP Technologies (Carrboro, NC, USA) was used for sample injection refrigerated at 7 °C during analysis. An API 3000 triple quadrupole from AB/MSD Sciex (Toronto, Canada) equipped with a TurboIonSpray interface was used as the detector. A Packard (Meriden, CT, USA) MultiPROBE1 II Plus HT liquid-handling system was used for all pipetting steps.

A 96-needle Apricot Personal Pipettor (Model PP-550MS-XH Apricot Designs, Inc., Monrovia, CA) was used for SPE.

A Turboflow Cyclone (50 mm × 0.5 mm, 60 μm) column (Cohesive Technologies, Inc., Franklin, MA, USA) was used as extraction column and an ACE C_{18} (50 mm × 4.6 mm, 5 μm) column (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) was used as analytical column.

2.2.1. LC–MS/MS conditions

Chromatography was performed on an ACE C_{18} (50 mm × 4.6 mm, 5 μm) column (Advanced Chromatography Technologies, Aberdeen, Scotland, UK) at room temperature with an injection volume of 10 μL. The mobile phase, consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile), was delivered at a flow rate of 1500 μL/min. The LC gradient started from 100/0% (A/B) and changed to 0/100% (A/B) in 2.0 min remaining constant to this ratio for 1 min. Then B% decreased to 100/0% (A/B), remaining constant to this ratio until 2.0 min that was the re-equilibration time.

2.2.2. On-line HTLC operative experimental conditions

The procedure we have applied to the direct injection analysis of crude plasma and/or hepatocytes samples basically can be described as four general steps: (1) loading, (2) transfer, (3) elution and (4) equilibration [3,13].

The high throughput liquid chromatography (HTLC) parameters are listed in the Table 1.

In the loading step (Fig. 1a) the plasma or hepatocyte samples are directly injected onto a narrow bore large particle size extraction column (50 mm × 0.5 mm, 60 μm) under turbulent flow conditions. During this step, the matrix components are quickly washed out and the analytes retained using an aqueous mobile phase at 1.25 mL/min flow rate; after sample loading 15% acetic acid mobile phase was introduced to remove proteins from the extraction column [3] and finally 0.1% FA aqueous mobile phase to eliminate the acetic acid and recreate the initial conditions.

The second step is to transfer the analytes onto a second column. In the transfer step (Fig. 1b), the analytes are eluted from the extrac-

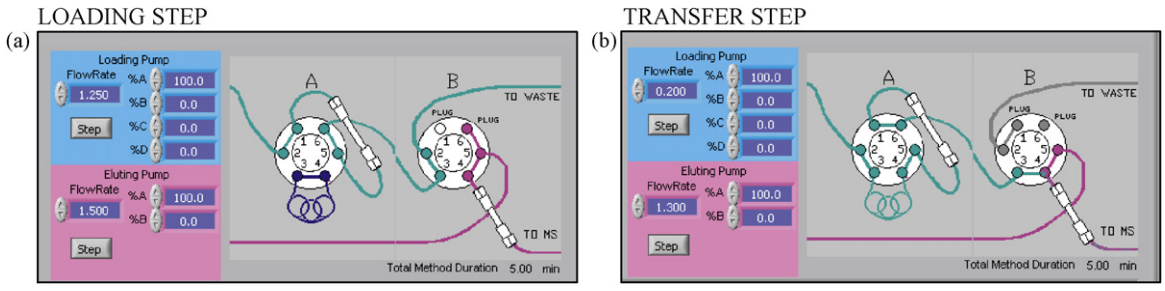

Fig. 1. Switching valves positions during loading and transfer steps.

tion column and transferred onto the reversed-phase analytical column using a 60% organic mobile phase contained in the elution loop (Fig. 1b). The flow rate for the loading pump was 0.2 mL/min and lasted for 30 s. An aqueous mobile phase is teed into the high organic mobile phase just before the analytical column, allowing the analyte to focus on the head of the analytical column. In the elution step, the analytes are separated on the analytical column and eluted to the mass spectrometer for determination [3]; during the analytical column elution step, the TFC column is washed and the elution loop is filled with the 60% of organic phase for the next injection. In order to efficiently wash the extraction column, we have divided the analytical gradient time in four time segments allowing in this way to clean the extraction column with two different mobile phases, B and D, in both flow and counter flow direction without increasing the total run time.

Finally in the equilibration step, both TFC and analytical systems are equilibrated for the next run.

The isopropanol and the acetic acid were added to extend the TFC column lifetime [3]. A 15% acetic acid and 90% IPA were respectively used as mobile phases C and D to increase the protein solubility and to prevent the accumulation of lipids. These eluents along with the autosampler washing solvent composition (Wash A: acetonitrile/isopropanol/acetone, 40/30/30 (v/v/v); Wash B: 1% acetic acid) were sufficient to obtain a carryover <0.1%. This was determined by comparing the peak area of the analyte in the blank after the analysis of the highest standard in plasma (5000 ng/mL).

2.2.3. Mass spectrometry conditions

The mass spectrometer was operated in electrospray positive or electrospray negative ion modes. Nitrogen delivered from a high-pressure dewar served as the nebulizer, auxiliary, source exhaust, curtain and collision gas. The capillary voltage was set at 5500 V in positive ion mode or −4500 V in negative ion mode, while cone voltage and collision settings were compound-dependent. The instrument was operated under unit resolution. Peak detection mode was selected reaction monitoring (SRM) with dwell time of 50 ms.

Analyte concentrations were determined by weighted linear least-squares regression analysis, using Analyst Quantitation Wizard software version 1.4.1.

2.3. Plasma sample preparation

2.3.1. APPP procedure

The standard solutions and quality control samples were prepared by serial dilution in plasma.

A stock solution of analyte was prepared in DMSO at a concentration of 1.0 mg mL^{-1}.

The stock solution was further spiked into rat plasma to make CC and QC working solutions at 50 μg/mL in plasma. A Packard Liquid-handling system was programmed for the preparation (with necessary dilutions) of calibration standards in control plasma, QC samples, and clinical samples, in a 96-well extraction plate. Using a liquid-transfer performance file and procedure for each step, 50 μL aliquots of the working solutions, standard and QC, respectively, were transferred into the assigned wells and diluted with blank plasma to obtain calibration standards and QC solutions over the 0.305–5000 and 2–5000 ng/mL concentration ranges, respectively. An aliquot of 50 μL of each calibration standard and QC solution as well as clinical samples, was transferred into a new 96-well plate and added of 20 μL working internal standard and of 200 μL acetonitrile. The samples were then centrifuged for 20 min at 3000 rpm and the supernatant was dried under N_2 current. The samples were reconstituted in 150 μL H_2O/ACN 10/90 and finally injected onto the LC–MS/MS system.

Table 2
TFC eliminates plasma sample preparation.

Protein precipitation	TURBOFLOW
Add internal standard to plasma samples	Add internal standard to plasma samples
Add acetonitrile	Centrifuge
Centrifuge	Inject to column
Evaporate to dryness	
Reconstitute	
Injection to column	

2.3.2. TFC plasma sample preparation

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A comparison between two different procedures is summarized in the Table 2.

2.4. Hepatocytes samples preparation

Hepatocytes (rat or human) were incubated in Hepatocyte Basal medium (Clonetics, CC3199) with the test compound for an appropriate time period. At different time points, aliquots of the cell suspension were removed, transferred in 96 deep well plates, deproteinized by the addition of a equal volume of acetonitrile and centrifuged at 1450 × *g* for 10 min. The supernatant was subsequently dried, reconstituted in H_2O 0.1% F.a. and cleaned-up by SPE before LC–MS/MS analysis or injected directly onto TFC-LC–MS/MS system.

2.4.1. SPE

SPE was performed using Apricot Personal Pipettor PP-550MS-XH.

A 96-well extraction plate packed with 30 mg of Oasis HLB resin was used for SPE.

SPE procedure for hepatocyte study sample clean-up prior to MS analysis is specified in the following scheme:

SPE procedure for sample clean-up prior to MS analysis	
Equilibration	1 mL MeOH 1 mL H_2O
Sample load	200 μL in H_2O 0.1% F.a.
Wash	5 mL H_2O 0.1% F.a. (1 mL for 5 times)
Elution	1 mL ACN 0.1% F.a.

The eluate was dried and reconstituted in 150 μL H_2O/ACN 90/10 (v/v) for LC–MS/MS analysis.

2.4.2. TFC

The sample preparation scheme has been highly simplified using the novel developed TFC method, in fact, after quenching

Table 3
Intra-day and inter-day precision and accuracy of compound B in rat plasma in the TFC-LC–MS/MS technique ($n = 6$ per day, 3 days).

Standard concentration (ng/mL)	Mean		SD		Precision (%RSD)		Accuracy (%RE)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
2	2.052	2.274	0.318	0.185	15.50	8.14	2.6	13.7
5	5.052	5.232	0.280	0.627	5.54	11.98	1.0	4.6
10	9.026	8.936	0.846	1.348	9.37	15.09	−9.7	−10.6
100	88.343	92.478	3.615	11.542	4.09	12.48	−11.7	−7.5
1000	927.981	934.734	35.871	97.216	3.87	10.40	−7.2	−6.5
5000	4878.228	4789.977	209.538	476.262	4.30	9.94	−2.4	−4.2

The intra-day precision and accuracy were between 3.87–15.5% and −11.7–2.6% (RE), respectively. The inter-day precision and accuracy were between 8.14–15.09% and −10.6–13.7% (RE), respectively. %RSD = (SD/mean) × 100. %RE = [(mean-nominal)/nominal] × 100.

the samples with acetonitrile we can inject them directly onto TFC-LC–MS/MS system following the disappearance of the parent compound. With this methodology the complex SPE multistep procedure can be bypassed.

4-MU was used as a positive control to test the hepatocyte functionality.

3. Results and discussion

3.1. PK studies

A direct plasma injection method using TFC on-line with mass spectrometry was developed in alternative to the protein precipitation routinely used in our laboratory to support quantitative bioanalysis, with the intent of reducing sample handling and increasing the throughput [15]. The on-line TFC-LC–MS/MS assay for the determination of several compounds in rat plasma was validated, and linearity, accuracy, precision, recovery and matrix effect were evaluated.

Precursor ions for each compound were determined from Q1 spectra obtained during infusion of a neat solution at 1 μg/mL in ACN/H_2O 1/1 (v/v) 0.1% formic acid via the TurboIonSpray source (TIS) into the mass spectrometer operated in positive or negative ionization mode with the collision gas off. The ionspray voltage was 5500 V in positive ion mode and −4500 V in negative ion mode. The TIS interface temperature was maintained at 450 °C. Nitrogen was used as nebulizer, curtain and collision gas.

Instrument settings were optimized for each analyte to maximize the response for precursor/product ion transitions for the multiple reaction monitoring (MRM).

3.1.1. Linearity

The assay showed an excellent linearity over a 0.305–5000 ng/mL range. The limit of quantification for the various compounds was 1.22 ng/mL with the correlation coefficient of 0.99 for the calibration curves in rat plasma. The curves were fit by the linear regression method using a $1/x^2$ weighting.

3.1.2. Precision and accuracy

The precision of the method was defined as the percentage relative standard deviation (%RSD) calculated from replicate measurements. The accuracy of the assay was defined as the percent relative error (%RE) of the mean of the replicate measurements from theoretical values. Precision and accuracy studies using 10 representative compounds were conducted by analyzing quality controls (QCs) prepared in biological matrix at six levels (2, 5, 10, 100, 1000, 5000 ng/mL) with six replicates on 3 separate days. Our target was to have at least 67% (4 out of 6) of QC samples within 20% of their respective nominal value.

To ensure that no error occurred in the preparation of stock solutions, quality control samples were made from a stock solution separate from that used to prepare standards.

Table 3 summarizes the intra-day and inter-day precision and accuracy for one representative compound for the TFC-LC–MS/MS procedure. All intra- and inter-day precision and accuracy values were acceptable and spanned the entire concentration range, which indicated the method was very reproducible and robust to support the quantitative analysis of diluted plasma samples. These results were representative of the other compounds (%RSD and %RE were ≤20%) [17].

3.1.3. Recovery % and matrix interference

The specificity of this TFC-LC–MS/MS assay was evaluated by testing six batches, each of them corresponding to a pool of 10 blank rat control plasma. No endogenous matrix interferences were found at the retention times for the selected compounds.

To determine the extraction efficiencies or recoveries for the analytes, the peak area ratios measured for spiked plasma samples using TFC extraction were compared to the peak area measured by direct injection (bypassing TFC extraction) of aqueous samples. Total recovery of this method is a cumulative evaluation of both the extraction efficiency and matrix effect [18]. Usually between 85 and 95% of recovery was obtained.

3.1.4. PK profiles by TFC-LC–MS/MS: comparison with the conventional ACN PPT procedure

The new HTLC/MS/MS method previously validated (Sections 2.2.2 and 3.1) was compared to the protein precipitation (sample-to-solvent ratio of 1:4) sample preparation method routinely used in our laboratory [15]. This comparison was carried out for several compounds that were dosed at 3 mpk both intravenously (IV) and orally (PO). The plasma samples were collected at 0.0333, 0.0833, 0.25, 0.5, 1, 2, 3, 4, 5, 6, and 24 h post-IV dose and at 0.0833, 0.25, 0.5, 1, 2, 3, 4, 5, 6, and 24 h post-PO dose. There were three animals in the IV and PO groups, respectively. A total of 69 plasma samples (33 IV and 30 PO plus 6 pre-doses) were analyzed under both procedures.

In this paper we report two representative compounds: A and B, analyzed after these two different procedures. Fig. 2a and b shows representative plasma concentration–time profiles after IV and PO dosing in animals using the APPP and the TFC methods. In both IV and PO dosages, the same PK profiles were observed, regardless of the sample preparation procedure. PK parameters derived from the plasma concentrations for these two different methodologies were found to be comparable for each compound, while greater sensitivity was obtained by the on-line extraction approach.

It is important to note that the capacity of TFC in focusing the analyte on the column head allowed us to increment sensitivity.

As shown in Fig. 3, at 4.88 ng/mL (LOQ) in rat plasma, the signal-to-noise (S/N) ratio achieved for compound B was 6, while it became 23.5 by using TFC methodology, with a fourfold improvement of sensitivity (1.22 vs. 4.88 ng/mL). This allowed us to profile candidate B after PO dosing up until 6 h, conversely it was not possible through APPP because all time points were BLQ (Fig. 2b and Table 4).

Table 4
Comparison of pharmacokinetic parameters of compound A and compound B after IV and PO dosing.

	Dose 3/mg/kg	Compound A		Compound B	
	PK Parameter	Protein Precipitation	TFC	Protein precipitation	TFC
IV 3 mg/kg	AUC μM × h	10.0	10.0	1.8	1.7
	Clearance (mL/min/kg)	13.0	12.5	58	55.7
	Volume of distribution (L/kg)	4.0	3.5	18.0	18.3
	T1/2 (h)	5.3	5.1	4.1	7.1
PO 3 mg/kg	AUC μM × h	2.9	4.0	PK parameters not calculated because all concentrations were BLQ	0.1
	C_{max} (μM)	0.4	0.4		0.01
	T_{max} (h)	3.0	3.2		2.3
	F%	29	40.3		7.8

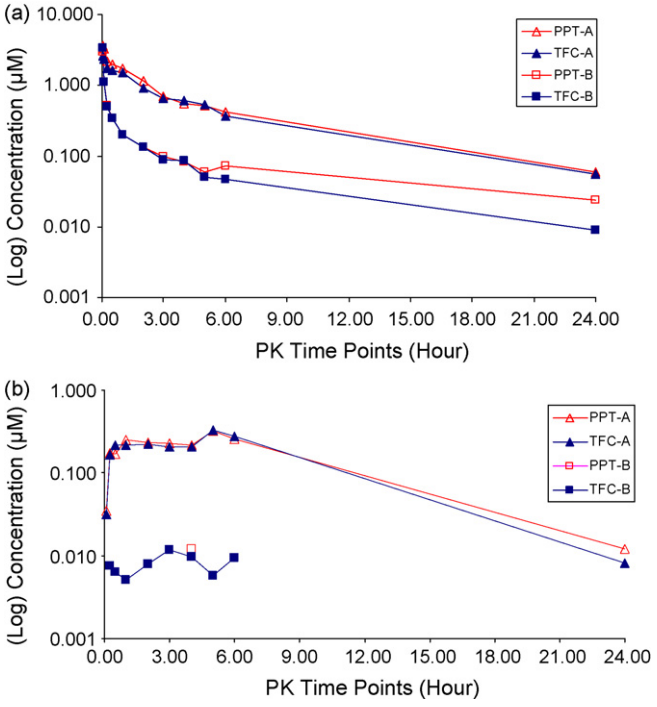


Fig. 2. (a) Direct comparison of mean concentration–time profiles of two compounds (A and B) corresponding to intravenous dosing. Concentrations ($n = 3$ per time point) were determined by protein precipitation LC–MS/MS approach and TFC-LC–MS/MS procedure. (b) Direct comparison of mean concentration–time profiles of two compounds (A and B) corresponding to an oral dosing. Concentrations ($n = 3$ per time point) were determined by protein precipitation LC–MS/MS approach and TFC-LC–MS/MS procedure.

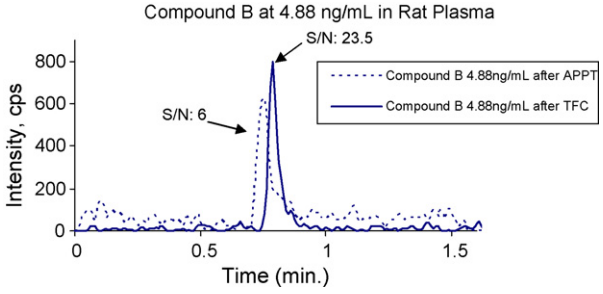


Fig. 3. Compound B: S/N ratios obtained by protein precipitation LC–MS/MS technique and TFC-LC–MS/MS technique.

For compound A better sensitivity was also achieved after TFC procedure. An LOQ of 0.305 ng/mL was obtained after TFC procedure and a LOQ of 2.44 ng/mL after ACN ppt.

3.1.5. Throughput

In order to satisfy demand in terms of throughput, the TFC Aria TX2 System was set up using two parallel columns (two TFC columns for on-line extraction at turbulent flow and two analytical columns). In this configuration each system operates independently, permitting multiple methods to run simultaneously and hence halving the analysis time. This is accomplished through the combination of the software controlling the Cohesive TX2 system and the associated valve-switching module.

The pool after dosing, coupled to this parallel configuration, allowed us to increase the throughput fourfold that of the single analysis procedure providing an efficient use of the mass spectrometer.

Four selected compounds were sorted for multiple-component LC–MS/MS analysis according to the molecular weight of the parent compound and to the molecular weight of some potential metabolites in order to avoid interferences or cross-talk between the analytes. Two compounds were assigned to two different pools based on the criterion that the mass difference between any parent compounds and potential metabolites should be 3 Da or larger (for compounds containing chlorine or bromine, it should be 5 Da or larger).

After having developed a specific and accurate LC–MS/MS method for analysis in pool, the four selected compounds were initially analyzed singly and the pharmacokinetic parameters obtained were then compared to those obtained in the pool after dosing analysis. After sample preparation according to Table 2, the compounds were run onto the ARIA System: one batch of samples on system 1 and another on system 2, each of which equipped with a TFC column Cyclone 0.5 mm × 50 mm, 60 μm and an analytical column ACE C_{18} 50 mm × 4.6 mm ID, 5 μm.

Fig. 4 shows representative plasma concentration–time profiles of compounds C and D after IV dosing in animals determined by TFC-LC–MS/MS technique in single and in pool after dosing. Table 5 shows that the PK parameters of compounds C and D after both IV and PO dosing are comparable by single and pool procedures.

This approach increased fourfold the throughput compared to single analysis procedure (14 h vs.56 h).

Another important aspect is that the TFC allows to focus the analyte on the column head, leading to an increment of sensitivity and improving the peak shape, which is particularly useful when plasma samples are analyzed in pool after dosing. In fact a disadvantage of this procedure is that the plasma concentration of each compound is diluted when the samples are combined before simultaneous compound analysis by LC–MS/MS.

In this case the LOQ obtained (1.22 ng/mL in single and 2.44 ng/mL in pool for compound C and 2.44 ng/mL in single and

Table 5
Comparison of pharmacokinetic parameters of compound C and compound D after IV and PO dosing.

	Dose 3 mg/kg	Compound C		Compound D	
	PK parameter	TFC single	TFC pool	TFC single	TFC pool
IV 3 mg/kg	AUC μM × h	5.7	8.3	1.8	1.9
	Clearance (mL/min/kg)	19	13.0	59	56
	Volume of Distribution (L/kg)	7.9	4.6	7.1	7.3
	T1/2 (hr)	6.9	6.7	2.1	2.0
PO 3 mg/kg	AUC μM × h	0.8	0.5	0.5	0.4
	C_{max} (μM)	0.1	0.1	0.1	0.1
	T_{max} (h)	1.6	1.0	2.3	1.2
	F%	14	6	28	24

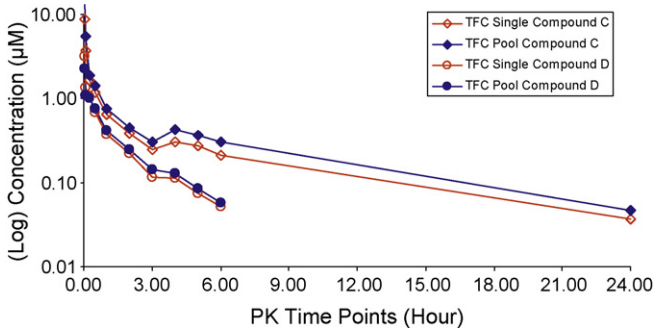


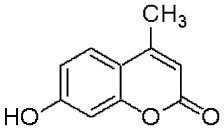
Fig. 4. Direct comparison of mean concentration–time profiles of two compounds (C and D) from an intravenous dosing. Concentrations ($n = 3$ per time point) were determined by TFC-LC–MS/MS technique in single and in pool after dosing.

4.88 ng/mL in pool for compound D) allowed the analytes to be observed until 24 and 6 h.

3.2. Hepatocyte stability studies by TFC-LC–MS/MS

3.2.1. Development and evaluation of the analytical procedure

Metabolic stability studies were conducted incubating freshly isolated or cryopreserved hepatocytes with test compound(s) and monitoring the disappearance of the parent compound and the formation of major metabolites over time using fast, specific and sensitive LC–MS/MS analysis. To reduce sample preparation time [14,16], we have developed an analytical method that permits the direct injection of supernatant onto the TFC-LC–MS/MS [19,20] system, adopting the in-loop dilution injection avoiding the SPE procedure that so far has been always performed in our laboratory to obtain appropriate samples to be analyzed by LC–MS/MS.



4-MU

Hepatocyte samples typically contain 50% acetonitrile after quenching. Injecting sample rich of acetonitrile onto a 20 μL loop, the hydrophilic compounds will be lost due to a very early elution. We covered this issue injecting the sample onto a 500 μL loop filled with polar solvent where the acetonitrile percent results dramatically reduced.

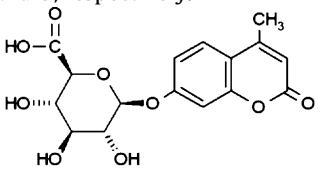
3.2.2. On-line loop dilution

A dilute mixture of 4-methylumbelliferone (4-MU) and the internal standard umbelliferone (U), used to probe the glucuronidation phase II hepatocytes activity, was prepared in acetonitrile at a concentration of 5 μg/mL. Using an automated TX2 System, 50 injections of sample (10 μL) were subjected to turbulent flow extraction at 1.25 mL/min by a 0.5 mm × 50 mm Cyclone column (Cohesive Technologies) followed by focusing into a 4.6 mm × 50 mm ACE C_{18} 5 μm column (Table 1).

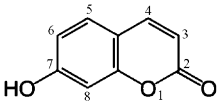
An acetonitrile gradient separated and eluted the analytes to a TurboIonSpray-API3000 system (AB/SCIEX), which permitted multiple reaction monitoring (MRM) of each analyte: 4-MU (174.8/119.1) and U (160.7/132.8) in negative ion mode.

We repeated the experiments after having modified the system to induce on-line dilution by forcing the injected samples (10 μL) through a 500 μL loop (inverting the plumbing configuration column-carrier/mobile phase). In this way the sample solution results sufficiently diluted within the loop.

Without on-line dilution, 50 injections of mix prepared with acetonitrile produced analyte peak areas that were less than half of those from the mix injected with turbulent flow dilution. This result confirms that if the supernatant is directly injected onto the TFC column, the amount of ACN causes a low recovery of the hydrophilic compounds (4-MU and U), while the injection of the supernatant by the on-line dilution loop shows that the diluted amount of ACN avoids an early elution from the TFC column. Moreover the robustness, measured as relative standard deviation (%RSD) on 50 injections with dilution loop procedure, was 5.28 and 4.69 for 4-MU and U, respectively.



Gluc-4MU



U

3.2.3. Linearity and LLOQ determination

The recovery, sensitivity (LLOQ), matrix effect and robustness of this approach were evaluated.

Testosterone and 4-methylumbelliferone (4-MU) were used as probes for cytochrome P450 3A-mediated metabolism and glucuronidation activity of hepatocyte suspension.

To assess the precision and accuracy of the TFC–MS/MS method, the corresponding 6β-hydroxytestosterone and the glucuronide of 4-MU were quantified. Eleven points standard curves were prepared in the medium containing hepatocyte suspension and 50% of acetonitrile for each analyte to verify the linearity and the sensitivity. Standard curves were processed identically to samples.

4-MU, Gluc-4MU, 6βOH-testosterone were chosen as representative compounds to assess the precision and accuracy of the TFC–MS/MS method because they are used as probes to test hepatocyte functionality. Eleven points standard curves were prepared

Table 6
4-MU, 6 βOH-test and Gluc-4MU calibration curves in the medium containing hepatocyte. Acceptance criteria and evaluation parameters.

	4-MU	6 βOH-test	Gluc-4MU
CC ng/mL	Accuracy	Accuracy	Accuracy
0.97	N/A	N/A	N/A
1.95	N/A	N/A	99.4
3.9	91.5	104	97.7
7.8	106	98.9	110
15.6	114	86.6	98.5
31.2	114	101	91.8
62.5	99	101	96.2
125	112	104	106
250	90.8	104	106
500	87.4	99.8	104
1000	84.5	101	91.7
Corr. Coeff	0.9906	0.9981	0.9977
LLOQ (ng/mL)	3.9	3.9	1.95
Recovery%	102	105	94.4
Matrix effect (dev. from 100%)	110	97	102

in the medium containing hepatocyte for each analyte to verify the linearity and the sensitivity.

A dynamic range of 0.9–1000 ng/mL was chosen. Calibration curves were calculated from the peak area ratio of analyte/internal standard using least square linear regression of the area ratio vs. the theoretical concentration of the standards.

Acceptance criteria included a correlation coefficient ≥0.99 for the CC with the accuracy value within 100 ± 20% (Table 6).

3.2.4. Recovery

For each analyte a spiked solution (500 ng/mL) in buffer containing hepatocyte was extracted adding acetonitrile 0.1% and formic acid (1:1, v/v) (Sol. A). A second solution of analyte at the same concentration was prepared in the supernatant coming from the extraction of buffer containing hepatocyte (Sol. B). The peak area ratio of analyte between Sol. A/Sol. B × 100 was used in the recovery calculation (Table 6) [18].

3.2.5. Matrix effect

For each analyte a spiked solution (500 ng/mL) was prepared both in the supernatant coming from the extraction of buffer containing hepatocyte (Sol. B) and in the aqueous solution (Sol. C) [18].

The effect of the hepatocyte matrix on the response for each analyte was calculated by applying the formula shown below. A deviation from 100% suggests that the hepatocyte matrix has contributed to the suppression (<100%) or enhancement (>100%) of analyte response (Table 6):

$$\frac{\text{Peak area analyte hepat. buffer}}{\text{Peak area analyte aqueous solution}} \times 100$$

3.2.6. Robustness

The assay robustness was evaluated with a continuous series of 150 injections of 4-MU (250 ng/mL) spiked in the supernatant coming from the extraction of buffer containing hepatocyte (Sol. B). The same method was followed for Gluc-4MU.

The relative standard deviation %RSD was 4.62% for 4-MU and 5.15% relative to Gluc-4MU.

3.2.7. Application: human and rat hepatocytes stability → TFC vs. SPE

Rat and human hepatocytes stability studies for many structurally diverse NCEs from different Merck programs were determined by LC–MS/MS assay using the TFC procedure and compared to data that had been previously generated using the conventional Solid Phase Extraction procedure.

As part of the validation of the overall metabolic stability protocol, degradation of 4-methylumbelliferone and formation of its corresponding glucuronide were followed.

4-MU was used as a positive reference control at a starting incubation concentration of 5 μM. The procedure involved duplicate sampling at 0, 30, 60, 120, 180 and 240 min post-incubation. The resulting acetonitrile-quenched samples were analyzed by two methodologies (SPE extraction and TFC), and the percentage of 4-MU remaining (normalized against the 0 min concentration) as well

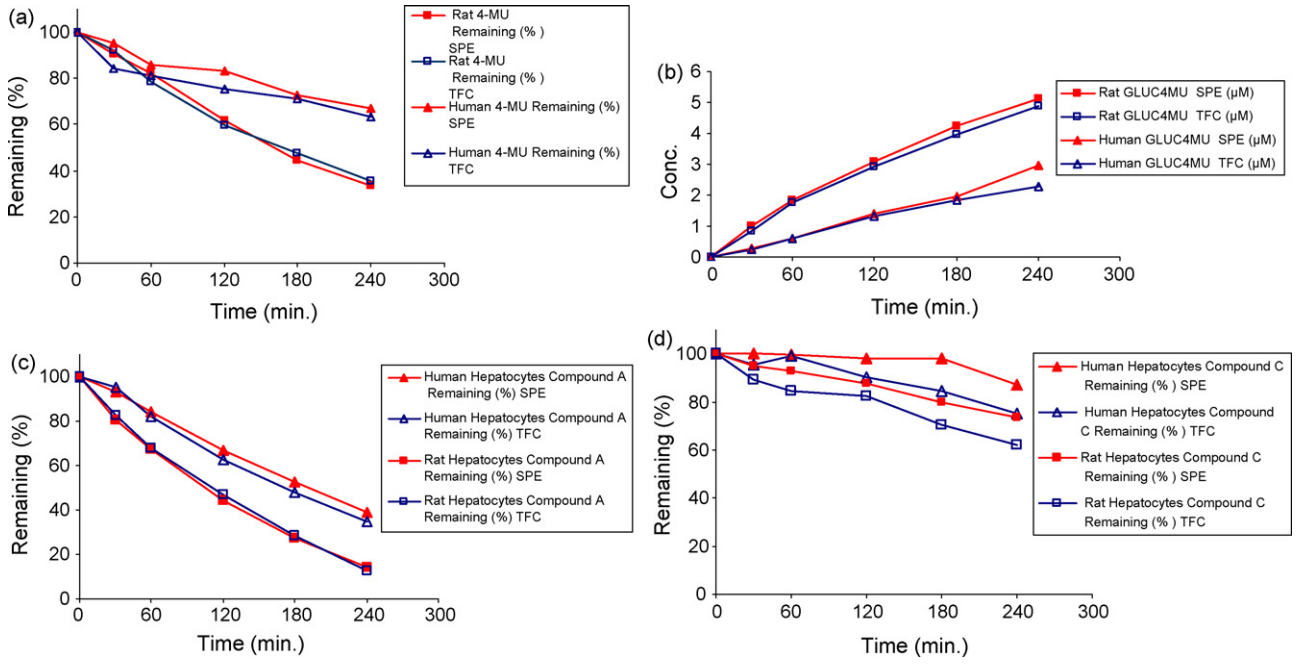


Fig. 5. (a) 4-Methylumbelliferone rat and human hepatocyte stability SPE vs. FC. (b) 4-Methylumbelliferone glucuronide formation in rat and human hepatocyte SPE vs. FC. (c) Compound A rat and human hepatocyte stability SPE vs. TFC. (d) Compound C rat and human hepatocyte stability SPE vs. TFC.

as the formation of its glucuronide was monitored as a function of time.

The results obtained after the two different procedures were found comparable both in human and rat hepatocytes (Fig. 5a–d).

Regarding the NCEs, in this paper we report the human and rat hepatocyte stabilities of two representative compounds A and C. The data emerging from the comparison between two different methodologies, TFC vs. SPE, are outlined in Fig. 5c and d. The results obtained with the new TFC assay were very similar to those of the conventional SPE assay. For compound A, the incubation data showed that the percentage remaining at 4 h in human hepatocytes was 38.97% by SPE and 34.86% by TFC, while in rat hepatocytes it was 14.03% and 12.86% by SPE and TFC respectively.

For compound C, the incubation data showed that the percentage remaining at 4 h in human hepatocytes was 87.18% by SPE and 74.85% by TFC, while in rat hepatocytes it was 73.37% and 62.09% by SPE and TFC, respectively.

4. Conclusion

The use of Turbulent Flow Chromatography in conjunction with LC–MS/MS techniques has proved to be an excellent tool for high throughput analysis of drug candidates in biological matrices, in both *in vivo* and *in vitro* studies.

A simple on-line extraction system has been described to analyze compounds in plasma that minimizes sample preparation, and increases sensitivity and productivity.

Further improvement in throughput was achieved by introducing two separate LC-systems. Each system operates independently, permitting multiple methods to run simultaneously and hence halving the analysis time.

In addition to PK measurements, the TFC-LC–MS/MS system has also been used to support analysis of samples generated from hepatocyte stability studies and an analytical method has been developed for this purpose. With the introduction of the on-line in-loop dilution technique, the sample preparation procedure was dramatically simplified.

This methodology could be more broadly applied for the analysis of a wide variety of cellular systems and biofluids.

References

[1] J.L. Herman, Rapid Commun. Mass Spectrom. 16 (2002) 421–426.
[2] W. Zeng, A.Q. Wang, A.L. Fisher, D.G. Musson, Rapid Commun. Mass Spectrom. 17 (2003) 2475–2482.
[3] W. Zeng, A.L. Fisher, D.G. Musson, A.Q. Wang, J. Chromatogr. B 806 (2004) 177–183.
[4] M. Takino, S. Daishima, T. Nakahara, Rapid Commun. Mass Spectrom. 17 (2003) 383–390.
[5] C. Chassaing, H. Stafford, J. Luckwell, A. Wright, A. Edington, Chromatographia 62 (2005) 17–24.
[6] R.P. Grant, C. Cameron, S. Mackenzie-McMurter, Rapid Commun. Mass Spectrom. 16 (2002) 1785–1792.
[7] J.L. Herman, Int. J. Mass Spectrom. 238 (2004) 107–117.
[8] J.L. Herman, Rapid Commun. Mass Spectrom. 19 (2005) 696–700.
[9] R. Krebber, F.J. Hoffend, F. Ruttmann, Anal. Chim. Acta 637 (2009) 208–213.
[10] H.M. Quinn, J.J. Takarewski, Int. Pat., WO 97/16724, 1997, p. 91, chapter 4.
[11] T. Edge, Bioanalytical separations, in: I.G. Wilson (Ed.), Handbook of Analytical separations, Elsevier Science, B.V., 2003, p. 91, chapter 4.
[12] T. Edge, Chromatogr. Sep. Technol. (CAST) 23 (2002) 10–16.
[13] W. Zeng, D.G. Musson, A.L. Fisher, A.Q. Wang, Rapid Commun. Mass Spectrom. 20 (2006) 1169–1175.
[14] V.S. Ong, K.L. Cook, C.M. Kosara, W.F. Brubaker, Int. J. Mass Spectrom. 238 (2004) 139–152.
[15] N. Sadagopan, B. Pabst, L. Cohen, J. Chromatogr. B 820 (2005) 59–67.
[16] D. Zimmer, V. Pickard, W. Czembor, C. Müller, J. Chromatogr. A 854 (1999) 23–35.
[17] FDA, Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, May 2001.
[18] B. Matuszewski, M. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[19] H.K. Lim, K.W. Chan, S. Sisenwine, J.A. Scatina, Anal. Chem. 73 (2001) 2140–2146.
[20] J. Smalley, A.M. Marino, B. Xin, T. Olah, P.V. Balimane, J. Chromatogr. B 854 (2007) 260–267.