

# Quantitative determination of a novel dual PPAR $\alpha/\gamma$ agonist using on-line turbulent flow extraction with liquid chromatography–tandem mass spectrometry

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## Abstract

Turbulent flow chromatography (TFC) is a technique for the direct and efficient analysis of drugs and metabolites in biological matrices. We report here TFC on-line with an HPLC–MS/MS assay for the determination of 5-[2,4-dioxothiazolidin-5-yl)methyl]-2-methoxy-*N*-[[4-(trifluoromethyl)phenyl]methyl]benzamide (**I**, MK-0767, KRP297, Fig. 1) in plasma. Samples were transferred using an automated system followed by the addition of internal standard (**II**), prepared in 0.1 M ammonium acetate (pH 4.0). The plasma samples were directly injected onto a C18 turbulent flow column on-line with an HPLC–MS/MS system, and the analytical column used was a ThermoHypersil Keystone C18. Detection was achieved by MS/MS, using positive ionization on a TurboIonSpray® probe, operated in multiple reaction monitoring (MRM) mode. The linear range was 4–2000 ng/mL for **I** when using 50  $\mu$ L of plasma. The method exhibited good linearity and reproducibility. The method also showed good selectivity and ruggedness when applied to clinical samples, and was successfully cross-validated with a conventional off-line SPE, LC–MS/MS method.

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**Keywords:** Turbulent flow chromatography; Sample preparation; Validation; MK-0767; KRP 297

## 1. Introduction

Quantitation of drug candidates in biological fluids is playing an increasingly important role in drug discovery and development. Exploring new methodologies to perform bioanalysis rapidly and accurately will help to speed up the drug development process. Because of its speed and selectivity, HPLC–MS/MS has become one of the dominant tools for bioanalysis. A conventional method using off-line sample preparation becomes rate limiting during an LC–MS/MS analysis when there are large numbers of clinical samples to be analyzed.

To reduce time and effort, on-line sample preparation (direct sample injection) based on turbulent flow chromatography (TFC) offers an alternative. Sample preparation using

TFC is fast, easy and less labor intensive compared to the traditional off-line sample preparation methods [1,2]. TFC first emerged in 1966 [3]. Coupled with API mass spectrometric detection, TFC has been applied for quantitative analysis in biological matrices [1]. TFC could be operated under either the single-column mode [2] or the dual column mode [4]. TFC on-line with LC–MS/MS has demonstrated its utility and potential in the bioanalytical support of preclinical [4–14] and clinical studies [15–23].

Compound **I** is a dual peroxisome proliferator-activated receptor (PPAR)  $\alpha/\gamma$  agonist for the potential treatment of type II diabetes [24]. In the previous validated method, an automated solid phase extraction (SPE) off-line with LC–MS/MS was used for sample cleanup and detection (unpublished data). Sample preparation involved SPE wash and elution steps, sample concentration and reconstitution; these steps were eliminated with the current TFC on-line method. In this paper, we discuss a validated method for the deter-

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mination of compound **I** using direct injection TFC on-line with HPLC–MS/MS. Compared to conventional SPE off-line with LC–MS/MS, labor involvement was extensively reduced.

## 2. Experimental

### 2.1. Materials and reagents

Compound **I** and its internal standard (**II**) (Fig. 1) were obtained from Merck Research Laboratory (Rahway, NJ, USA). The 2 mL 96-deep well collection plates and 96-square well plate sealer were purchased from Waters Corp (Milford, MA, USA). Ammonium acetate (ultra grade) and formic acid (99%) were purchased from Sigma (St. Louis, MO, USA). Methanol and Acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Control human plasma containing sodium heparin was purchased from Biological Specialty (Colmar, PA, USA). Deionized water (18 M $\Omega$ ) was obtained from a Millipore Milli-Q ultra pure water system (Bedford, MA, USA).

### 2.2. Instrumentation

A Cohesive 2300 HTLC Turboflow System (Cohesive Technology, Franklin, MA, USA), which included two Agilent 1100 binary pumps and a valve module with focusing

mode loop (200  $\mu$ L) was used for on-line extraction. A Beckman Allegra 6R Centrifuge (Beckman Instruments, Fullerton, CA, USA) was used in the centrifugation steps. A LEAP PAL autosampler with refrigerated sample compartment (set at 10 °C) from LEAP Technology (Carrboro, NC, USA) was used as the injector. The mass spectrometer was a PE Sciex API 365 (Concord, Ont., Canada) triple quadrupole equipped with a TurboIonSpray<sup>®</sup> interface. The data were collected and quantitated using Sciex Analyst software (Version 1.1). The standard and sample transfer were conducted using a Quadra 96 Model 320 robotic liquid handler (Tomtec, Hamden, CT, USA), and a Packard MultiPROBE II<sup>ex</sup> Robotic Liquid Handling System from Packard (now PerkinElmer, Wellesley, MA, USA).

### 2.3. Chromatographic conditions

The on-line extraction was done using the dual column mode. The TFC parameters are listed in Table 1. Fig. 2 illustrates the system configuration during sample loading (cleanup), sample transfer, sample elution, loop re-filling and column re-equilibration to aqueous conditions, respectively.

The first column, a 1.0 mm  $\times$  50 mm Turboflow C18 column (Cohesive Technology, Franklin, MA) packed with 50  $\mu$ m porous particles (60 Å pore), was used as the extraction column to elute plasma matrix components and retain the analytes. The chromatographic separation was achieved on the second column, an ODS Hypersil analytical column

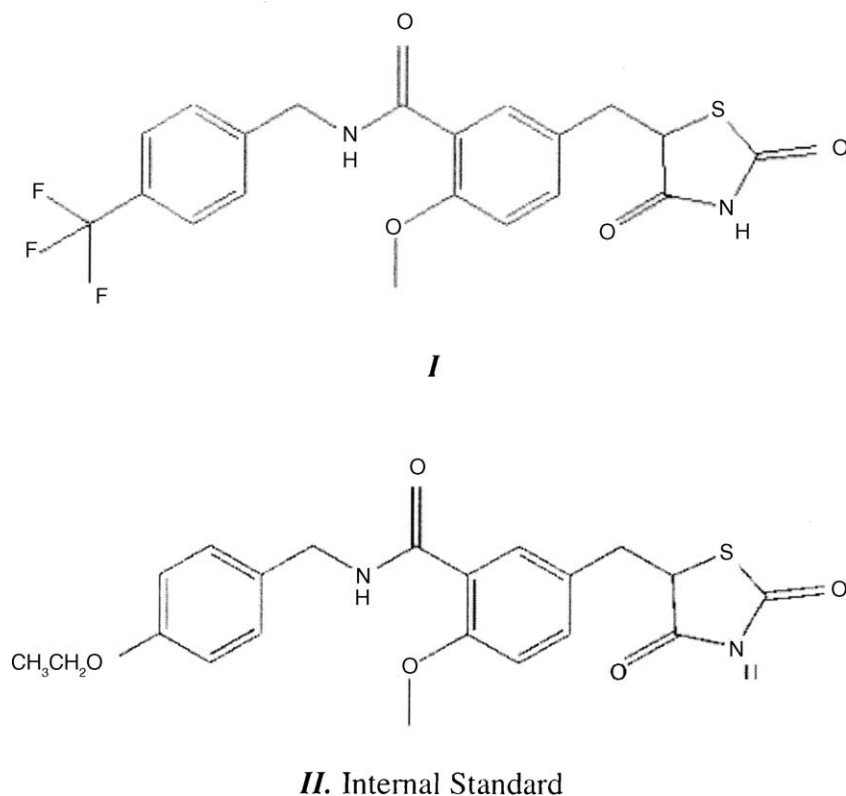


Fig. 1. Structures of **I** and **II**.

Table 1  
On-line extraction method for Cohesive 2300 Turboflow System

Step	Time (s)	Flow (mL/min)	Grad	%B	Tee	Loop	Eluting pump <sup>b</sup>		
							Flow (mL/min)	Grad	%B
I. Loading	60	4.00	Step	0	–	Out	1.00	Step	15.0
II. Transfer	40	0.20	Step	100.0	T	In	0.80	Step	0
III. Cleaning and elution	50	4.00	Step	100.0	–	Out	1.00	Step	60.0
IV. Elution and loop refilling	55	4.00	Step	55.0	–	In	1.00	Step	95.0
V. Re-equilibration to aqueous conditions	65	4.00	Step	0	–	Out	1.00	Step	15.0

<sup>a</sup> A: 0.3% formic acid (FA) in water; B: 0.1% formic acid (FA) in acetonitrile; extraction column: C18, 1.0 mm × 50 mm, 50 μm.

<sup>b</sup> A: 0.1% FA in water; B: acetonitrile; analytical column: C18; Hypersil, 4.6 mm × 30 mm, 5 μm.

(30 mm × 4.6 mm, 5 μm) purchased from Thermo Hypersil-Keystone (Bellefonte, PA, USA). The loading and extracting solvents consisted of 0.3% formic acid in water and 0.1% formic acid in acetonitrile. The eluting solvent was a mixture of acetonitrile and 0.1% formic acid under the gradient conditions listed in Table 1. The flow rate was 1 mL/min and was introduced into the TurboIonSpray® interface after a 1:3 split. In order to keep the samples stable throughout the injection period, the autosampler sample tray was kept at 10 °C.

#### 2.4. Standard solutions and QCs

A stock solution of **I** was prepared at 100 μg/mL in methanol and stored at –20 °C. This solution was further diluted to prepare a working plasma standard curve at con-

centrations of 4, 10, 40, 100, 400, 1000 and 2000 ng/mL. Calibration curve standards were prepared on the day of sample analysis using a serial dilution method. All samples were transferred using a Packard MultiPROBE II<sup>ex</sup> Robotic Liquid Handling System.

The internal standard (**II**) working solution was prepared at 50 ng/mL in 0.1 M ammonium acetate (pH 4.0). This solution was stored at 4 °C.

The quality control (QC) stock solution for **I** was prepared separately at a concentration of 100 μg/mL in methanol. This stock solution was diluted with methanol–water (50:50, v/v) to generate working QC stock solutions. Plasma QC samples were prepared by adding the appropriate volume of working QC stock solutions into control human plasma to yield final concentrations of 8, 160 and 1600 ng/mL for **I**, samples were stored at –20 °C.

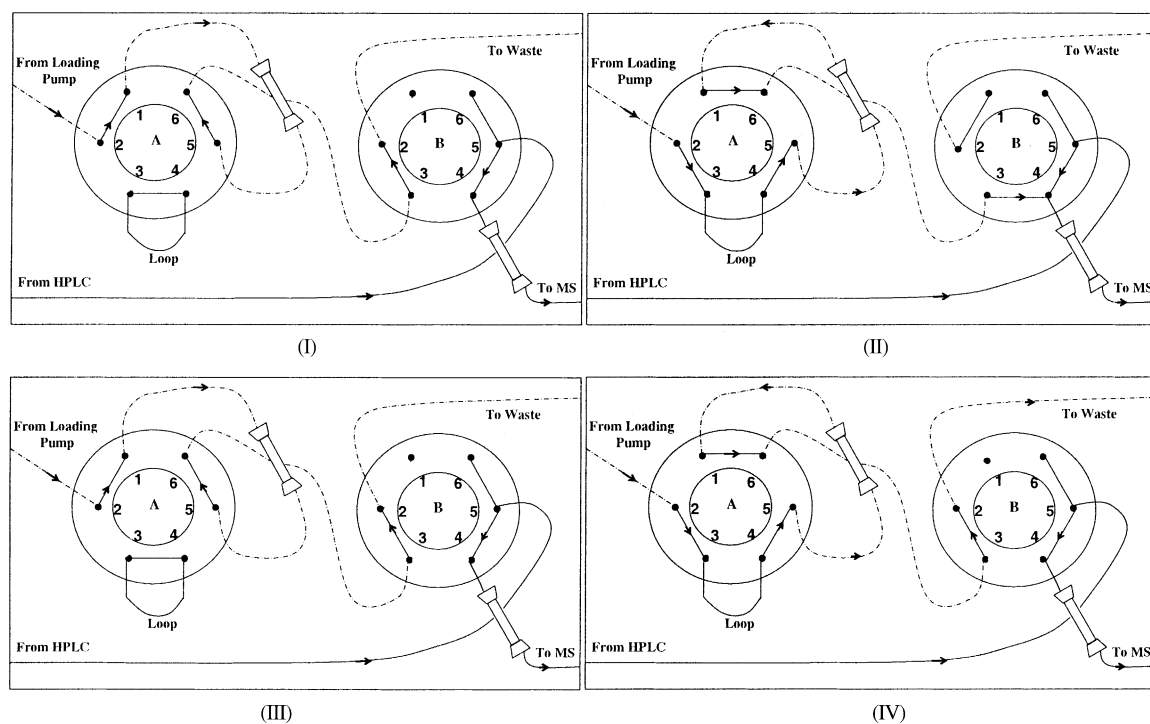


Fig. 2. System configuration during I: sample loading; II: sample transfer; III: cleaning and elution and IV: loop refilling and elution, respectively. Sample re-equilibration to aqueous conditions step (step V) followed the same flow scheme and flow rate as the sample loading step (step I), therefore, was not included in the following figures. (Arrows demote the direction of mobile phase flow. Dot line: extraction column process; solid line: analytic column process.)

### 2.5. Sample preparation for TFC

The frozen plasma samples were thawed completely at room temperature, vortexed and centrifuged at 3000 rpm ( $\sim 2056$  g) for 5 min prior to preparation in order to sediment the clot suspending in the plasma samples. A 50  $\mu$ L aliquot of each plasma sample was placed into a 96-well plate followed by 200  $\mu$ L of internal standard (**II**) prepared in 0.1 M ammonium acetate at pH 4.0. The above steps were done using the Packard MultiPROBE II<sup>ex</sup> Robotic Liquid Handling System. The plate was vortexed and then sealed with a mat. The sample preparation steps were reduced to centrifugation, sample transfer and IS addition. Centrifugation was essential in order to protect the column from particles, which might cause early plugging.

### 2.6. Mass spectrometry and data evaluation

The mass spectrometer used was a PE Sciex API 365 in positive ionization, multiple reaction monitoring (MRM) mode. The TurboIonSpray<sup>®</sup> conditions were as follows: 400 °C temperature setting, 80 psi supply N<sub>2</sub> gas pressure, 3500 V ion spray voltage, 7.5 L/min auxiliary gas flow, 0.9 L/min curtain gas flow rate and 0.9 L/min nebulizer gas flow rate. The product ion spectra of compounds **I** and **II** are shown in Fig. 3. The MS/MS transition selected to monitor **I** was from  $m/z$  439  $[M+H]^+$  to a product ion at  $m/z$  264. The protonated molecules were fragmented by collision-activated

dissociation (CAD) using nitrogen as the collision gas at instrument setting 5. The collision energy (CE) was set at 33 eV. The internal standard (**II**) was monitored using the transition from  $m/z$  415 to 264. The linear dynamic range of the calibration curve of **I** was 4–2000 ng/mL when 20  $\mu$ L of treated plasma were injected into the TFC HPLC–MS/MS system.

In each analytical run, a standard curve was constructed from the peak area ratios of **I** to the internal standard **II** versus the nominal concentration of the standards. Unknown plasma sample concentrations were calculated from the equation ( $y = mx + b$ ) as determined by the weighted ( $1/x$ ) linear regression of the standard curve.

### 2.7. Cross validation

Since TFC on-line with LC–MS/MS is an alternative method for the determination of **I**, human clinical study samples were analyzed by using previously validated reference SPE assays and also by TFC on-line with LC–MS/MS for cross validation purposes.

## 3. Results and discussions

### 3.1. Mass spectra and chromatograms of drug (**I**) and IS (**II**)

During tuning, both positive and negative ionization of **I** and **II** provided a good response. However, positive ionization using formic acid as the mobile phase modifier provided relatively higher response.

### 3.2. Turbulent flow chromatography

Most chromatography systems are operated under laminar-flow conditions. However, if the flow rate is increased to a very high level, a plug-type flow profile as opposed to a parabolic profile will be formed [2,14,25]. This altered flow profile is achieved at high flow rates by the particles in the packed bed causing the formation of eddies, which in return causes flow equalization across the column. A moderate back-pressure can be achieved at high flow rates by using columns packed with larger particles ( $>50$   $\mu$ m particle; pore size, 60 Å), compared to columns packed with conventional particles. Consequently, large protein molecules can easily pass through these columns as waste.

Initially, the method was developed on a 96-well SPE plate; however, a direct plasma injection method using TFC on-line with mass spectrometry was developed with the interest in reducing human labor. This method is based on the dual column TFC approach. The first column is a turbulent flow column, which separates the compound of interest from the biological matrix. Several TFC loading columns with C18, C8, Phenyl, PolarPlus<sup>TM</sup>, Cyclone, and Cyclone-P stationary phases were evaluated, and C18 was selected after the comparison based on elution recovery and sensitivity. With the help of the eluting mobile phase, the compounds are trans-

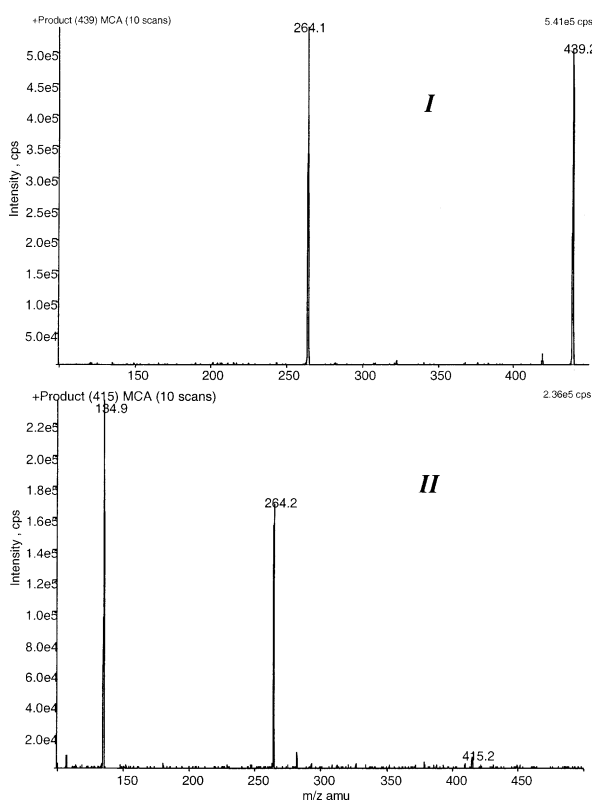


Fig. 3. Product ion spectra of **I** and **II** (IS).

ferred to and focused on the analytical column. Several analytical columns, Luna C18, Mercury C18, HeRes C18 and Hypersil C18 were tested. Hypersil C18 provided the best result, based on the separation, the system back-pressure, the analyte peak shape and the column life time. For the loading mobile phase, 0.3% formic acid provided the best result regarding sensitivity and peak shape. The peak area response was 80% less without an acidic modifier. The loading mobile phase was 0.3% formic acid in water. A 90% aqueous (or 10% organic modifier) loading was also evaluated, and the use of 10% organic modifier provided slightly better sensitivity. This improved sensitivity could possibly be caused by the increased wettability of the stationary phase. The column life was dramatically shortened by possible protein precipitation when 10% organic modifier was used in the loading step. The sample loading flow rate was also evaluated. The signal response increased with the increase of flow rate, while no improvement was observed when the flow rate was higher than 4 mL/min. Therefore, 4 mL/min was chosen as the loading flow rate. A sample loading time from 30 to 80 s (increased by 10 s increments) was also evaluated. Sixty seconds provided the best response; meanwhile, the analytical column achieved adequate equilibrium.

Once the sample was loaded, it was then transferred onto the analytical column. The transfer step was critical. During this step, the loading flow rate was decreased to 0.2 mL/min to ensure the organic percentage in this part was low enough in order to avoid breakthrough prior to being transferred onto the analytical column at the Tee connection (Fig. 2, step II). Loading pump transfer was done by 55% B in loop surrounded by aqueous mobile phase pushed by 100% B (Table 1). A 40–90 s transfer time was evaluated, and there was no difference in the peak area response. The 40 s period was adequate to move all analytes off the extraction column and into the vicinity of the mixing T. An aqueous mobile phase at a flow rate of 0.8 mL/min was introduced from the eluting pump (Table 1, transfer step for eluting pump) and was used to dilute the eluting plug from the loading column (Table 1) at the mixing Tee prior to the analytical column. This was to ensure the environment was at a low percentage of organic modifier to facilitate sample-focusing and to obtain nice peak shape thereafter.

Due to the large system void volume (about 200  $\mu$ L on each of the binary pump systems) and the dramatic change in mobile phase composition (from 0% to 100% organic percentage) at 0.2 mL/min flow rate, together with the limited time, it was difficult to reach the organic percentage required to elute the samples off the column at the initial loading step. Hence, a loop system was used to quickly and efficiently discharge organic solvents. Therefore, loop refilling was very important in providing adequate solvent strength to elute samples off the column at 0.2 mL/min flow rate. The loop refilling was also a very critical step in reducing peak broadening on the analytical column and enhancing sensitivity. During method development, 55–70% organic solvent in the loop provided nice peak shape.

Table 2

Intra-day precision and accuracy for the determination of **I** in five lots of human plasma using TFC–MS/MS

Nominal concentration (ng/mL)	Mean concentration <sup>a</sup> (ng/mL, <i>n</i> = 5)	Precision <sup>b</sup> CV (%)	Accuracy <sup>c</sup> (%)
4.0	3.7	13.5	92.5
10	9.8	9.2	98.0
40	39.7	8.8	99.3
100	104.6	6.7	104.6
400	429.3	1.5	105.2
1000	1001.6	4.8	100.2
2000	1965.3	4.0	98.3

<sup>a</sup> Mean concentrations calculated from the weighted (1/*x*) linear least-squares regression curve using all five replicates at each concentration.

<sup>b</sup> Expressed as coefficient of variation (CV) of peak area ratios.

<sup>c</sup> Expressed as [(mean observed concentration/nominal concentration)  $\times$  100%].

### 3.3. Validation results

The intraday accuracy and precision of the assay were evaluated by analyzing five replicates of each calibration standard in five different lots of plasma. By using five different lots of plasma for each individual curve, the relative matrix effect could also be evaluated. The data are shown in Table 2. The precision (%CV, *n* = 5) ranged from 1.5% to 13.5% and the accuracy was between 92.5% and 105.2% of nominal value; these data meet the criteria from the FDA Guidance [26]. No endogenous interferences were found at the retention times for either **I** or the internal standard (**II**) by testing five batches of blank control samples in the same run. Based on the precision and accuracy from each of the standard curve concentration points, the relative matrix effect of this assay is not significant. Chromatograms are presented in Fig. 4. Representative chromatograms at the LLOQ (4 ng/mL) of **I** and **II** (50 ng/mL) are presented in Fig. 5.

Samples initially used as QCs were used to evaluate reinjection stability. After the initial injection/analysis, the plate containing the samples was left on the autosampler for 24 h and the samples were then reinjected. The reinjected QC concentrations were calculated against the original standard curve. The initial plasma QC and autosampler reinjection stability data were acceptable and are shown in Tables 3 and 4, respectively.

Table 3

Initial quality control samples for **I** in human plasma

Nominal concentration (ng/mL)	Mean found concentration <sup>a</sup> ( <i>n</i> = 5)	Precision <sup>b</sup> CV (%)	Accuracy <sup>c</sup> (%)
8.0	8.1	2.8	101.3
160	173.3	6.0	108.3
1600	1582.8	3.4	98.9

<sup>a</sup> Mean concentrations calculated from the weighted (1/*x*) linear least-squares regression curve.

<sup>b</sup> Expressed as coefficient of variation (%CV) of peak area ratios.

<sup>c</sup> Expressed as [(mean found concentration/nominal concentration)  $\times$  100%].



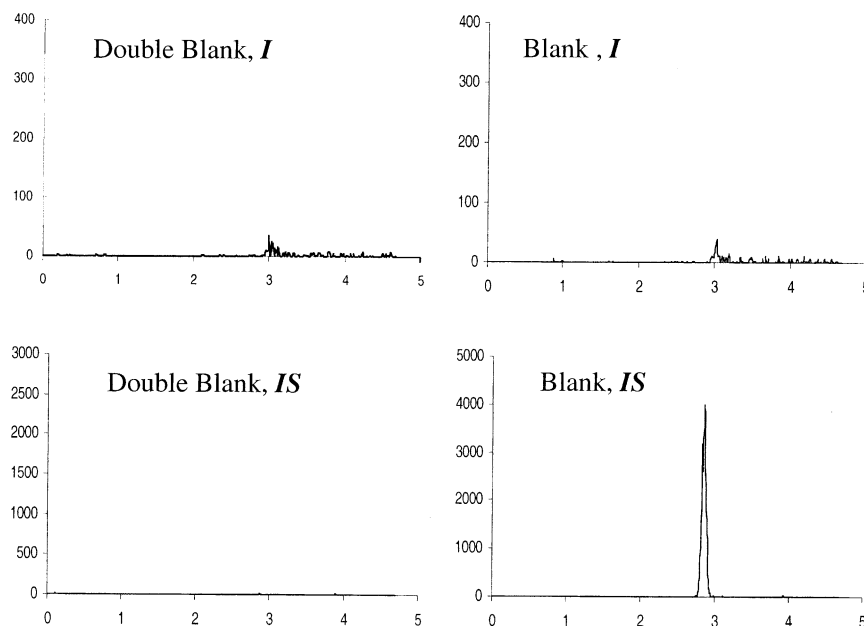


Fig. 4. Representative extracted ion chromatograms of a human plasma double blank and blank with IS.

Long term freezer stability and room temperature plasma stability experiments were not conducted they were performed in the original SPE LC–MS/MS method (unpublished data).

Due to the complicated nature of evaluating recovery for an on-line technique, the recovery in this paper was defined slightly different from the traditional definition described in the FDA guidance [26]. The recovery was calculated by comparing the peak area of the neat standard with the extraction

column to the peak area of the neat standard without the extraction column. Recoveries of **I** and **IS** are shown in Table 5.

According to FDA guidance [26], inter-day accuracy and precision of these assays were evaluated by analyzing five replicates of each calibration standard in plasma on five different days. The data are shown in Table 6. The precision (%CV,  $n=5$ ) ranged from 6.3% to 9.6% and the accuracy was from 91.8% to 106.3% of nominal value.

### 3.4. Cross validation results from in-vivo human samples

Samples from a clinical study of **I** in human were extracted using a conventional SPE method as well as an on-line TFC LC–MS/MS assay on the same working day. In order to evaluate whether the differences between these two methods were significant, regression analysis [27] and paired *t*-test were performed [28]. The fitted linear regression equation

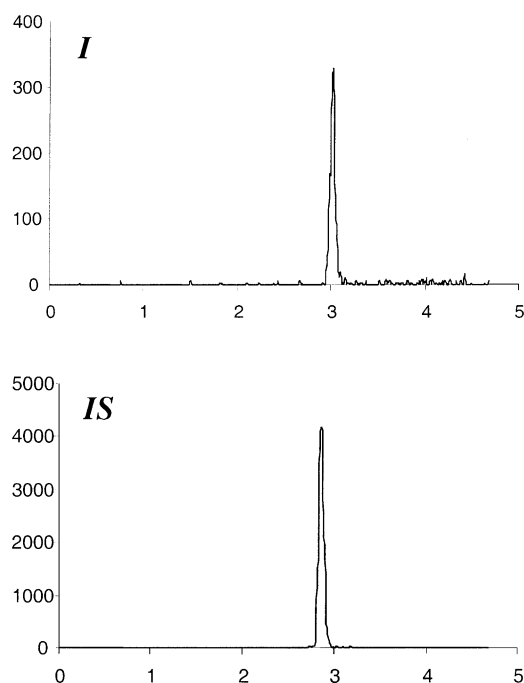


Fig. 5. Representative extracted ion chromatogram at LLOQ (4 ng/mL) of **I** and internal standard **II** (50 ng/mL) in human plasma.

Table 4  
Autosampler reinjection stability

Nominal concentration (ng/mL)	24 h on autosampler		
	Mean found concentration <sup>a</sup> (n = 3) (ng/mL)	Precision <sup>b</sup> CV (%)	Accuracy <sup>c</sup> (%)
8.0	7.3	6.9	91.0
160	171.6	1.3	107.0
1600	1479.4	5.1	92.0

<sup>a</sup> Processed QC samples in autosampler (10 °C) for 24 h against original curve. Mean concentrations calculated from the weighted (1/x) linear least-squares regression curve.

<sup>b</sup> Expressed as coefficient of variation (%CV) of peak area ratios.

<sup>c</sup> Expressed as [(mean found concentration/nominal concentration) × 100%].

Table 5  
Recovery of **I** and **II** (IS)

Nominal concentration (ng/mL)	Mean peak area without extraction column <sup>a</sup>	Mean peak area with extraction column <sup>b</sup>	Recovery (%) <sup>c</sup>
4.0	611	449	73
100	10323	7723	75
2000	184056	145035	79
50 (IS)	14260	10316	72

<sup>a</sup> Peak area of the neat standard ( $n=5$ ) without the extraction column.

<sup>b</sup> Peak area of the neat standard ( $n=5$ ) with the extraction column.

<sup>c</sup> Calculated as [(mean peak area without extraction column)/(mean peak area with extraction column)  $\times$  100%] at each concentration.

Table 6  
Inter-day precision and accuracy from five plasma curves over five days

Nominal concentration (ng/mL)	Mean concentration <sup>a</sup> (ng/mL, $n=5$ )	Precision <sup>b</sup> CV (%)	Accuracy <sup>c</sup> (%)
4	3.7	9.1	91.8
10	9.9	8.1	98.6
40	41.5	6.3	103.7
100	106.5	7.3	106.3
400	390.6	9.6	97.7
1000	1022.6	9.2	102.3
2000	1974.1	9.0	98.7

<sup>a</sup> Mean concentrations calculated from the weighted ( $1/x$ ) linear least-squares regression curve using all five replicates at each concentration.

<sup>b</sup> Expressed as coefficient of variation (CV) of peak area ratios.

<sup>c</sup> Expressed as [(mean observed concentration/nominal concentration)  $\times$  100%].

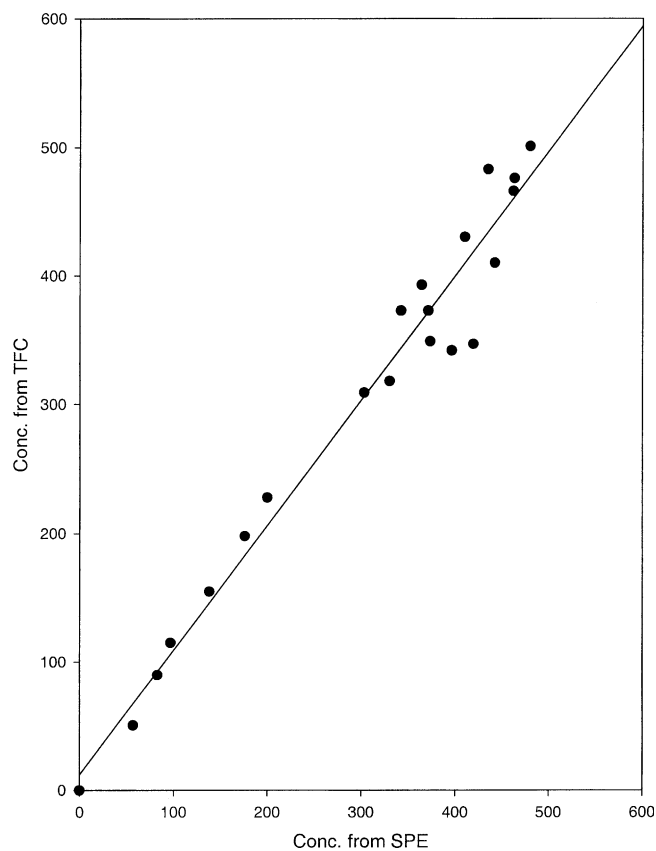


Fig. 6. Cross validation: comparisons of measured concentrations of **I** in human plasma using 96-well SPE and on-line TFC LC–MS/MS (regression results:  $Y=0.9700X+12.1889$ ;  $r^2=0.9640$ ).

was  $Y=0.9700X+12.1889$  and the determination coefficient  $r^2$  was 0.9640 (Fig. 6). The slope of the regression was not statistically different from unity with 95% confidence interval limits including unity (0.88–1.06). The intercept was not statistically different from zero with the 95% confidence interval limits including zero (–49.1 to 73.5). Paired  $t$ -test (two-tail test) also showed no significant differences ( $P=0.63$ ,  $\gg 0.05$ ). Hence, the analyzed concentration values using the on-line TFC LC–MS/MS method and the SPE method were comparable.

### 3.5. Ruggedness and life-time of columns

Column ruggedness was evaluated, and over 600 plasma injections could be achieved on a single turbulent flow column. The analytical column also achieved this goal by using a guard column.

## 4. Conclusions

Turbulent flow chromatography is a useful technique for the direct analysis of drugs in biological fluids. TFC eliminates the need for time consuming sample cleanup in the laboratory. The on-line TFC LC–MS/MS assay for the determination of compound **I** in human plasma was developed and validated. The same assay sensitivity and standard curve working range were achieved compared to the original off-line, SPE LC–MS/MS method. The quality of the TFC assay, as measured in term of accuracy and precision, compared well with the reference SPE assay. TFC on-line with LC–MS/MS proved to be rugged and selective.

The on-line TFC LC–MS/MS method offered an automated way to quantify compound **I** in human plasma that required less human involvement.

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