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Laser diode thermal desorption–positive mode atmospheric pressure chemical ionization tandem mass spectrometry for the ultra-fast quantification of a pharmaceutical compound in human plasma

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ABSTRACT

An ultra-fast, reliable and sensitive analytical method enabling high-throughput quantitative analysis of pharmaceutical compounds in human plasma is described. The quantitative work was performed on one of our compound currently under clinical trial by employing a deuterated internal standard (IS). Plasma samples were treated on solid phase micro-extraction (SPME) plates prior their analysis by laser diode thermal desorption and atmospheric pressure chemical ionization coupled to tandem mass spectrometry (LDTD/APCI-MS/MS) in positive mode. The sample analysis run time was 10 s as compared to the 7 min obtained for the validated LC–MS/MS method. The limit of quantification (LOQ) of the method was estimated at 1 ng/mL. The calibration graphs were linear with a regression coefficient $R^2 > 0.997$. The data of the partial validation show that LDTD/APCI-MS/MS assay was highly reproducible and selective. In addition, the deviations for intra and inter assay accuracy and precision data were within 15% at all quality control levels. The LDTD/APCI-MS/MS method was successfully applied to the analysis of clinical samples and the data obtained were consistent with those found with a validated LC–MS/MS assay. This work demonstrates that LDTD/APCI-MS/MS could be used for the ultra-fast and reliable quantitative analysis of pharmaceutical compounds in human plasma without using the separation step commonly associated with the LC–MS/MS assay.

1. Introduction

The development of any new drug is a long process that requires multiple steps. In this process, *in vivo* studies in animals and humans along with *in vitro* studies are essential to understand the behavior of a new compound. Generally, biological matrices such as blood, plasma and urine are collected for the quantitative analysis of a desired compound. Samples from these biological matrices are traditionally analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS) because of its selectivity, sensitivity and high precision [1–3]. The LC-separation time can range between 2 and 10 min and with such a run time it is quite challenging to have a high throughput method. Recently, various groups have developed rapid LC–MS/MS methods (run time less than 2 min) by using sub-2 μm particle columns [4,5], ballistic gradients [6], fast isocratic LC/MS runs [7], high flow rates using shorter columns [8] and fused-core HPLC columns [9,10]. To better characterize the throughput of a LC–MS/MS method, the total time from one injection to the next has to be considered. This total time includes the LC–MS/MS time and the overhead time. The LC–MS/MS time is needed for the HPLC gradient, MS data acquisition, column washing and equilibration. The overhead time is required for the auto-sampler washing cycles and software initialization for the next injection. Recently, it has been showed that for a LC–MS/MS method with a total run time of 0.9 min, the overhead time accounted for more than 50% of the total run time [11]. As a consequence, a method capable of determining compound concentrations in biological matrices without the need of injection and auto-sampler washing steps would offer obvious advantages.

During the last decade, several rapid techniques have been introduced for the direct analysis of samples. Among these new mass-spectrometry based methods, direct analysis in real time [12], desorption electro-spray ionization [13–15], atmospheric pressure matrix-assisted laser desorption/ionization [16–18] and low temperature plasma [19] have been mostly applied for quantitative work. One drawback of these methods is essentially the modest quantitative accuracy making these assays most useful for semi-quantitative work. In addition, the matrix suppression effect associated with these assays can lead to erroneous quantitative data, as previously reported [19]. Recently, a novel sample

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introduction method, the LDTD/APCI-MS/MS has been used for the analysis of hormones in wastewater [20]. With this method the authors were able to achieve low detection limits and intra-day and inter-day precision data below 20%. In another study, LDTD/APCI-MS/MS has been successfully applied to the quantification of sulfonamide residues in a much more complex matrix such as dairy milk [21]. LDTD/APCI-MS/MS has been applied for the quantitative analysis of metformin in plasma from different species (mouse, rat, dog and human). For the later study, the deviations for intra-assay accuracy and precision across the four species were less than 30% at all calibration and quality control levels [22]. The principles and the thermal desorption mechanisms of the LDTD–APCI source have been extensively discussed [20,23–25]. One benefit of the combination between LDTD–APCI source and tandem mass spectrometry results in fast analysis as compared to traditional LC–MS/MS. In addition, operating under the SRM (selected reaction monitored) mode which is specific to each targeted analyte allows the detection of compounds of interest in complex matrix without interference from the others. The use of LDTD/APCI-MS/MS would then dramatically reduce the interfering matrix background observed with other rapid method such as MALDI assay [16].

The purpose of the present study was to assess the capabilities of LDTD/APCI-MS/MS for the quantification of a small molecule in a complex matrix such as plasma with accuracy and precision data within 15% at all level concentrations. In addition, a partial validation of the LDTD/APCI-MS/MS method was performed by testing the reproducibility, selectivity, dynamic range and carry-over. In addition, LDTD/APCI-MS/MS was applied to the analysis of human clinical samples in order to compare LDTD/APCI-MS/MS data with those obtained with a previously validated LC–MS/MS method.

2. Experimental

2.1. Chemicals and reagents and stock solutions

The compound used for the LDTD/APCI-MS/MS evaluation was synthesized in house. The compound has a molecular weight of 305.4 Da, a $C\log P$ of 3.615, and has a moderate solubility in water. The pK_a of hydroxyl group and the pyrimidine, the two ionizable groups of compound-1 are 7.90 and 2.60, respectively. For confidentiality reasons, the compound structure cannot be disclosed. Throughout this manuscript, this compound was named compound-1. The deuterated (D_6) internal standard (IS) was synthesized in house. The HPLC grade methanol, formic acid, ammonium acetate, acetonitrile and ammonia were obtained from Merck KGaA (Darmstadt, Germany). MilliQ grade water was produced by a Millipore system (Bedford, MA, USA). The different human plasma batches used for the preparation of Cs and QCs were obtained from the internal blood bank.

2.2. Preparation of stock and working solutions

Compound-1 stock was prepared in water/methanol (50/50, v/v) to give a final concentration of 250 μg/mL. Individual working calibration standard (Cs) solutions with concentrations of 20, 100, 1000, 2000, 5000, 8000, and 10,000 ng/mL were prepared after serial dilutions of the stock solution in water/methanol (50/50, v/v). The working Quality Control sample (QCs) solutions with concentrations of 20, 60, 6000 and 9000 ng/mL were prepared in the same manner. The working solutions were freshly prepared on each day of the analysis.

2.3. Preparation of working standard, and Quality Control (QC) solutions

Two different batches of human plasma were used for the preparation of Cs and QCs. The Cs samples were prepared by spiking 25 μL of each compound-1 individual working Cs solution into 475 μL blank human plasma. This yielded Cs concentrations of 1 (LOQ), 5, 50, 100, 250, 400 and 500 ng/mL. The QCs were prepared in the same manner to give final concentrations of 1, 3, 300 and 450 ng/mL.

2.4. Sample preparation

Prior to the plasma sample extraction, 200 μL of each of the calibration standards, quality control, blank, zero samples and unknown samples were mixed together with 50 μL IS at a concentration of 100 ng/mL prepared in methanol/water (50/50, v/v). Then a volume of 400 μL 2% ammonia aqueous solution was added in the tubes. The tubes were vortexed briefly and the diluted plasma samples were submitted to the extraction as described below.

2.5. SPME

The SPME was performed on Oasis micro-elution MAX 96 well-plate (Waters Inc., Milford, MA, USA). The SPME cartridges were conditioned with 250 μL methanol followed by 250 μL water. A volume of 600 μL samples was loaded on the cartridges column at a flow rate of 2–3 mL/min by applying negative pressure using a mechanical pump. After the loading step, the cartridges were washed with 300 μL 5% ammonia in water followed by 300 μL methanol. Compound-1 and its IS were eluted in collecting plates with 100 μL formic acid 2% in methanol, followed by 100 μL water. The collecting plates were then briefly centrifuged at 2000 × *g* for 5 min at 10 °C. A volume of 100 μL of the supernatant was injected for the LC–MS/MS analysis and 6 μL were spotted on LazWell plate™ for LDTD/APCI-MS/MS analysis.

2.6. LC–MS/MS

Sample analysis was performed on a LC–MS/MS system consisting of an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ interface from Applied Biosystems, (Foster City, CA, USA). The MS system was connected to a HTS CTC PAL auto-sampler (Zwingen, Switzerland) and to an Agilent 1100 pump system (Wilmington, DE, USA). Chromatographic separations were performed at a flow rate of 0.6 mL/min on a Zorbax XDB C_{18}, column 50 mm × 4.6 mm (Wilmington, DE, USA) equipped with a Phenomenex C_{18} Guard Cartridge 4.0 mm × 3.0 mm I.D. (Utrecht, The Netherlands). A binary gradient with a mobile phase consisting of 2 mM ammonium acetate (A) and acetonitrile (B) was used for the LC-separation. The mobile phase (A) was acidified with 0.1% formic acid. The elution gradient program was as follow: [time (min), (% mobile phase B): (0, 20) (1, 20) (4.5, 90) (5.5, 90) (5.7, 20) (7, 20)]. The column temperature was maintained at 60 °C using a column heater. The system was operated in electrospray positive ionization using SRM mode. The other MS conditions were as follows: turbo ion spray 4000 V; source temperature 650 °C; collision activated dissociation 5; curtain gas 40 psi; Gas1 40 psi; Gas2 60 psi; entrance potential 10 V; dwell time 150 ms; collision energy 47 V; declustering potential 41 V (compound-1) and 61 V IS and collision cell exit potential 10 V (compound-1), and 6 V IS.

2.7. LDTD/APCI-MS/MS

Ionizations of compound-1 and that of its IS were achieved with the LDTD/APCI source, developed and manufactured by Phytronix Technologies (Quebec, QC, Canada), coupled to an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ interface from Applied Biosystems, (Foster City, CA, USA).

The LDTD/APCI sample optimization for MS and MS/MS conditions in positive ionization mode was performed using the

manual-tune function in the Analyst 1.4.2 software (Foster City, CA, USA) with compound-1 and its IS dissolved at a concentration of 1 mg/mL in methanol/water (50/50, v/v). An aliquot of this solution (2 μL) was spotted onto the LazWell™ plate and the methanol/water mixture was evaporated to dryness at room temperature prior the sample analysis by LDTD/APCI-MS/MS. The LDTD/APCI source parameters were set to the following values: curtain gas pressure 10 psi, entrance potential 10 V, collision cell exit potential 15 V, declustering potential 80 V.

Physical parameters of the LDTD/APCI source were optimized with spiked aliquots of human plasma (compound-1 at 1 μg/ml) in order to account for matrix on the deposition volume into plate wells and on the laser power (laser pattern). Following the LDTD/APCI source parameters optimization, the deposition volume was set up to 6 μL while the laser pattern programming consisted of a 3 s ramp from 0% to 45%, held for 2 s at 45%, a 1 s and decreased to 0% that was held for 4 s before the next analysis. The other LDTD/APCI ionization source settings: corona discharge needle voltage (3 μA), vaporizer temperature (ambient) and carrier gas air flow rate (3 L/min) were those recommended by the manufacturer. The LDTD/APCI source was controlled by the LazSoft 4.0 software (Phytronix Inc., Quebec, Canada).

3. Results and discussion

3.1. LDTD/APCI-MS/MS parameters optimization

The mass spectrometer was operated in positive ion selected reaction monitoring mode. The main precursor ion of compound-1 was monitored at a mass of 306.5 and the product ion at mass of 160.4 (Fig. 1A) with collision energy of 47 V. The main precursor ion of IS was monitored at a mass of 312.8 and the product ion at mass of 166.5 (Fig. 1B) with collision energy of 47 V. These precursor ions were identical to those used for the quantitative analysis by LC–MS/MS in electrospray mode ionization (data not shown).

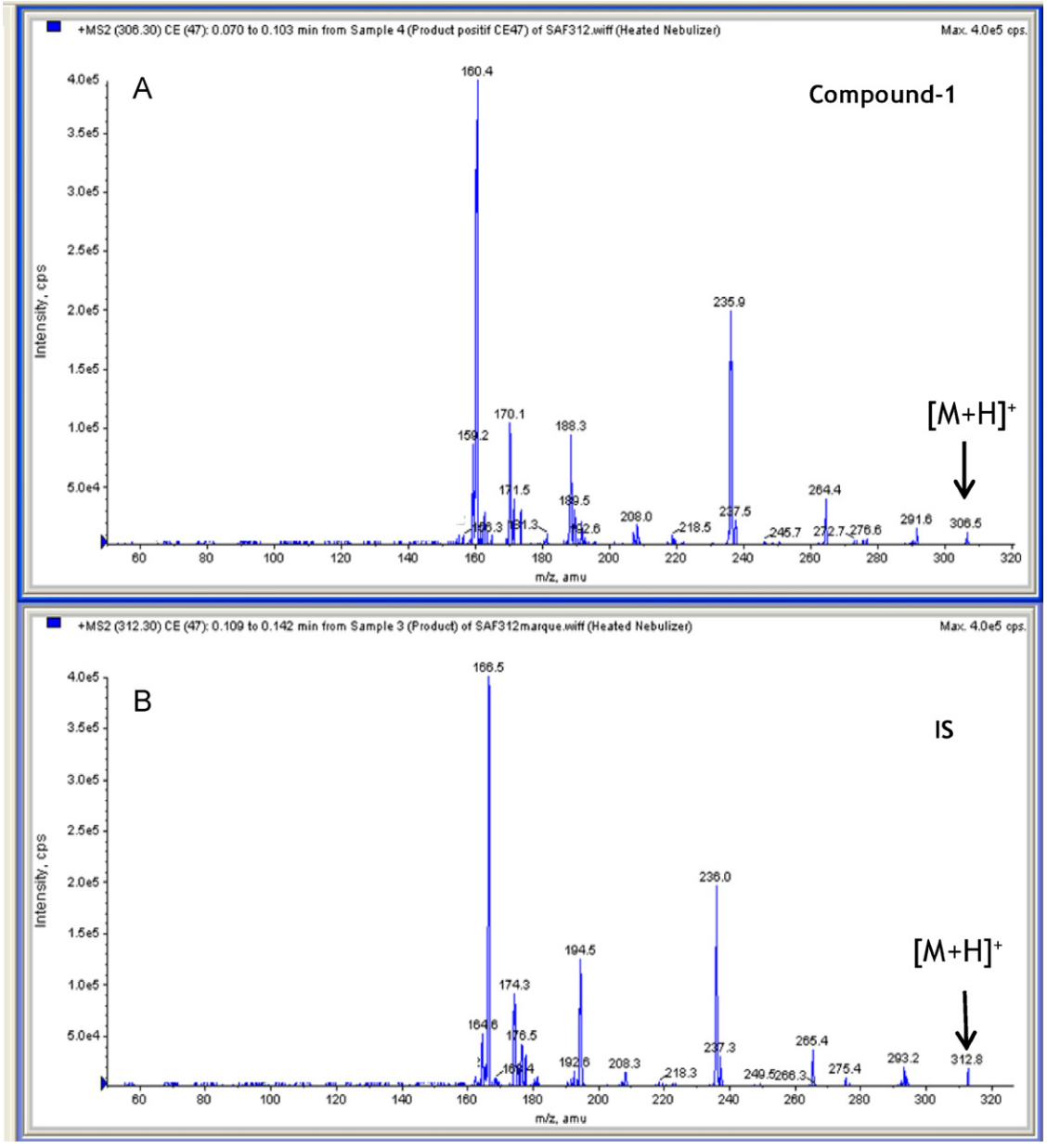

Fig. 1. MS/MS spectra showing (A) the precursor ion of compound-1 at *m/z* 306.5 and its product ion at *m/z* 160.4, (B) the precursor ion of IS at *m/z* 312.8 and its product ion at *m/z* 166.5.

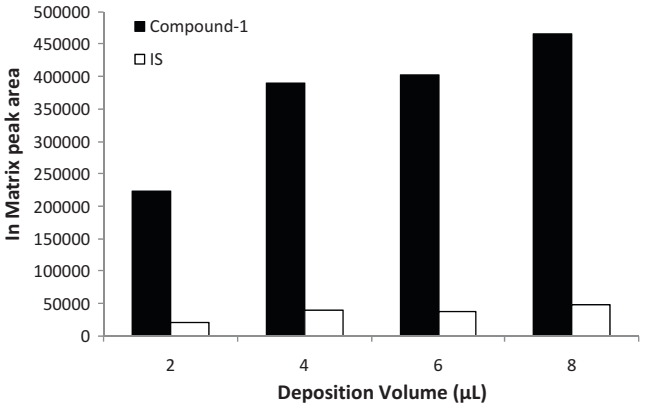

Fig. 2. Effects of deposition volume change on method sensitivity in spiked human plasma. The concentrations used for this assessment were 1 μg/mL (compound-1) and 0.1 μg/ml (IS). The laser pattern was set at 3.45.

During the LDTD/APCI/MS-MS method development we have realized two main parameters need to be optimized: the deposition volume of sample onto the plate and the laser pattern.

The deposition volume will influence the amount of material loaded into the sample well and this could affect the overall MS signal intensity for a given compound. One could argue that adding more samples into the sample well cavities would lead to an increase of MS signal. To test this hypothesis, four different deposition volumes (2, 4, 6 and 8 μL) were compared. Fig. 2 represents the effect of deposition volume on peak area intensities for compound-1 and for its IS. As expected, the peak areas of compound-1 and its IS increase with increasing deposition volume from 2 to 4 μL, whereas no significant signal increasing was observed for a deposition volume above 4 μL.

The laser pattern is used to control the power of the laser diode radiation (maximum 20 W) applied to the back of the metal well during a short period of time (<10 s). Therefore, increasing the percentage of laser power will increase the laser radiation power hitting the back side of the sample holder and ultimately the amount of energy transferred to the sample. In order to optimize the LDTD/APCI source laser pattern (0–35% (pattern 3.35), 0–45% (pattern 3.45), 0–55 (pattern 3.55) and 0–65% (pattern 3.65)) were tested under the following conditions: 3 s ramp, held at the maximum power for 2 s, then a 1 s decrease to 0% and held for 4 s before the next analysis.

The increase in MS peak area of compound-1 and that of its IS was only observed between the laser pattern 3.35 and 3.45 (Fig. 3A and B) regardless to the deposition volume. However, there was no clear effects on the MS peak area signal intensity of compound-1 and its IS when the laser pattern was increased from 3.45 to 3.65 (Fig. 3A and B). For the present study we have decided to use 6 μL and 3.45 for the deposition volume and the laser pattern, respectively. This laser pattern allows us to work under "mild" desorption conditions as opposed to the "hard" conditions obtained with the laser patterns 3.55 and 3.65. Working under mild condition maintains low the background signal generated by the matrix components. As a consequence, better desorption peak shapes were obtained for compound-1 and for its IS (data not shown). Under our selected experimental conditions, the analysis time was set to 10 s.

3.2. Validation

A SPME LC–MS/MS method was previously validated for quantitative determination of compound-1 in human plasma in our laboratory. Therefore LDTD/APCI-MS/MS was only tested for

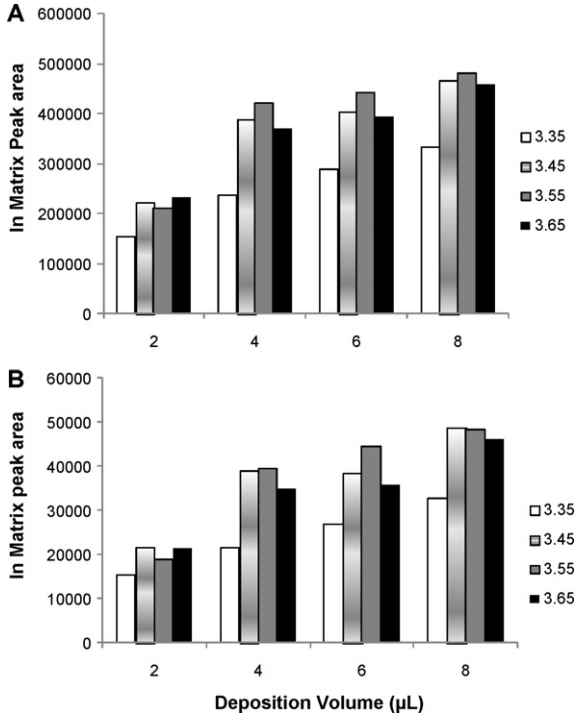

Fig. 3. Effects of laser power (laser pattern) on method sensitivity in spiked human plasma samples for (A) compound-1 and its (B) IS. For each deposition volume, the laser power applied for the sample desorption from the Lazwell plate™ was ramped in 3 s is as follows: from 0–35% (pattern 3.35), 0–45% (pattern 3.45), 0–55 (pattern 3.55) and 0–65% (pattern 3.65), then held for 2 s and then brought back to 0% in 5 s. To assess the effect of laser power, four deposition volumes were tested.

reproducibility, selectivity, carry-over, calibration, precision and accuracy, and its ability to produce comparable results to our validated LC–MS/MS method.

3.2.1. Reproducibility

Under our experimental conditions, a spiked plasma sample was extracted and spotted 21 times on the LazWell plate™ prior the APCI/LDTD-MS/MS analysis. The relative standard deviation obtained for the MS peak area of compound-1 was below 9% (Fig. 4), demonstrating the high reproducibility LDTD/APCI-MS/MS for compound-1 measurement in plasma samples. One should note that the given reproducibility is taking into account both the extraction and the instrument variations.

3.2.2. Selectivity and LOQ

Since no chromatography separation is associated to our method, it was essential to assess the selectivity of our method. This was established by assaying 6 lots of human blank plasma and comparing the response of each blank relative to the lowest calibration standard. The signal found on the compound-1 SRM channel of the blank extracted human plasma sample was less than 20% of the one obtained on human plasma spiked with 1 ng/ml (LLOQ) of compound-1 (Fig. 5A and B). The signal found on the compound-1 SRM channel of the zero sample was similar to the one obtained in the blank extracted sample (Fig. 5C and D), indicating that the IS does not contribute to compound-1 signal. We also observed that Compound-1 did not contribute to the signal of the IS (data not shown). This demonstrates that our method is highly selective for both compound-1 and its IS. This selectivity is mainly due to the SRM mode which allows the detection of compounds of interest in complex matrix without interference from the others. Also, the fact that the laser pattern was not set too high in power might have reduced the matrix components' thermal degradation, sus-

Table 1
Daily variation of calibration parameters in human plasma.

Run date	Slope (a)	Intercept (b)	Regression coefficient (R^2)	LLOQ (ng/mL)	ULOQ (ng/mL)
22-Mar-2010	0.04274	0.008199	0.9974	1	500
12-Apr-2010	0.04695	0.006023	0.9982	1	500
n	2	2	2		

ceptible to produce additional interfering components. The LOQ for the quantification of compound-1 was 1 ng/mL, as defined by the lowest concentration of analyte with accuracy within 15% and a precision <15%.

3.2.3. Carryover

Carry-over represents one of the most critical problems to assess during the LC–MS/MS development method. To avoid this type of issue during the LC-analysis, different solvents are used to wash the auto-sampler needle [26]. This will then increase the LC-analysis time as, the washing cycle before and after the injection could take

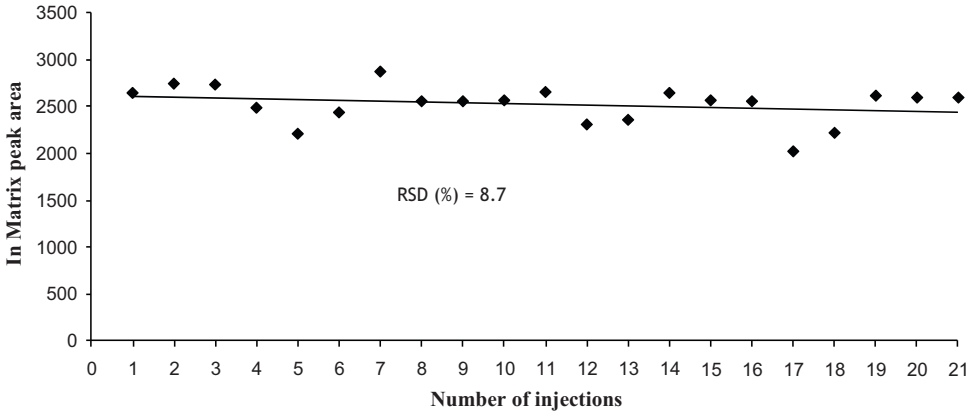

Fig. 4. LDTD/APCI-MS/MS reproducibility obtained on extracted human plasma. The plasma sample was extracted according to our sample preparation procedure and spotted 21 times on Lazwell plate™. Compound-1 was analysed 21 times by LDTD/APCI-MS/MS and the instrument response (peak area) was determined as the ratio of the analyte area to that of the isotropically labeled IS.

Table 2
QCs precision and accuracy data.

Run date	Compound-1 nominal concentration (ng/mL)			
	1	3	300	450
Measured concentration (ng/mL)				
22-Mar-2010	0.967	3.08	298	434
	0.909	2.84	290	429
	0.947	3.07	315	424
	0.964	2.78	293	424
	0.933	3.00	301	433
	0.998	3.07	300	439
Intra-run mean (ng/mL)	0.953	2.97	300	431
Intra-run (%CV)	3.2	4.4	2.9	1.4
Intra-run (%bias)	−4.7	−1.0	0.0	−4.2
n	6	6	6	6
12-Apr-2010	NP[b]	3.25	[a]208	441
	NP[b]	3.10	297	434
	NP[b]	3.22	309	425
	NP[b]	3.08	282	444
	NP[b]	3.16	290	443
	NP[b]	3.27	297	424
Intra-run mean (ng/mL)		3.18	281	435
Intra-run SD (ng/mL)		0.0792	36.6	8.98
Intra-run (%CV)		2.5	13.0	2.1
Intra-run (%bias)		6.0	-6.3	-3.3
n		6	6	6
Mean concentration (ng/mL)		3.08	290	433
Inter-run (%CV)		4.8	9.4	1.8
Inter-run (%bias)		2.7	-3.3	-3.8
n		12	12	12

[a] Bias > 15%.
[b] NP: not performed.

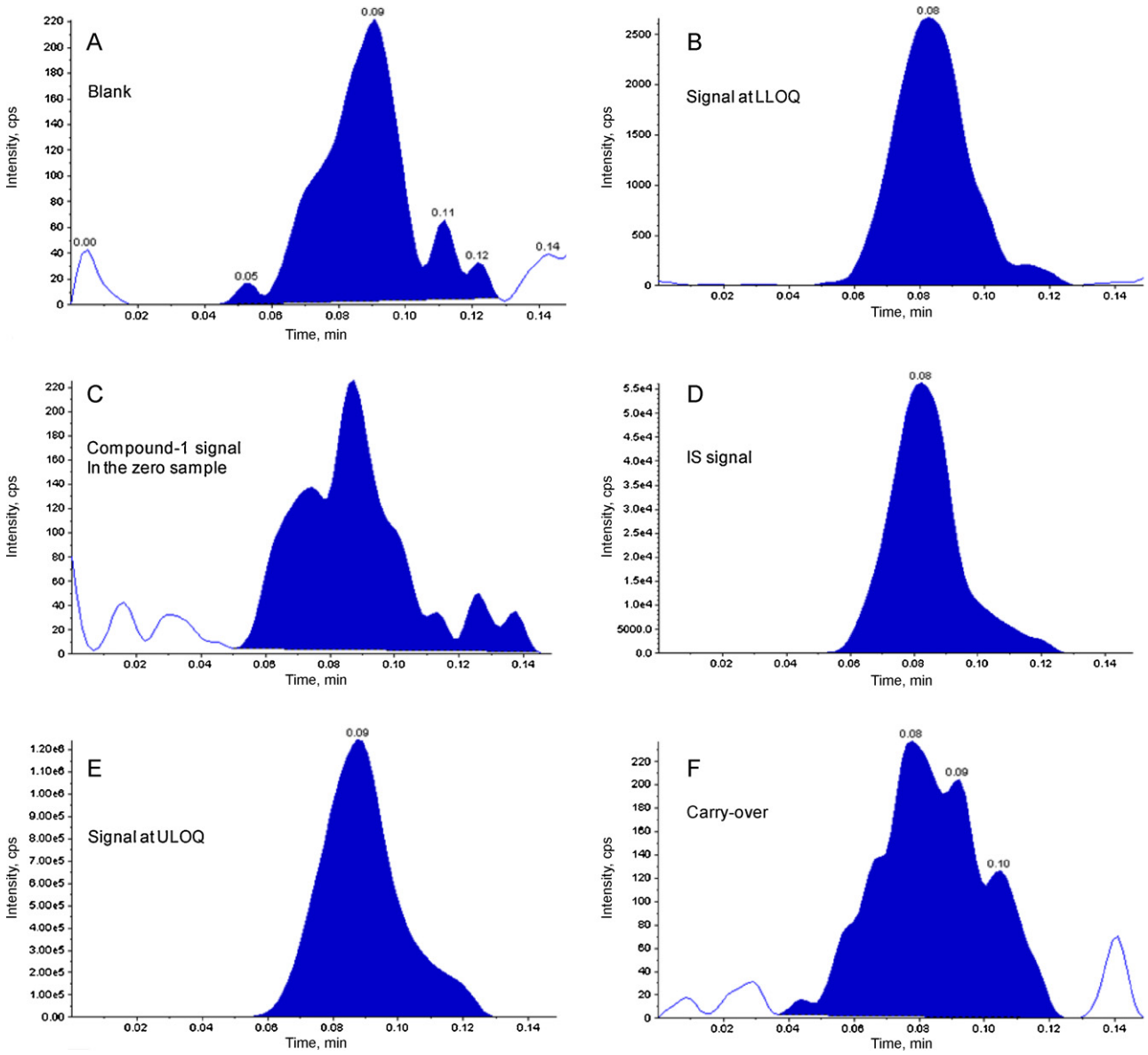

Fig. 5. Reconstructed LDTD/APCI-MS/MS desorption peaks showing SRM transition signal of compound-1 for (A) extracted double blank plasma sample; (B) extracted plasma sample spiked with compound-1 at a concentration of 1 ng/mL (LOQ); (C) compound-1 in the zero extracted sample; (D) extracted blank plasma sample spiked with IS; (E) extracted plasma sample spiked with compound-1 at a concentration of 500 ng/mL (ULOQ) and (F) blank extracted sample injected right after the ULOQ.

more than 1 min. With the LDTD–APCI-MS/MS method, we have tested the carry-over by analyzing a blank extracted plasma sample right after the ULOQ concentration (Fig. 5E). The peak found on this blank sample was similar to the one obtained in the blank extracted sample (Fig. 5F), suggesting the absence of carry-over.

3.2.4. Calibration

The plasma calibration curve was constructed using peak area ratios of compound-1 to that of its IS and applying a weighted ($1/x^2$) least-squares quadratic regression analysis. The daily variations of calibration parameters obtained in human plasma are shown in Table 1. As it can be noticed, regression coefficients (R^2) of duplicate calibration curves were >0.997. The calibration curves were linear over the concentration range of 1–500 ng/mL demonstrating its suitability for the quantification of compound-1 in human plasma samples.

3.2.5. Precision and accuracy

Precision (expressed as percent relative standard deviation, %CV) and accuracy (expressed as percent error, %bias) were calculated for the four QCs (concentrations of 1, 3, 300 and 450 ng/mL). At least five replicates of each QC point were analyzed every day (on the second day, the QC at the LOQ was not analysed) to determine the intra-day accuracy and precision. This process was repeated over 2 days in order to determine the inter-day accuracy and precision. The intra-run QCs accuracies were all within ±15% at all concentration levels except for 1 QC at 300 ng/mL (Table 2). The inter-run precision and accuracy data for QCs ranged from 1.8 to 9.4% ($n = 12$) and from −3.8 to 2.7% ($n = 12$), respectively (Table 2).

3.2.6. Pharmacokinetics of compound-1 in human: in vivo experiment

Single oral dose of compound-1 was given to patient and blood samples were taken by either direct venipuncture or an indwelling cannula inserted in a forearm vein in a coated K_3-EDTA tubes at

Table 3
Individual and mean pharmacokinetic parameters of compound-1 in two different patients after its oral administration at 50 mg/kg.

PK parameters	LDTD			LC–MS/MS		
	Patient 1	Patient 2	Mean	Patient 1	Patient 2	Mean
Tmax	2	2	2	2	2	2
Cmax	169	148	159	163	136	150
Cmax/dose	3.38	2.96	3.17	3.26	2.72	2.99
AUC	1664	1229	1447	1606	1222	1414
AUC/dose	33	25	29	32	24	28

Units: Tmax (h); Cmax (ng/mL); Cmax/dose (ng/mL)/(mg/kg); AUC (ng h/mL); AUC/dose (ng h/mL)/(mg/kg); AUC interval (0–24 h).

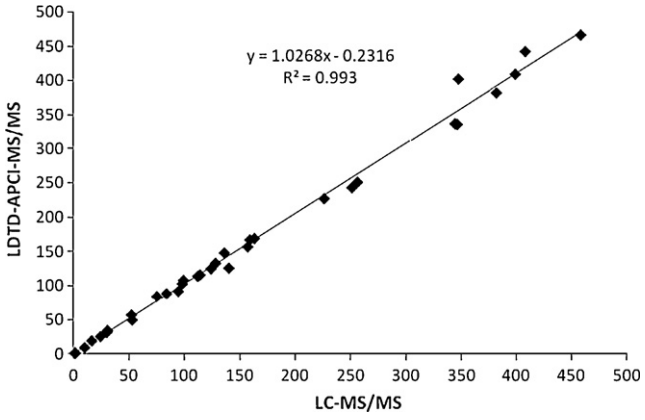

Fig. 6. Correlation between LC-MS/MS and LDTD/APCI-MS/MS on compound-1 concentration determined in clinical samples ($n = 30$).

0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 12 h and 24 h post-dose. A 5-mL sample of venous blood was collected into pre-cooled EDTA-containing tubes. Immediately after each tube of blood was drawn, it was inverted gently several times to ensure the mixing of tube contents. Within 15 min after collection, the samples were centrifuged for 10 min at 3000 g and at 4 °C, then the top layer (about 2 mL of plasma) was transferred to pre-cooled storage vials. The tubes were kept frozen below −20 °C for pending analysis.

3.2.7. Comparison between LC–MS/MS and LDTD/APCI-MS/MS for human PK study of compound-1

Having demonstrated that the method was accurate and precise within the defined acceptance criteria, the assay was applied to a human pharmacokinetic study. The individual concentrations of compound-1 in plasma were determined by both LDTD/APCI-MS/MS and LC–MS/MS, There was an excellent correlation between the results obtained with the LDTD–APCI-MS/MS and the LC–MS/MS systems as shown by a coefficient of correlation higher than 0.99. Moreover, with a slope value of 1.02 (Fig. 6), it confirms the accuracy between both systems. The pharmacokinetic parameters derived from the analysis are listed in Table 3. The Cmax was 159 ng/mL and occurred at 2 h. The area under the plasma concentration-time curve was 1447 ng h/mL. The results are consistent with the values obtained with a validated LC–MS/MS assay (Table 3).

4. Conclusions

A high-throughput and ultra-fast and sensitive method for the quantitative analysis of a pharmaceutical compound in human plasma using LDTD/APCI-MS/MS has been successfully developed. The overall performances (recovery, precision, accuracy and calibration) of the method were comparable to that of a previously validated LC–MS/MS method but offer the advantage of ultra-fast analysis (10 s per sample). The fast analysis time was achieved without using chromatography, which would then reduce the cost and environmental impacts of the column and solvent consumption. The comparative data between our method and LC–MS/MS assay demonstrated that LDTD/APCI-MS/MS is efficient approach for the quantitative analysis of pharmaceutical compound in biological matrices. The high throughput nature of LDTD/APCI-MS/MS method would alleviate the clinical sample analysis and therefore speed up the clinical trials process. At the time of publication, quantitative bioanalytical method using LDTD/APCI-MS/MS has been developed and applied to support more than 10 different molecules in our laboratory.

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