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Ultra-high capacity liquid chromatography chip/quadrupole time-of-flight mass spectrometry for pharmaceutical analysis

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ABSTRACT

Nanoflow liquid chromatography/mass spectrometry (nano-LC/MS) has attracted increasing interest in virtue of high sensitivity, low sample consumption, and minimal matrix effect. In this work a HPLC-Chip/quadrupole time-of-flight (Q-TOF) MS device with a new ultra-high capacity small molecule chip (UHC-Chip) which features a 500 nL enrichment column and a 150 mm × 75 μm analytical column, was evaluated with a drug mixture covering a wide range of polarities. Excellent chromatographic precision with 0.1–0.5% RSD for retention time and 1.7–9.0% RSD for peak area, low limit of detection, good chip-to-chip reproducibility and linearity were obtained by using this UHC-Chip. Compared with the standard HPLC-Chip with 40 nL trapping column, the UHC-Chip showed higher enrichment capability and hence gave a higher response in signal detection. Additionally, 4–30 times increase in sensitivity was obtained compared with conventional LC/MS, which indicated that UHC-Chip/MS was a valuable tool for the quantitative analysis of low level impurities and degradation products in pharmaceuticals. Moreover, satisfactory results obtained from trace drug analysis of serum samples further proved its practicality and potential for use in drug testing and development.

1. Introduction

The quantitative analysis of active pharmaceutical ingredients (API) including their related impurities and degradation products is one of the most active areas in modern analytical chemistry [1–4]. The purity profiling for API in any pharmaceutical product is crucial to ensure the quality of the drug while API quantitation in preclinical and clinical samples plays an important role in drug discovery and development. In recent years, great effort has been made to improve both separation and detection techniques for pharmaceutical analysis. Among various methods for pharmaceutical analysis [5–7], high performance liquid chromatography/mass spectrometry (HPLC/MS) is the most commonly used technique on account of high sensitivity, robustness and reliability.

Recently, nanoflow LC/mass spectrometry (nano-LC/MS) has generated increasing interest because of the minimal sample consumption and the high sensitivity [8–11]. Several representative applications of chip-based nano-LC/MS for the analysis of small molecules have also been reported [12–14]. In order to get a more stable and robust nano-LC/MS platform, a new generation of HPLC-Chip/MS system has been developed. All functional components, including enrichment column, analytical separation column and nano-electrospray emitter, were integrated onto this microfluidic device, which can significantly reduce the possibility of leaks and dead volumes, and greatly improve the ease of use during analysis [15,16]. Moreover, the miniaturized analytical platform results in less sample consumption, dilution and improved overall analyte ionization efficiency, and ultimately leads to the pronounced sensitivity advantage compared to conventional LC/MS [15–19]. At present, most of the applications of new HPLC-Chip/MS system have been focused on proteomic and oligosaccharide analysis [20–23]. However, the high sensitivity, ease of use and reusable nature of this device make it attractive for pharmaceutical analysis, especially in the area of trace analysis. Because of smaller injection volume on the HPLC-Chip, it is highly desirable to have an enrichment column with enhanced trapping capability to further improve its sensitivity.

In this work, a new ultra-high capacity small molecule HPLC-Chip (UHC-Chip), including a 500 nL enrichment column and a 75 μm (i.d.) × 150 mm (L) separation column that were both packed with ZORBAX 80 Å SB-C18 5 μm stationary phase, was coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometry, and used

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for the analysis of drug mixture covering a wide range of polarities and the highly complex serum samples containing imipramine to investigate the usefulness of this HPLC–UHC-Chip/MS device. Our results showed good precision, linearity, high sensitivity and comparable selectivity to results obtained from regular HPLC/MS methods.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile was purchased from Merck Corporation (Merck, Germany), HPLC grade formic acid was supplied by TEDIA Corporation (Tedia, USA). Deionized water (18.2 MΩ) was prepared using an in-house water purification system (Millipore, USA). All the drug standards (atenolol, caffeine, N-acetylprocainamide, propranolol, methoxyverapamil, imipramine, theophylline, bumetanide, aspartame, cortisone, reserpine, Pfizer drug A and potential degradation products) were provided by Pfizer Global R & D (USA).

2.2. Sample preparation

Stock solutions (2 mg/mL) of each compound were prepared in ACN/water, 50:50 (v/v). To obtain different concentrations, compounds were diluted in the same mobile phase as used for sample loading.

For the determination of imipramine in serum samples, five Kunming mice weighing 30–40 g each were orally administrated with imipramine (10 mg/kg dosage), serum samples were collected from mouse's eye after dosing 30 min, 60 min and 120 min, respectively. All serum samples were centrifuged at 4000 rpm for 5 min, 100 μL supernatant was mixed with 0.5 mL hexane and centrifuged at 4000 rpm for 5 min, and then the supernatant was evaporated. The dried samples were redissolved in 100 μL loading mobile phase, and used for HPLC-Chip/Q-TOF MS analysis (20 μL) and regular HPLC/Q-TOF MS analysis (80 μL). All the samples including the standards were centrifuged at 5000 rpm for 10 min before HPLC-Chip analysis.

2.3. HPLC-Chip/Q-TOF MS analysis

The Agilent 1200 Series Nanoflow LC system comprised a microwellplate autosampler with sample thermostat, microdegasser, capillary pump and nanoflow pump. The Agilent HPLC-Chip/MS interface provided all connections between the HPLC-Chip, capillary pump, autosampler, nanoflow pump and Agilent 6510 quadrupole time-of-flight mass spectrometer (Agilent, USA). The HPLC-Chip, including the enrichment column, separation channel and nano-electrospray emitter, is interposed between the rotor and stator with a 6-position valve so that in one position flow from the liquid autosampler is directed through an enrichment column to waste (Fig. 1a, connect enrichment column via rotor groove 5–4 to waste via rotor groove 1–6), when the rotor travels 60°, the flow from the nanopump enters the enrichment column and elutes the sample onto the analytical column (Fig. 1a, connect enrichment column via rotor groove 2–1 to separation column via rotor groove 4–3). Here all the chips were operated in back-flush mode (with sample loading at port 5 and waste line at port 6) for best peak shapes.

All three types of chips were provided by Agilent. The standard chip has a 40 nL enrichment channel and a 75 μm (i.d.) × 43 mm (L) separation channel. The Direct-Injection chip is equipped with a 14 nL loop with no packing material and a 75 μm (i.d.) × 43 mm (L) separation channel. The ultra-high capacity chip (UHC-Chip, Fig. 1b) was custom-made by Agilent, including a 500 nL enrichment column and a 75 μm (i.d.) × 150 mm (L) separation channel. All chips including the enrichment columns were packed with ZORBAX 80 Å SB-C18 5 μm stationary phase.

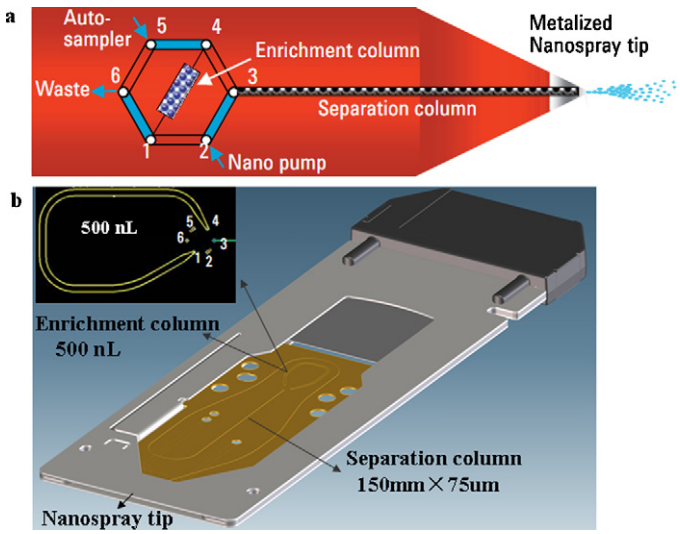

Fig. 1. Schematic illustration of HPLC-Chip (a) and artwork of ultra-high capacity small molecule chip (b).

Analyses were performed with an Agilent HPLC-Chip/Q-TOF MS system. The mobile phases A and B were 0.1% formic acid (FA) in water (A) and 0.1% FA in acetonitrile (B), respectively. The capillary pump (for sample loading) delivered 98% A isocratic mobile phase at 4 μL/min, after 1 μL sample injections, the enrichment column was washed for 1 min with the loading mobile phase. Following sample injection, the chip cube microvalve was switched automatically from enrichment mode to analysis mode with simultaneous start of the nanoflow pump gradient and MS data acquisition. For the separation of drug mixture (mix-1) including atenolol, caffeine, N-acetylprocainamide, propranolol, methoxyverapamil and imipramine, the nanoflow pump gradient (for sample analysis) consisted of 0–3 min, 2–30% B; 3–6 min, 30–38% B; 6–12 min, 38–78% B; 12–13 min, 78–95% B, held for 2 min to flush out thoroughly all potential column contaminants. Post run time was 9 min at 2% B and the flow rate was 0.3 μL/min. For the analysis of Pfizer drug A and potential impurities, the nanoflow pump gradient was as follows: 0–5 min, 12–20% B; 5–10 min, 20–30% B; 10–12 min, 30–80% B; 12–15 min, 80–95% B.

The mass spectrometer was operated in positive ionization mode, other settings were as follows: gas temperature, 300 °C; drying gas, 4 L/min; nebulizer, 0 psi; ref nebulizer, 0 psi; capillary voltage, 1800–1900 V; fragmentor, 230 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V. Centroid mass data within mass range 100–1000 m/z was acquired with 1 spectrum/s, and the data analysis was performed using Mass Hunter workstation (Agilent, USA).

2.4. HPLC/Q-TOF MS analysis

HPLC/Q-TOF MS analysis was performed with Agilent 1200 Rapid Resolution LC system (Agilent, USA). Zorbax SB-C18 column (1.8 μm, 3.0 × 100 mm, Agilent, USA) was used, the injection volume was 10 μL and the flow rate was 0.3 mL/min. The elution after column was directly introduced into Agilent 6510 quadrupole time-of-flight mass spectrometer equipped with dual electrospray ionization source without splitting the flow. The mobile phases A and B were 0.1% formic acid (FA) in water (A) and 0.1% FA in acetonitrile (B), respectively. The pump gradient was as follows: 0–3 min, 2–30% B; 3–6 min, 30–38% B; 6–12 min, 38–78% B; 12–13 min,

Table 1
Determination of intra-day ($n = 5$) and inter-day ($n = 3$) precision, chip-to-chip reproducibility ($n = 3$) and LOD with 6 pharmaceutical compounds.

Compound	log *P* value[a]	Precision (intra-day)		Precision (inter-day)		Chip-to-chip reproducibility		LOD (pg/μL)	
		RSD% (RT)	RSD% (PA)	RSD% (RT)	RSD% (PA)	RSD% (RT)	RSD% (PA)	Chip/MS[b]	HPLC/MS[c]
Caffeine	−0.23	0.08	4.74	0.06	3.25	0.06	6.23	0.3	5.0
Propranolol	3.03	0.29	3.64	0.11	2.95	0.14	8.51	0.5	2.0
Atenolol	0.57	0.52	4.83	0.13	6.57	0.29	12.27	0.5	2.0
N-acetylprocainamide	1.42	0.30	3.89	0.28	5.20	0.20	6.93	0.5	2.0
Imipramine	4.53	0.14	2.35	0.05	8.97	0.10	10.74	0.05	1.5
Methoxyverapamil	5.23	0.11	1.72	0.08	2.47	0.08	13.71	0.5	1.5

[a] From DrugBank, predicted using ALOGPS.
[b] Injection volume: 1 μL.
[c] Injection volume: 10 μL.

78–95% B. The mass spectrometer was operated with the following settings: ionization mode, positive; gas temperature, 350 °C; drying gas, 11 L/min; nebulizer, 45 psi; ref nebulizer, 5 psi; capillary voltage, 4000 V; fragmentor, 230 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V. Centroid mass data within mass range 100–1000 m/z was acquired with 1 spectra/s rate; and the data analysis was performed using Mass Hunter workstation (Agilent, USA).

3. Results and discussion

With tightened scrutiny on drug safety, advances in pharmaceutical analysis nowadays battle in two frontiers: specificity and sensitivity. Sub 2-μm columns and nano-spray mass spectrometry detection are the latest state-of-art practical analytical endeavors to push the limits of separation efficiency and detection, respectively. Effectively applying nano-LC/MS to routine pharmaceutical drug analysis is the focus of this evaluation.

Compared with the conventional nano-LC/MS, the HPLC-Chip/MS design eliminates all connections and micro-fittings, incorporates trapping, separation column and micro-valve connections all on a polyimide-based microfluidic chip. Due to the integrated design with reduced dead volumes and post-column peak dispersion, a five-times increase in sensitivity was obtained by using the HPLC-Chip/MS compared with conventional nano-LC/MS [20]. Moreover, the Chip Cube MS interface device first positions the chip between a stator and a rotor with the 6-position valve right in front of the MS inlet, then further connects external fluidic flow from capillary or nano pump to the chip, hence completely automates the microfluidic chip with highly versatile flow paths while providing robust operation and ease-of-use for users [15,16].

3.1. Evaluation of HPLC-Chip/MS with a commercial pharmaceutical drug mixture

To evaluate the HPLC-Chip/MS performance, a mixture of commercial pharmaceutical drugs (mix-1, consisting of 6 components including atenolol, caffeine, N-acetylprocainamide, propranolol, methoxyverapamil and imipramine) with wide range of polarity (as shown in Table 1) were first selected to investigate its general applicability to retain and separate diverse drug compounds.

The standard chip with a 40 nL enrichment column was first tested for the separation of mix-1 (Fig. 2a). While propranolol, imipramine and methoxyverapamil were well retained and detected, the three polar components were all missing. The problem was likely related to the poor sample enrichment of this chip. During chip operation, adequate volume of mobile phase (1–4 μL), usually an isocratic elution at very low % B, had to be pumped through the enrichment column to remove contaminants potentially interfering with MS analysis after sample loading. Since the retention capacity of a compound on the C18 enrichment column depends on the hydrophilicity of the molecule, hydrophobic compounds are better trapped, whereas very polar compounds with a weaker retention are washed off. This is confirmed by an experiment with a Direct-Inject chip. Without any stationary phase in the 14 nL injection loop, all compounds of mix-1 were well retained and separated with relatively weak responses (Fig. 2b).

However, even for clean samples without strong matrix interference, enrichment is often highly desired to attain ultimate sensitivity in many analyses. This is particularly true for the HPLC-Chip, although merely 14 nL is needed, a minimum of 0.2 μL sample has to be injected to fill the loop in order to minimize sample dilution. Rather than wasting majority of the sample, the enrichment would allow further to push down the detection limit. For this reason, the UHC-Chip was specially designed with a 500-nL enrichment column. To better match the enrichment, the analytical column length had to be extended to 150 mm to maintain decent separation efficiency. Fig. 2c showed the enrichment and analysis result of the separation. All 6 drug compounds were well trapped, separated, and detected with high responses.

In order to assess the intra-day and inter-day precision of this system, the mix-1 (100 pg/μL each compound) was consecutively injected five times in the same day and in three consecutive days. Table 1 shows the relative standard deviation (RSD, %) of retention time (RT) and peak area (PA) of each compound. Even without any internal standards, low RSD values of 0.05–0.52% for RT and 1.72–8.97% for PA were obtained for all compounds in the intra-day and inter-day precision test. Moreover, the chip-to-chip reproducibility was evaluated with three different UHC-Chips, acceptable results were also obtained with 0.06–0.29% RT RSD values and 6.23–13.71% PA RSD values for above compounds (Table 1). With the same mix-1, the lifetime of the UHC-Chip was determined and more than 400 injections were obtained from all three UHC-Chips.

The sensitivity of UHC-Chip/Q-TOF MS system was investigated by determining limits of detection (LOD) of above 6 compounds. The LOD of each compound under the present chromatographic conditions was determined at signal-to-noise ratios (S/N) of 3. As shown in Table 1, the LOD for all 6 drug compounds were all below 0.5 pg/μL. A comparative study was also carried out to assess the sensitivity enhancement against conventional LC/MS under the optimum chromatographic conditions. Here one would easily appreciate the ease of use of the chip cube MS interface for nano-LC/MS. Switching between Chip Cube MS interface and a regular ESI source took only a few minutes, allowing the use of the same Q-TOF MS instrument for direct comparison of both methods. The detailed condition for HPLC/Q-TOF MS analysis was shown in Sections 2.3 and 2.4. As shown in Table 1, 4–30 times improvement in sensitivity was observed with the HPLC-Chip/MS approach.

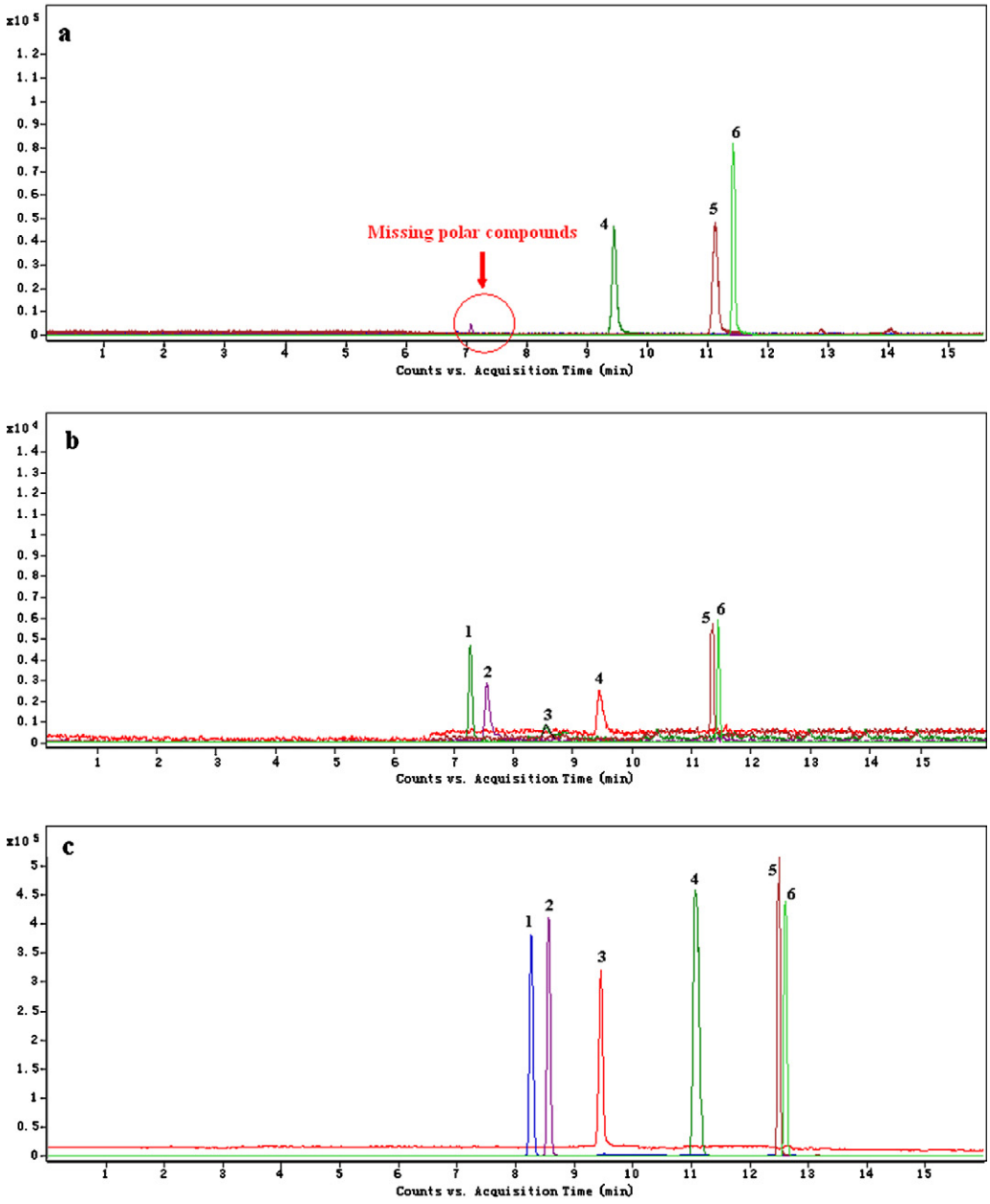

Fig. 2. Separation of mix-1 with (a) standard chip; (b) Direct-Inject chip; (c) UHC-Chip. 1, Atenolol, 100 pg/μL; 2, N-acetylprocainamide, 100 pg/μL; 3, caffeine, 100 pg/μL; 4, propranolol, 100 pg/μL; 5, imipramine, 50 pg/μL; 6, methoxyverapamil, 100 pg/μL.

With the observed good reproducibility, sensitivity, separation efficiency, combined with the ease of use, the microfluidic chip/MS method proved to be superior to the conventional LC/MS for accurate and sensitive quantitation of the small drug molecules, which laid the foundation for the subsequent trace analysis of impurity and pharmacokinetic metabolites.

3.2. Purity analysis of new pharmaceutical drug

Purity analysis of pharmaceutical drug substances is essential for the quality control and safety assessment in the drug development, highly sensitive methods are needed.

An HPLC-Chip/MS method for the analysis of a Pfizer proprietary new drug A and its potential impurities was developed. With the buffer of ammonium acetate (10 mM, pH 6.5) and acetic acid (0.1%, pH 3.2), the drug A and potential impurities cannot be separated (data not shown). When adding 0.1% formic acid (pH 2.1), drug A and 4 potential impurities (Fig. 3) were separated well under the chromatographic conditions described in Section 2.3.

The relative standard deviation (% RSD) values for retention time and peak area for each compound with 5 repeated injections were calculated, the LOD of each compound under the present chromatographic condition was determined at S/N of 3 (Table 2). The experimental results showed RSD values of 0.05–0.44% for RT and 0.92–6.77% for PA, respectively, and the LODs were at pg level for all compounds. In order to evaluate the linear dynamic range of HPLC–UHC-Chip/MS, four compounds with a wider concentration range (fourteen concentrations levels 1, 10, 20, 30, 50, 100, 200, 500, 800, 1000, 1500, 2000, 2500, 3000 pg/μL) were analyzed, finally nine concentrations levels were applied for evaluating their linear calibration, the correlation coefficient and linear dynamic ranges of these compounds were shown in Table 2. HPLC–UHC-Chip/MS approach was capable to detect compounds with greater than three orders of dynamic range in complex systems.

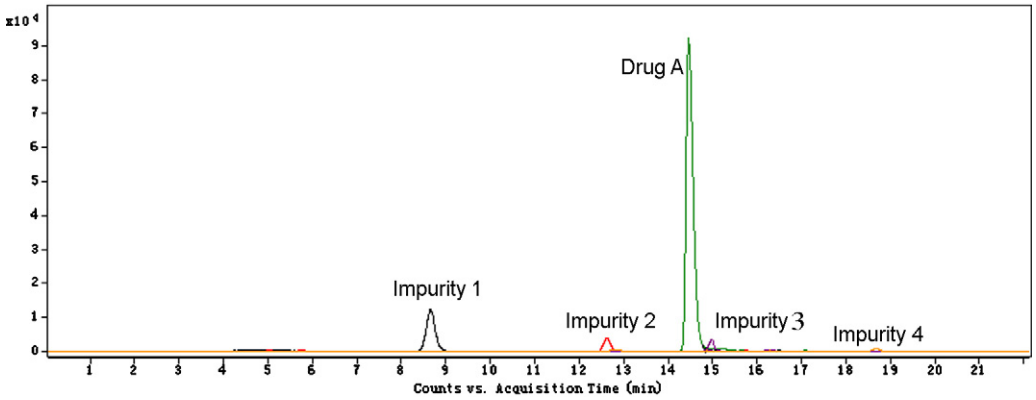

Fig. 3. Separation of Pfizer Drug A (1 ng/μL) and potential impurities. Impurity 1, 10 pg/μL; impurity 2, 5 pg/μL; impurity 3, 10 pg/μL; impurity 4, 10 pg/μL.

Table 2
Intra-day precision ($n = 5$), LOD, and linear range determination with Drug A and potential impurities.

Compound	RSD% (PA)	RSD% (RT)	LOD (pg/μL)	r^2	Linear range (pg/μL)[a]
Impurity 1	0.92	0.44	0.5	0.9942	1–1000
Impurity 2	6.77	0.11	0.5	0.9937	10–1000
Drug A	3.10	0.17	5	0.9906	10–1000
Impurity 3	3.62	0.16	0.5	0.9923	10–2500
Impurity 4	4.27	0.05	3	–	–

[a] Injection volume: 1 μL.

3.3. Quantitative analysis of imipramine in serum samples

The high sensitivity of nano-electrospray LC/MS in combination with the ability to automate sample enrichment from very small amount of biological samples (such as urine, blood, cerebrospinal fluid, saliva, etc.) has the potential to immensely reduce both cost and data variability in the *in vivo* drug metabolism and pharmacokinetics (DMPK) laboratory. In order to evaluate the potential performance of HPLC–UHC-Chip/MS with real samples, a linearity curve of imipramine spiked in serum samples was examined, a good linear relationship was obtained (1–20 pg/uL) with a regression coefficient better than 0.99. A simple animal model oral administrated with imipramine was set up, serum samples were collected after dosing at 30 min, 60 min and 120 min and were processed according to DMPK workflows (see Section 2.2), imipramine was quantitatively analyzed by HPLC–UHC-Chip/MS. The concentrations of imipramine in serum samples after dosing 30 min, 60 min and 120 min were 5.74 ± 0.60 pg/μL, 4.38 ± 0.34 pg/μL, and 3.71 ± 0.15 pg/μL, respectively. Compared with the results using routine HPLC/MS method (5.17 pg/μL, 4.21 pg/μL and 3.25 pg/μL), relative differences were 3.9–12.4%, which indicated the suitability of using chip-HPLC/MS system for quantitative analysis of imipramine in serum samples. As a new concept of nano-electrospray LC/MS, HPLC–UHC-Chip/MS provided high sensitivity and unequivocal capability to handle smallest sample volumes, and opened a new era for DMPK laboratories. Reduced sample volumes in animal or pediatric studies commensurate with ethical arguments could yield substantial cost-savings and higher quality results.

4. Conclusions

In this study, a new UHC-Chip, including a 500 nL enrichment column and a 75 μm (i.d.) × 150 mm (L) separation column that were both packed with ZORBAX 80 Å SB-C18 5 μm stationary phase, was custom-made and used here for pharmaceutical analysis, the HPLC–UHC-Chip/Q-TOF MS system was evaluated for qualitative and quantitative analysis of different pharmaceutical drugs covering a wide range of hydrophilicity ($\log P$ −0.23 to 5.23). Compared with the standard chip, UHC-Chip shows higher enrichment capability, comparable chromatographic precision and separation performances. Good linearity and low LOD were also obtained by using the HPLC–UHC-Chip/Q-TOF MS. When compared with conventional LC/MS, 4–30 times increases in sensitivity were obtained for all compounds. It was clearly demonstrated that the HPLC–UHC-Chip/Q-TOF MS system could be a valuable tool for the quantitative analysis of active ingredients and impurities with a wide range of polarities and abundance levels in pharmaceuticals, satisfactory results of serum samples also proved its practicality and potential for use in drug testing and development.

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