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Short communication

Development of a LC–ESI-MS^3 method for determination of nitrendipine in human plasma

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ABSTRACT

A novel and sensitive method utilizing high performance liquid chromatography coupled with electrospray ionization source tandem mass spectrometry (LC–ESI-MS^3) was developed for the first time in order to analyze nitrendipine in human plasma samples. Human plasma samples were prepared by protein precipitation with acetonitrile and well resolved on a 100 mm reversed-phase column in gradient elution with 0.05% (*v*/*v*) formic acid in acetonitrile as the mobile phase. Determination was performed in MS^3 scan mode for nitrendipine and in the multiple reaction monitoring (MRM) mode for nimodipine (internal standard). This method, having a lower limit of quantification (LLOQ) of 0.05 ng/mL when using a 100 μL sample aliquot (5 pg/sample), is acceptable for calibration of the linearity and repeatability and is of better sensitivity than the reported methods (>0.25 ng/mL). The major advantages of the method are that small sample volume (100 μL) is required, simple sample processing technique, high sensitivity and excellent selectivity is guaranteed by the MS^3 detection. The proposed validated method has been successfully applied to a clinical study on nitrendipine.

1. Introduction

Nitrendipine (NTD), a calcium channel blocker with marked vasodilator action, is a commonly antihypertensive agent. The concentrations of NTD is relatively low as a result of extensive first-pass metabolism and poor bioavailability (10–20%) [1,2]. In the past, many analytical techniques have been reported [3–8] for the quantitation of NTD in human plasma. However, the LC-UV method [3,4] failed to give adequate sensitivity and the use of the GC-ECD [5] method may result in thermal decomposition of NTD. LC–MS [6] or LC–MS/MS [7,8] method might be the best choice considering its sensitivity, specificity and stability. To the best of our knowledge, very few methods are reported in the literature which can detect NTD up to 0.25 ng/mL (LLOQ) with acceptable accuracy and precision with LC–MS or LC–MS/MS [6–8].

However, sample preparations methodologies [6–8] are time consuming, such as liquid–liquid extraction (LLE) and solid phase extraction (SPE) with large volumes (500–1000 μL) of human plasma. And the procedures are usually cumbersome and have many pitfalls. Multiple extraction steps are commonly needed to obtain cleaner extracts but to decrease analyte recovery. Thus, it is necessary to use a smaller amount of plasma and a simpler sample preparation method to high-throughput measurement such as pharmacokinetic study, which requires a higher sensitivity and selectivity.

In the following work, we propose another alternative to improve selectivity and sensitivity in quantitative applications, using MS^3 on a hybrid quadrupole-linear ion trap (QqLIT) [9–11]. As far as we know, we may be the first to use the LC–ESI-MS^3 for the application of quantify NTD in human plasma. In present method small volumes (100 μL) of plasma sample was cleaned by a simple sample preparation of protein precipitation and a dynamic (0.05–50.0 ng/mL) linearity range that can cover the plasma concentrations following a single oral dose of NTD. The novel method had been validated by evaluating the precision, accuracy and other validation parameters for human plasma samples. A corresponding pharmacokinetic study has been conducted with the developed method.

2. Experimental

2.1. Chemicals and reagents

NTD and nimodipine (Internal Standard IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (purity > 99%, Beijing, China). Acetonitrile, acetic acid and formic acid were purchased from Dikma Technology (CA, USA). Water was obtained from a Milli-Q plot water

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purification system (MA, USA). Other chemicals used were of analytical grade. Drug free and drug-containing plasma were obtained from 24 volunteers, and stored below −80 °C until used for analysis.

2.2. Apparatus and software

LC was performed on an Agilent 1200 system equipped with an autosampler, a degasser and a quaternary pump (Agilent, USA). The system was coupled to a 3200 Q TRAP high performance hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) via a TurboIonspray ionization (ESI) interface for mass analysis and detection. Data acquisition was performed with Analyst 1.4.2 software (Applied Biosystems).

2.3. Chromatographic conditions

The analyte was separated on a 3 μm Sepax GB-C_{18} column (100 mm × 2.1 mm) at room temperature with an injection volume of 20 μL of pretreated sample. The mobile phase consisting of acetonitrile (A) and 0.05% (*v*/*v*) formic acid (B) was used at a flow rate of 300 μL min^{-1}. The following gradient was used for quantitation: 0–0.5 min, 70–95% A (linear); 0.5–4 min, 95% A; 4–4.1 min, 95–70% A (linear).

2.4. Mass spectrometric conditions

All measurements were carried out with mass spectrometer operated under positive ESI mode. MS^3 and multiple reaction monitoring (MRM) were used for data acquisition of NTD and IS, respectively. The operating conditions were as follows: curtain gas, 25 psi; nebulizing gas, 40 psi; auxiliary gas (nitrogen), 50 psi; ion spray voltage and temperature, 5500 V and 500 °C. In the MS^3 experiment, the MS^3 ion transition monitored was *m*/*z* 361.2 → 315.1 → 298.0, 283.0, 269.1 in the linear ion trap (LIT) using a fixed LIT fill time of 50 ms and excitation time of 40 ms with a mass window of 3 amu, using a declustering potential (DP) of 23 eV, collision energy (CE) of 16 eV. The time required to perform the MS^3 was 476.5 ms. For the internal standard (IS) the MS/MS ion transition monitored was *m*/*z* 419.4 → 343.2 using a DP of 25 eV, a CE of 15, and a dwell time of 20 ms. The final MS cycle time, including both MS^3 and MRM experiments, was 499.5 ms.

2.5. Calibration standards and quality control samples

An individual stock standard solution of NTD and IS containing 500 μg/mL and 400 μg/mL was prepared by dissolving pure compounds in acetonitrile. Intermediate and working solutions were prepared from corresponding stock solutions by diluting with acetonitrile. All standard solutions were stored at 4 °C in the dark.

Human plasma calibration standards of NTD (0.0500, 0.100, 0.250, 0.500, 2.50, 5.00, 10.0, 25.0 and 50.0 ng/mL) were prepared by spiking the working standard solutions into a pool of drug-free human plasma. Quality control (QC) samples at 0.100, 5.00 and 40.0 ng/mL were prepared in bulk by adding appropriate working standard solutions from independent stock solutions to drug-free human plasma. The bulk samples were aliquoted (100 μL) into polypropylene tubes and stored at −80 °C for further measurement.

2.6. Sample preparation

To prevent the photodegradation of NTD, the entire experiment, including standard and QC preparation, plasma collection, sample preparation and instrumental analysis, was performed under dim red light. 100 μL of collected plasma sample was vortex-mixed for 1 min with 20 μL IS working solution (800 ng/mL) and 200 μL acetonitrile in 1.5 mL polypropylene tube. The sample was centrifuged at 12,235 × *g* for 10 min. 100 μL of the supernatant was transferred to an autosampler vial and 20 μL was injected into the LC–MS/MS system.

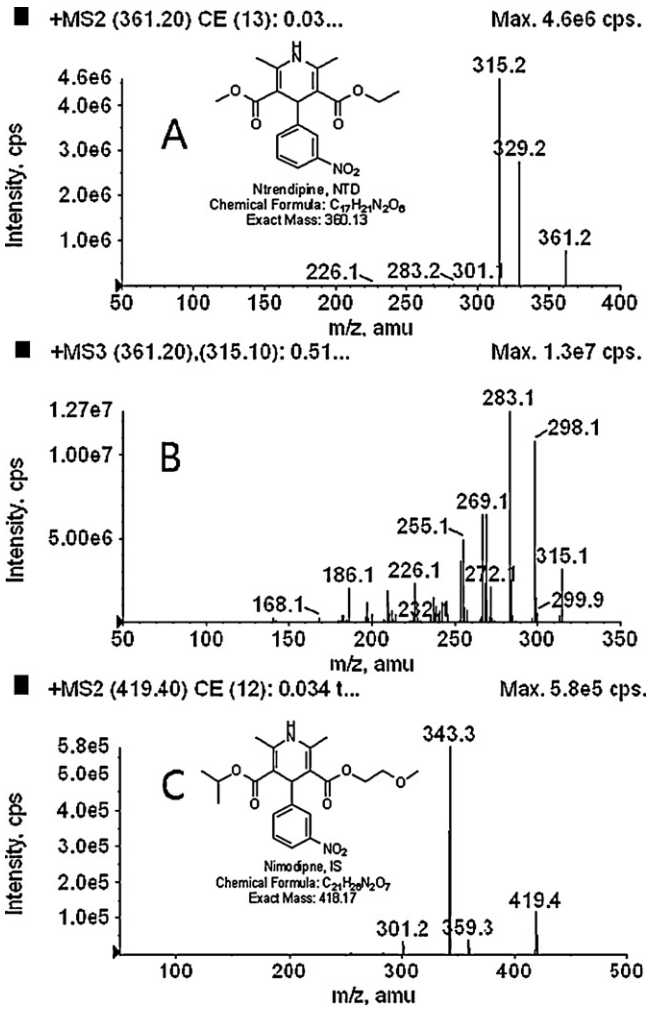

Fig. 1. MS^2 spectra of NTD (A), MS^3 spectra of NTD (B) and MS^2 spectra of IS (C).

2.7. Method validation

The method was validated in terms of linearity, specificity, LLOQ, extraction recovery, matrix effect, accuracy, precision and stability studies [12].

3. Results and discussion

3.1. Optimization of LC–MS^3 condition

3.1.1. Optimization of MS condition

A standard solution of NTD and IS was directly infused along with the mobile phase into the mass spectrometer in ESI source. The mass spectrometer was tuned in positive ionization mode for optimum response of NTD and IS. MS^2 spectra of NTD, MS^3 spectra of NTD and MS^2 spectra of IS are shown in Fig. 1.

In the positive precursor ion full-scan spectra, the most abundant ions were protonated ions $[M+H]^+$, *m*/*z* 361 and 419 for NTD and IS, respectively. Parameters such as gas flow, gas temperature, nebulizer pressure, fragmentor, capillary voltage and DP, were optimized for fine-tuning to obtain the best response. The product ion scan spectra showed high abundance fragment ions at *m*/*z* 315 for NTD and *m*/*z* 343 for IS which are illustrated in Fig. 1. The ion

spray voltage, temperature, collision gas, curtain gas, nebulizing gas and auxiliary gas, CE were optimized for maximum response of the fragmentation of *m/z* 315 for NTD and *m/z* 343 for IS. The ion transitions of *m/z* 361.2 → 315.1 and *m/z* 419.4 → 343.3 were chosen for NTD and IS, respectively. MS^3 spectrum showed an intense peak at *m/z* 298.0, 283.0, 269.1 in Fig. 1, and the MS^3 ion transition *m/z* 361.2 → 315.1 → 298.0, 283.0, 269.1 was monitored for the quantitation of NTD.

3.1.2. Optimization of LC condition

The chromatographic conditions were modified to obtain high sensitivity and sample throughput. The separation and ionization of NTD and IS were affected by the composition of mobile phase. The mobile phase system of acetonitrile–water was the same as used in the determination of NTD in human plasma in the previous study [8]. The chromatographic column, Diamonsil C_{18} (150 mm × 4.6 mm, 5 μm), Agilent Zorbax SB-C_{18} (150 mm × 4.6 mm, 5 μm) and Sepax GB-C_{18} (100 mm × 2.1 mm, 3 μm), were investigated in the chromatographic separation. The Sepax GB-C_{18} column was proved to be better than the others, which was tested to achieve shorter run time, better resolution and symmetric peak shapes for NTD and IS. Besides, gradient elution was performed on LC column to push the speed of analysis, which provided a better peak shape.

3.1.3. Mobile phase optimization

Direct injections of a composite solution of the NTD and IS at a concentration of 100 ng/mL were used to determine the optimum mobile phase modifier in water. Four concentrations of formic acid (0.01, 0.05, 0.1 and 0.2%) and four concentrations of ammonium acetate (0, 0.2, 1, 2 mM) were tested. The highest ionization efficiency for NTD and IS occurred when the concentration of formic acid was 0.05% (*v/v*).

3.1.4. Comparison of LC–MS^3 and LC-MRM

NTD was determined by LC–MS^3 and LC-MRM in blank samples and LLOQ samples (Fig. 2). As a result, the better response signal for NTD occurred with LC–MS^3 (*m/z* 361.2 → 315.1 → 298.0, 283.0, 269.1) with the *S/N* = 23 at LLOQ level while the NTD was not detected with LC-MRM (*m/z* 361.2 → 315.1) at LLOQ level.

3.2. Sample clean-up

According to the literature, LLE [3,6,7] and SPE [4,8] for sample clean-up have been used for determination of NTD in biologic samples. However, these methods required large volumes (500–1000 μL) of plasma. And the procedures became more complex. In present method, due to the highly sensitive LC–MS^3 method, small volumes (100 μL) of plasma sample was cleaned by protein precipitation, which fit the request of assay.

3.3. Method validation

3.3.1. Specificity and selectivity

Specificity and selectivity was evaluated by comparing chromatograms of six randomly selected blank plasmas with the spiked plasma to make sure there were no significant interfering peaks at retention time at LLOQ of the analytes. The representative chromatograms are shown in Fig. 3, which indicated that no endogenous interferences were observed at the retention time for NTD and the IS. Typical retention times were 1.93 and 2.13 min for NTD and IS, respectively.

Table 1
Precision and accuracy results for NTD in human plasma (*n* = 3 days, 6 replicates per day).

Concentration(ng/mL)	Intra-day RSD(%)	Inter-day RSD(%)	RE(%)
0.0500	1.0	10.4	0.71
0.100	14.4	5.9	0.22
5.00	9.3	7.3	4.4
40.0	11.3	3.9	−6.2

Table 2
Mean extraction recoveries and matrix effects of NTD and IS in human plasma (n = 5).

Spiked concentration (ng/mL)	Extraction recoveries (%)	RSD(%)	Matrix effect (%)	RSD(%)
0.050	95.3	6.3	96.4	9.5
0.100	93.4	5.7	93.6	4.2
5.00	99.5	8.3	100.9	7.4
40.0	94.3	4.8	97.1	6.3
Mean	95.6	2.5	97.0	2.7
160 (IS)	96.7	8.3	97.4	8.5

3.3.2. Linearity of calibration curves and lower limits of quantification

The linearity was determined in the range of 0.0500–50.0 ng/mL by plotting the peak area ratio (*y*) of NTD to IS versus the nominal concentration (*x*) of NTD in plasma. The average regression equation for the calibration curve (*n* = 5) was: $y = 61.06x + 0.3219$, $r = 0.9968$. The slopes of the regression equations were consistent for the calibration curves prepared on 5 separate days. The LLOQ was set at the level of the lowest calibrators for the analyte (0.05 ng/mL), and was evaluated as the other QC samples and individually spiked to at least 6 different lots of plasma, with precision and accuracy reported in Table 1 with RE% within ±20% and RSD lower than 20%.

3.3.3. Matrix effect and recovery

The absolute matrix effects for NTD and IS were assessed at four concentrations, i.e., 0.050, 0.100, 5.00, and 40.0 ng/mL as described in the literature [13]. The absolute matrix effect for NTD and IS was assessed by comparing the mean peak areas of the analyte spiked at three concentrations into the extracts originated from five different blank human plasma samples to the mean peak areas for neat solutions of the analytes in 70% acetonitrile. The extraction recovery of NTD was evaluated by comparing mean peak areas of NTD spiked pre-extraction into the same five different sources with those of NTD spiked post-extraction into different blank plasma lots at three concentrations. Summary of matrix effect and recovery data are presented in Table 2. It is demonstrated that the matrix effect and recovery were not independent of the concentration.

3.3.4. Precision and accuracy

Validation runs were conducted on three consecutive days. In each run, QC samples were evaluated in sets of six replicates to evaluate the intra- and inter-day precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the RSD served as the measure of accuracy and precision, respectively. Summary of Precision and accuracy data are presented in Table 1. These above values were all within the acceptable range.

3.3.5. Stability

QC samples (0.100, 5.00 and 40.0 ng/mL) of six replicates were subjected to the conditions below. Freeze–thaw stability was investigated after three freeze–thaw cycles (−80 °C to room temperature), with accuracy of 94.4–105.8% and precision of

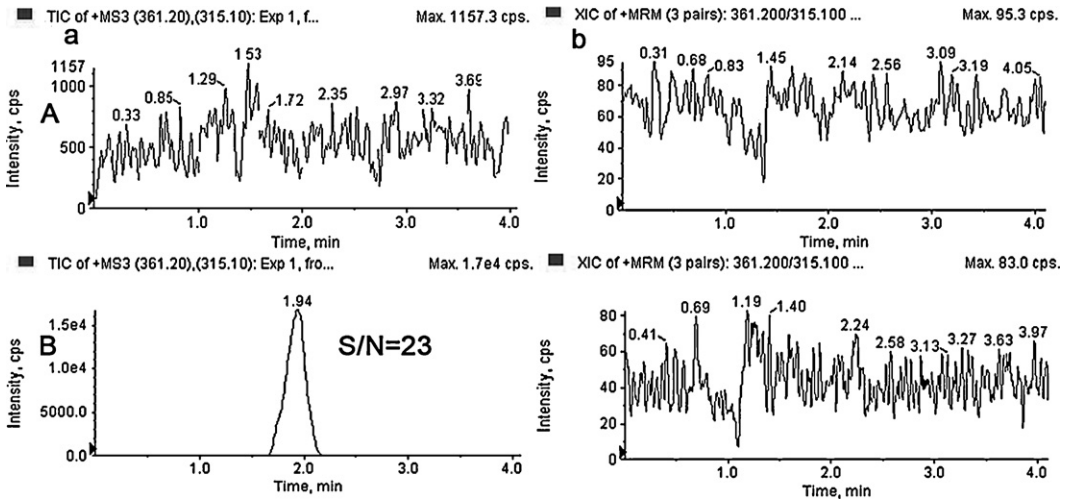

Fig. 2. LC–MS^3 (a) and LC-MRM (b) chromatograms of NTD (A – blank, B – LLOQ = 0.050 ng/mL).

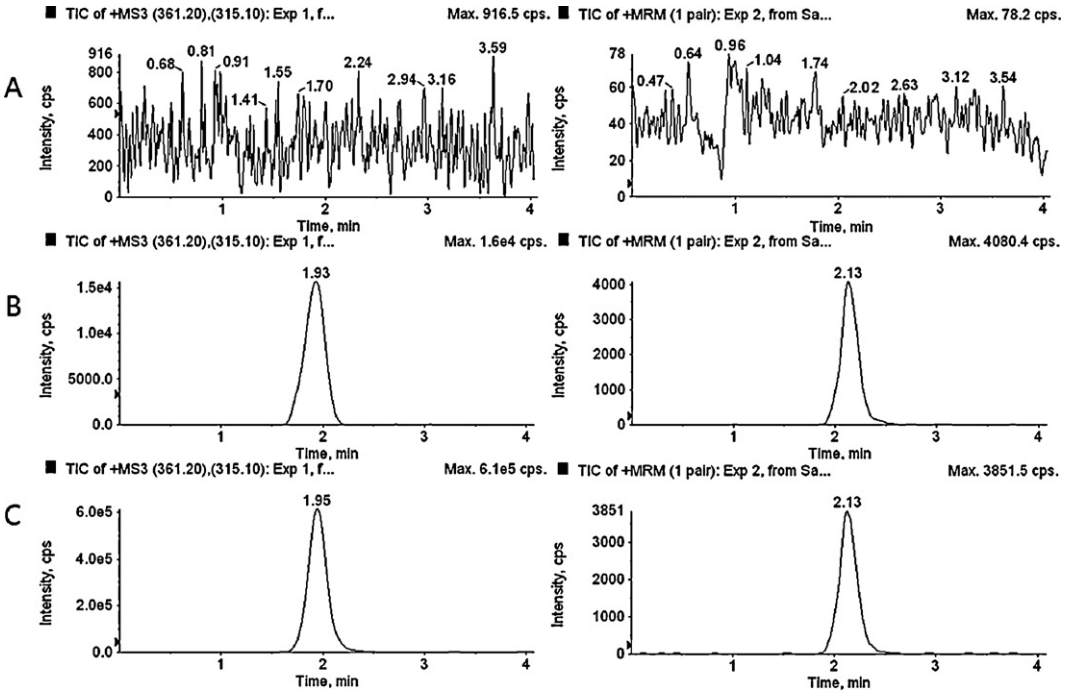

Fig. 3. Typical LC–MS^3 chromatograms of NTD (left) and LC-MRM chromatograms IS (IS, right) in human plasma samples (A) blank plasma sample; (B) plasma sample spiked with NTD (0.050 ng/mL) and IS (160.0 ng/mL); (C) a volunteer plasma sample 0.25 h with the concentration of NTD is 1.37 ng/mL after a single oral dose of 20 mg NTD tablets.

4.2–7.3%. Autosampler stability was assessed by analyzing processed QC samples kept in the autosampler at room temperature for 24 h to evaluate post-preparative stability, with accuracy of 95.2–103.3% and precision of 5.6–8.3%. Bench stability was assessed by analyzing QC plasma samples kept at room temperature for 8 h which exceeded the routine preparation time of samples, with accuracy of 97.5–106.4% and precision of 3.5–7.3%. Long-term stability was determined by assaying QC samples after storage at −80 °C for 21 days, with accuracy of 94.1–104.3% and precision of 3.9–6.7%. The standard solutions were investigated at three different levels (0.300, 6.00 and 120 ng/mL) after storage at 4 °C for 15 days, which were shown to be stable (RSD from 1.5 to 4.6%). The results were obtained by a comparison with freshly prepared solutions. NTD proved to be stable under all testing conditions. No significant degradation of NTD was observed under any of these conditions.

3.4. Application to a pharmacokinetic study

The pharmacokinetic study (No. YD2Y-090405) was approved by the Local Ethics Committee and carried out in the Second Hospital of Hebei Medical University (Shijiazhuang, China). All volunteers gave their signed informed consents to participate in the study according to the principles of the Declaration of Helsinki. Each participant received a single oral dose (20 mg) of test NTD tablets (10 mg each, Shijiazhuang, China). Blood samples were collected before and at 0.0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 11.0, 14.0, and 24.0 h post-dosing. Plasma was separated by centrifugation and stored at −80 °C. This validated LC–ESI-MS^3 method was applied to a pharmacokinetic study of NTD in healthy volunteers following a single oral administration.

Using DAS software (version 2.0, Chinese) analysis, the mean plasma concentration–time profile of NTD is illustrated in Fig. 4 and

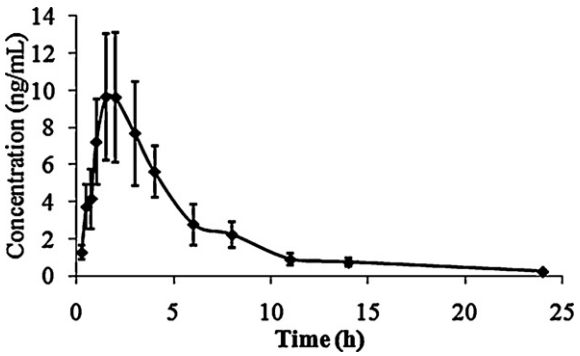

Fig. 4. Mean plasma levels ($n = 24$) of NTD after single dose administration of 20 mg NTD formulation to healthy volunteers.

Table 3
Pharmacokinetic parameters of NTD in Chinese volunteers (mean ± SD, n=24).

Parameters	Test	Literature [6]
$t_{1/2\beta}$ (h)	6.54 ± 2.12	4.74 ± 1.11
C_{max} (ng/mL)	9.62 ± 4.3	8.64 ± 3.86
T_{max} (h)	1.5 ± 0.9	3.0 ± 1.4
AUC_{0-t} (ng h/mL)	52.9 ± 25.2	59.29 ± 27.24
$AUC_{0-\infty}$ (ng h/mL)	55.1 ± 22.3	62.51 ± 29.02
$MRT_{0-\infty}$ (h)	6.51 ± 1.87	7.89 ± 1.71

the mean analysis pharmacokinetic parameters are listed in Table 3. These parameters were similar with those reported previously [6] (Table 3).

4. Conclusion

A novel sensitive LC–ESI-MS^3 method for quantification of NTD in human plasma was developed and validated for the first time. This method demonstrated high sensitivity (LLOQ: 0.05 ng/mL) with small volumes (100 μL) of plasma sample. This method is superior when compared to previously reported methods in sensitivity and speed. And the additional selectivity of the MS^3 scan mode is potent application of LC–MS in the clinical environment in future.

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