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Short communication

Simultaneous quantitative determination of paracetamol and its glucuronide conjugate in human plasma and urine by liquid chromatography coupled to electrospray tandem mass spectrometry: Application to a clinical pharmacokinetic study

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ABSTRACT

A specific, sensitive and rapid method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–MS/MS) was developed for the simultaneous determination of paracetamol (APAP) and its glucuronide conjugate (PG) in human plasma and urine. Plasma samples were precipitated with the mixture of acetonitrile and propylene glycol (90:10, v/v) solution and urine samples were diluted with the mobile phase, which were used to isolate the analytes from biological matrices followed by injection of the extracts onto a C_{18} column with isocratic elution. Detection was carried out on a triple quadrupole tandem mass spectrometer in multiple reaction monitoring (MRM) mode using positive electrospray ionization (ESI^+). The method was validated over the concentration range of 10–30,000 ng/mL and 100–6000 ng/mL for APAP in human plasma and urine as well as 10–15,000 ng/mL and 200–60,000 ng/mL for PG in human plasma and urine, respectively. Inter- and intra-run precisions of APAP and PG were less than 15% and the accuracy was within 85–115% for both plasma and urine. The average extraction recoveries were 93.1% and 89.1% for APAP, and 93.7% and 92.3% for PG in human plasma and urine, respectively. The linearity, recovery and stability were validated for APAP and PG in human plasma and urine. The method proved to be simple, robust and time efficient.

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1. Introduction

Paracetamol or acetaminophen (N-acetyl-p-aminophenol [APAP]) is a widely used analgesic worldwide. Study of paracetamol metabolism is important in the toxicological and pharmacokinetic studies of the drug. Paracetamol glucuronide (PG) is a major metabolite of paracetamol that can be detected in urine. It has been well documented that glucuronidation of APAP is the major metabolism pathway in most mammalian species [1]. Therefore, we considered the paracetamol as the substrate of the phase II metabolic enzymes (uridine diphosphate glucuronyl transferase, UGT).

In order to appraise the pharmacokinetic characteristics of the substrate (APAP) and its major metabolite (PG), establishment of a reliable method of analyzing drug conjugate in the plasma and urine is necessary but difficult to apply. Generally speaking, the method of measuring aglycone is utilized for analysis of conjugates, which is obtained by enzymatic or chemical hydrolysis. In many cases, however, hydrolysis does not proceed quantitatively due to different locations or types of conjugation [2]. In order to solve such problems, the use of LC–MS or MS–MS for the analysis of drug conjugates has been receiving more and more attention.

Paracetamol in biological fluids can be analyzed by a variety of electroanalytical, chromatographic and capillary electrophoretic methods including colorimetry, spectrophotometry [3], thin-layer chromatography (TLC) [4], gas chromatography (GC) and high-performance liquid chromatography (HPLC) [5–8] and several types of immunoassays [9]. For the simultaneous determination of paracetamol and its metabolites, mainly HPLC methods have been described [10]. GC–MS methods using derivatization have also been reported, for instance by using trifluoroacetic anhydride as the derivatization agent and electron-capture negative-ion chemical ionization (ECNICI) [11] or extractive methylation [12], or electron-capture pentafluorobenzyl bromide [13,14].

However, most of these assays are complex and include the use of additives like ion pairing agents or require time-consuming sample preparation. In recent years, although several LC–MS and LC–MS/MS methods have been reported for the analysis of

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paracetamol in plasma or its major metabolites in urine, either alone or in combination with other drugs in biological samples [15–21]. To the best of our knowledge, however, there is no other LC–MS/MS method for the simultaneous determination of APAP and PG with validation data in human plasma and urine up to date. In this work, a rapid and sensitive LC–MS/MS method for the determination of APAP and PG in human plasma and urine was developed and validated. Following validation, this method was successfully applied to the pharmacokinetics studies of APAP and PG in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and reagents

Paracetamol, the reference standard (purity >99.7%), and the internal standard, theophylline (purity >99.9%), was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). P-acetamidophenyl β-D-glucuronide sodium salt was purchased from Sigma Aldrich. Paracetamol 500 mg tablets were provided by Gunagzhou Ouhua Pharmaceutical Holdings Co., Ltd. (Guangzhou, China). HPLC-grade acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Formic acid and ammonium acetate, also of HPLC grade, were obtained from Tedia Company, Inc. (Fairfield, Ohio). Distilled water was deionized by using a Milli-Q Gradient system A10 (Millipore, Bedford, MA, USA) and was used throughout the study. Other chemicals were all of analytical grade.

2.2. Instrumentation

The LC–MS/MS system (Waters Corporation, Milford, Massachusetts) consisting of an Acquity High Performance LC and electrospray ionization tandem mass spectrometer (LC–ESI-MS/MS; Quattro Premier XE, Waters Corporation). Chromatographic analysis of APAP, PG and theophylline (I.S.) were performed on a C_{18} column (100 mm × 2.1 mm 3.0 μm, Welch Materials, USA). All data were acquired employing MassLynx V4.1 Quantitative Analysis version analyst data processing software.

2.3. LC–ESI-MS/MS conditions

The chromatographic separation was achieved on an Ultimate C_{18} column (100 mm × 2.1 mm 3.0 μm, Welch Materials, USA). The mobile phase composition was a mixture of acetonitrile–methanol–water containing (0.0875% formic acid) in a ratio 4:4:92 (v/v/v). Measurements were made at a flow rate 0.3 mL/min at 40 °C column temperature. Mass spectrometer was operated in the positive mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 151.6 → 109.4 for APAP, m/z 349.9 → 173.8 for PG and m/z 180.9 → 123.4 for theophylline (I.S.), respectively, with a scan time of 0.05 s per transition. The tuning parameters were optimized for APAP, PG and I.S. The optimal MS parameters obtained were as follows: capillary voltage was 1.0 kV, the ionization sources and the desolvation temperature were 120 °C and 400 °C, respectively; desolvation gas flow rate was 750 L/h; the optimized collision cone voltage for APAP, PG and I.S. were 28, 32 and 35 eV, and the optimized collision energies chosen for APAP, PG and I.S. were 15, 16 and 18 eV respectively. Fig. 1 shows the product ion mass spectra of $[M+H]^+$ of APAP, I.S. and $[M+Na]^+$ of PG.

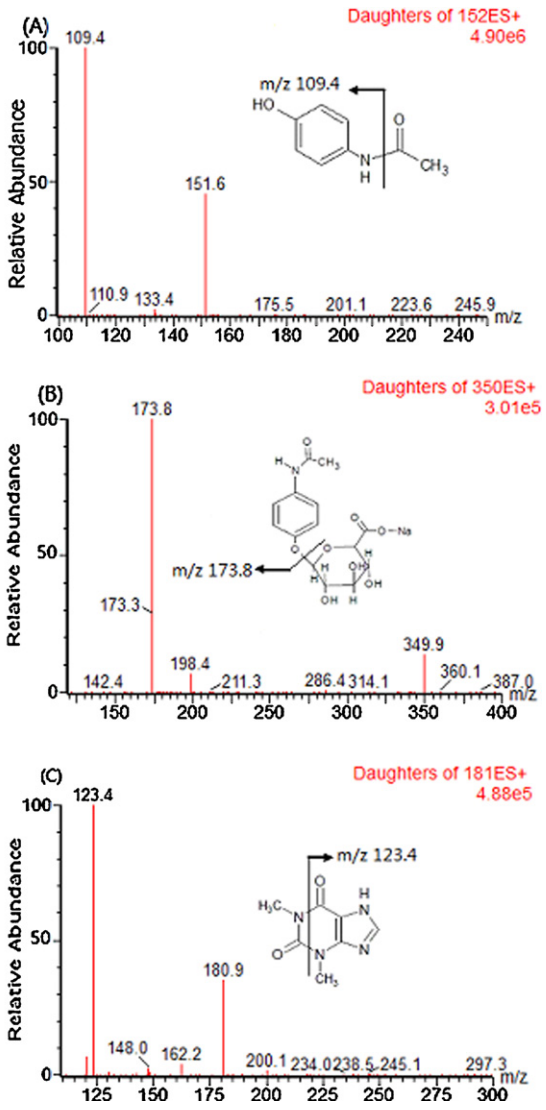

Fig. 1. Full scan product ion mass spectra of $[M+H]^+$ of APAP (A), I.S. (C) and $[M+Na]^+$ of PG (B).

2.4. Calibration standards (CS) and quality control (QC) samples in human plasma and urine

Stock solutions of APAP and I.S. for CS and QC were prepared separately in methanol: water (1:1, v/v), and stock solutions of PG were prepared in methanol stored at −25 °C. The concentrations of APAP and PG stock solutions were 1.0 mg/mL and 0.2 mg/mL, respectively. They were further diluted with methanol to yield working solutions at several concentration levels. CS and QC samples in plasma and urine were prepared by diluting corresponding working solutions with drug-free human plasma and urine, respectively. The final calibration curve range of APAP and PG were as follows: 10–30,000 ng/mL and 10–15,000 ng/mL in plasma; 100–6000 ng/mL and 200–60,000 ng/mL in urine, respectively.

The concentrations of QC sample of APAP and PG were as follows: 50, 1500 and 15,000 ng/mL and 50, 500 and 6000 ng/mL in plasma; 200, 1000 and 5000 ng/mL and 500, 5000 and 50,000 ng/mL in urine. The internal standard working solution was prepared using mobile phase. All of plasma and urine samples were stored at −75 °C.

2.5. Sample preparation

Frozen human plasma samples were thawed at ambient temperature. A 100 μL of the plasma sample and 50 μL of 5 μg/mL I.S. were added to 300 μL mixture of acetonitrile and propylene glycol (90:10, v/v) solution; frozen urine samples were thawed and 20 μL of 50 μg/mL I.S. was added to 20 μL of urine and then diluted 10-fold by adding 160 μL of the mobile phase. All the previously treated plasma and urine samples were then mixed by vortexing and centrifuged at 12,000 rpm at 4 °C for 5 min before injection. 100 μL of the upper layer was transferred to an injection bottle, which was loaded into autosampler cabinet and 3 μL aliquot was injected into the LC–MS/MS system.

2.6. Validation of the method

The selectivity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the APAP, PG and I.S. Calibration was performed by a least-squares linear regression of the peak area ratios of the drugs to the I.S. versus the respective standard concentration. The LLOQ was defined as the concentration of the lowest concentration standard in the calibration curve that was analyzed with accuracy within ±15% and a precision ≤15% [22]. In order to assess the intra- and inter-day precision and accuracy, complete analytical runs were performed on the same day and on three consecutive days. Each analytical run consisted of a matrix blank, a set of calibration standards and a set of low, medium and high concentration QC samples. Concentrations for the QC samples were calculated by reference to the calibration curve generated from the calibration standards.

The extraction recoveries of APAP and PG in the plasma and urine at three QC levels were determined by comparison of the peak areas of APAP and PG extracted from plasma and urine samples with that of dissolved in the mobile phase. Stability tests were performed for analyte-spiked plasma samples under various conditions: short-term storage (at ambient temperature), through freeze/thaw cycles, and for long-term storage (frozen for 90 days), autosampler storage (at 4 °C) by analyzing five replicates at low, medium and high QC concentrations. Matrix effects were evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's reconstituted solution (the final solution of the blank plasma after precipitation) with that of the analytes dissolved in the mobile phase. Three different concentration levels of the analytes were evaluated by analyzing five samples at each level Percent nominal concentrations, and estimated whether they were within the acceptable limits (85.0–115.0%) after evaluating six different lots of plasma.

2.7. Pharmacokinetic study

The method was applied to determine the plasma concentrations of APAP and PG from a clinical trial in which 12 healthy volunteers received an oral dosage 500 mg paracetamol tablet. The demographic data for these volunteers were mean age 23.7[1.8] years and mean weight 62.5[5.6] kg. Blood samples (4 mL each) were collected at 0, 10, 20, 30 and 45 min; then 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h after dosing. Urine samples were collected prior to the administration and 0–2, 2–4, 4–6, 6–8, 8–12, 12–24 h after administration. The blood and urine samples obtained were frozen at −75 °C until analysis. The SPSS version 13.0 (SPSS Inc., Chicago, Illinois) was used for calculation of the pharmacokinetic parameters.

The protocol of this study was approved by the Ethical Committee of Second Xiangya Hospital of Central South University (Changsha, China). All participants signed a written informed consent after they had been informed of the nature and details of the study.

3. Results and discussion

3.1. Mass spectrometry

An LC–MS–MS method for the determination of APAP, PG and I.S. in human and urine plasma was investigated. Firstly, the possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) source under positive ion detection mode was evaluated during the early stage of assay development. Results showed that ESI could offer higher sensitivity for the analytes than APCI. Attempts to perform negative ion MS, were unsuccessful because there was no response for the paracetamol. In positive ion MS, the protonated molecular ions of APAP, PG and the I.S. were produced by the protonation of the N-acetyl group at the N position producing secondary ammonium groups. Consequently, ESI was chosen as the source for further study. Parameters were optimized in order to obtain more abundant protonated molecules of the analytes. The ion source temperature was set at 120 °C which was to make sure the isocratic mobile phase thoroughly atomized and enhance the sensitivity as well. The quasi molecular ions with m/z 151.6, 349.9 and 180.9 represent APAP, PG and I.S., respectively. By introducing those $[M+H]^+$ ions into the second quadrupole (Q2) cell with the optimum collision energy, the MS/MS fragmentation was achieved. The major fragment ions observed in each product spectrum were at m/z 109.4, 173.8 and 123.4, respectively, after collision-induced dissociation. Additional tuning of the CID energy onto the transition of m/z 151.6 → 109.4 (APAP), m/z 349.9 → 173.8 (PG) and m/z 180.9 → 123.4 (I.S.) further improved the sensitivity. Therefore, they were selected for sensitive quantification of APAP, PG and I.S. Furthermore, as seen in Figs. 2 and 3, PG produced two MRM peaks at the same retention time as the main peak: one due to the monitored MRM transition (349.9 → 173.8 m/z ion) and another due to an additional transition of 151.6 → 109.4 m/z ion (because PG produce 349.9 and 151.6 m/z parent ion in the ion source). Because the method is set up to detect 151.6–109.4 m/z ion (as the primary transition for paracetamol), the additional transition of PG (which is the same as the primary transition of paracetamol) is also detected at their respective retention times (second trace of PG peaks in Figs. 2 and 3). This gave an additional degree of confirmation for PG (in addition to retention time and the primary MRM transition), increasing the specificity of the method for these two analytes.

3.2. Chromatography

Various mobile phase combinations of water and methanol or acetonitrile were investigated to optimize sensitivity, speed and peak shape. The results (data not shown) demonstrated that mixture of acetonitrile and methanol gave a better response than methanol or acetonitrile separately. Due to different concentrations of the ammonium acetate inhibit ionization of the analytes in varying degrees, we also have attempted different concentrations of the formic acid and found containing 0.0875% formic acid in water not only improved the efficiency of ionization and peak shapes but also promoted ionization of the analytes in the ESI^+ mode, at the same time, increase the sensitivity. Thus, HPLC separation was achieved in a total runtime of 6.5 min on an Ultimate C_{18} column with 0.0875% formic acid in water–acetonitrile/methanol isocratic.

3.3. The choice of processing method

After plasma samples were treated by solid-phase extraction and liquid–liquid extraction, PG was not found by monitoring.

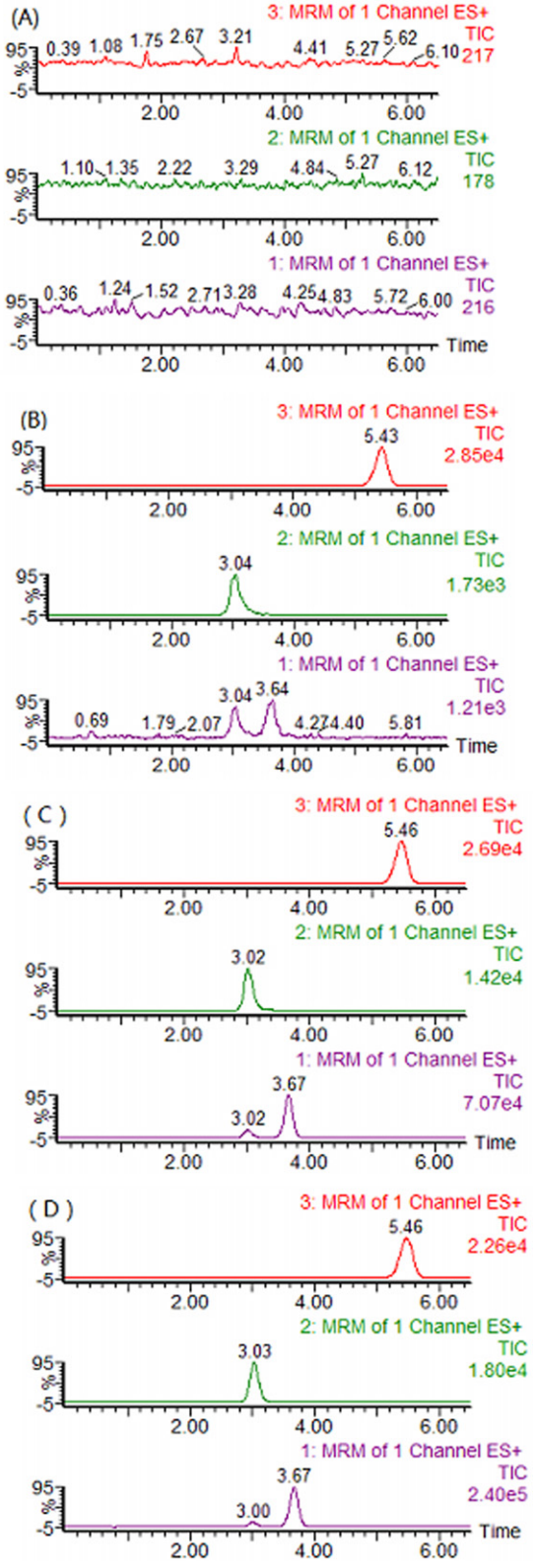

Fig. 2. Selective ion chromatograms (bottom-up order: channel 1: APAP m/z 151.6 → 109.4, collision energy 15 eV; channel 2: PG m/z 349.9 → 173.8, collision energy 16 eV; channel 3: I.S. m/z 180.9 → 123.4, collision energy 18 eV). (A) Representative chromatograms of extracted blank human plasma sample. (B) Chromatograms of the LLOQ plasma spiked with 10 ng/mL of APAP and 10 ng/mL of PG. (C) Chromatograms of the plasma sample spiked with 1500 ng/mL of APAP and 500 ng/mL of PG. (D) Representative chromatograms of human plasma after 1.0 h of administrated paracetamol tablet.

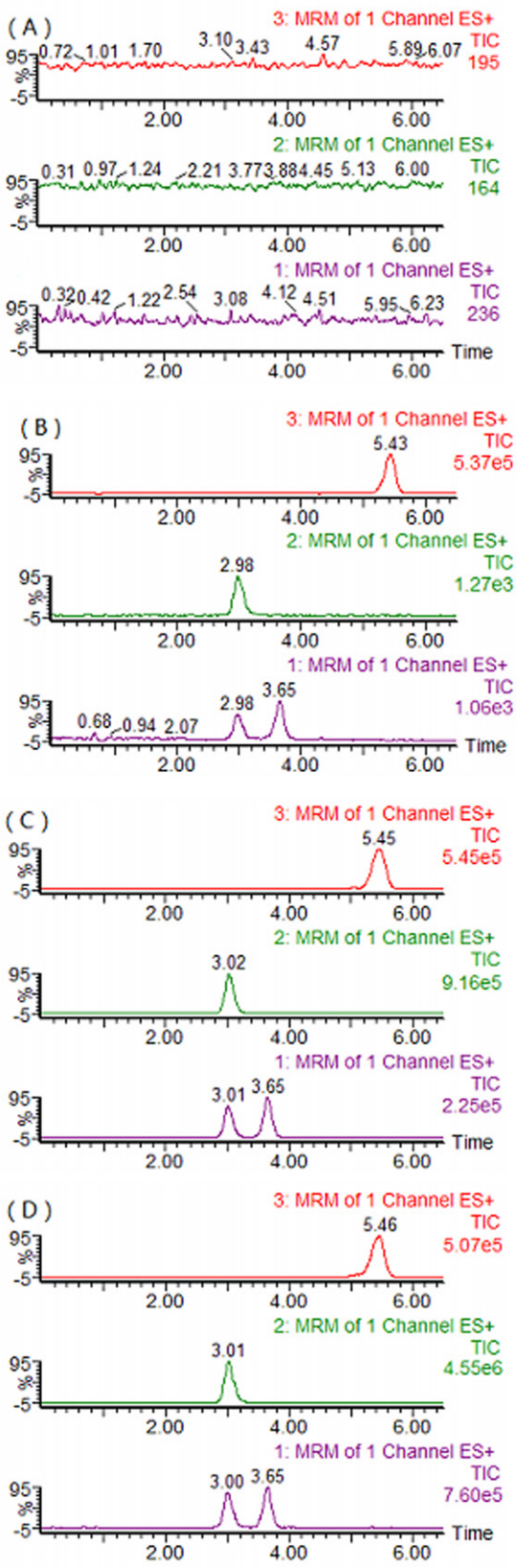

Fig. 3. Selective ion chromatograms (bottom-up order: channel 1: APAP, channel 2: PG, channel 3: I.S.). (A) Representative chromatograms of extracted blank human urine sample. (B) Chromatograms of the LLOQ urine spiked with 5 ng/mL of APAP and 5 ng/mL of PG. (C) Chromatograms of the urine sample spiked with 1000 ng/mL of APAP and 5000 ng/mL PG. (D) Representative chromatograms of human urine 4.0 h of administrated paracetamol tablet.

Table 1
Precision, accuracy and extraction recovery for APAP and PG in LLOQ and QC of plasma and urine samples.

Matrix	Components	Nominal concentration (ng/mL)	Inter-day (n = 5)			Intra-day (n = 15)			Extract recovery (%) (n = 5)
			Measured concentration (ng/mL)	RSD (%)	Bias (%)	Measured concentration (ng/mL)	RSD (%)	Bias (%)	
Human plasma	APAP	10	10.2 ± 0.6	5.4	2.0	ND	ND	ND	93.2
		50	49.8 ± 4.4	8.9	−0.4	50.4 ± 4.2	8.3	0.8	90.3
		1500	1536.3 ± 49.2	3.2	2.4	1489.6 ± 140.0	9.4	−0.7	93.5
		15,000	14,893.2 ± 1608.5	10.8	−0.7	15,023.5 ± 1592.5	10.6	0.2	95.6
	PG	10	9.7 ± 0.9	9.3	−3.0	ND	ND	ND	93.5
		50	48.7 ± 1.2	2.4	−2.6	48.6 ± 3.0	6.2	−2.8	91.4
		500	520.5 ± 39.0	7.5	4.1	498.7 ± 38.9	7.8	−0.3	92.1
		6000	6224.3 ± 130.7	2.1	3.7	6135.6 ± 687.2	11.2	2.3	97.7
Human urine	APAP	5	5.2 ± 0.6	11.5	4.6	ND	ND	ND	ND
		200	196.2 ± 10.2	5.2	−1.9	212.5 ± 8.9	4.2	6.3	85.4
		1000	1020.6 ± 12.2	1.2	2.1	946.8 ± 73.9	7.8	−5.3	93.2
		5000	5200.3 ± 530.4	10.2	4.0	4912.3 ± 63.9	1.3	−1.8	88.6
	PG	5	4.7 ± 0.4	8.5	−7.6	ND	ND	ND	ND
		500	489.6 ± 36.7	7.5	−2.1	486.7 ± 25.8	5.3	−2.7	88.1
		5000	4985.6 ± 613.2	12.3	−0.3	5289.8 ± 338.5	6.4	5.8	94.2
		50,000	48,899.2 ± 4694.3	9.6	−2.2	51,203.6 ± 3686.7	7.2	2.4	94.5

ND: not done.

The results showed the glucuronide conjugate of APAP was not remained in the solid-phase extraction column to separate and not extracted with organic solvents from plasma, which might be attributed to their high polarity and water-soluble features. Thus, we utilized a relatively simple method of protein precipitation to prepare the blood sample, but we found recovery of this method is too low, maybe solubility of the analytes in acetonitrile are poor, we tried different types and different proportions of cosolvent and found that 5% DMSO or 10% propylene glycol in acetonitrile can increase the recovery nicely. Considering the toxic effects of DMSO, we used acetonitrile containing 10% propylene glycol as protein precipitant with the extraction recovery rate reached more than 90%, whereas there was a little solvent consumed procedure, and the time of protein precipitation and centrifugation was also short. Therefore, the procedure exhibited excellent performance in terms of recovery and sample pre-treatment, which met the pharmacokinetic requirements of the experiment very well.

3.4. Method validation

The calibration curves had good linearity within the linear range, with their correlation coefficients of all calibration curves were more than 0.997.The accuracies, intra- and inter-day precisions for the two analytes in human plasma and urine samples are shown in Table 1. All the RSD were below 12.3% (n = 5). Meantime, all the RSD of the stabilities under various conditions for the two analytes in human plasma and urine samples were below 10.7% (n = 5) (data not shown). The LLOQs for APAP and PG were 10 and 10 ng/mL in human plasma and were 5 and 5 ng/mL in human urine, respectively. Traditionally, the lowest concentration of the standard calibration curve is used to define the LLOQ value because researchers usually want to quantify the analyte as low as possible. In our study, the lowest concentration of the analyte was just higher than its LLOQ in plasma, so the minimum plasma concentration was in agreement with the lower limit of quantification. In urine samples, however, the lowest concentration was much higher than the lower limit of quantification in methodology, so the minimum urine concentration was inconsistent with the lower limit of quantification. Mean extraction recoveries for APAP were ranged from 90.3% to 95.6% in human plasma and ranged from 85.4% to 93.2% in human urine, respectively. For PG, the recovery was ranged from 91.4% to 97.7% in human plasma and ranged from 88.1% to 94.5% in human urine (n = 5), respectively. All recoveries had RSD better than 11.5% throughout the entire standard concentration ranges, showing good consistency. The matrix effects were within the acceptable limits (86.3–102.3%) after evaluating six different lots of plasma. The same evaluation was performed on I.S. (105.3%) and no significant peak area differences were observed. Thus, the ion suppression/enhancement and low variability of each analyte was negligible and the quantifications of them were not affected by the matrix effect.

3.5. Application of the method to pharmacokinetic study in healthy volunteers

This validated analytical method was used to study the pharmacokinetic profiles of APAP and PG in human plasma and urine after an oral administration of a 500 mg paracetamol tablet. Profiles of The mean plasma concentration–time curve and urine cumulative amount–time curve of APAP and PG were shown in Figs. 4 and 5, respectively. The parameters were calculated by a non-compartmental model. The values of C_{max} and T_{max}

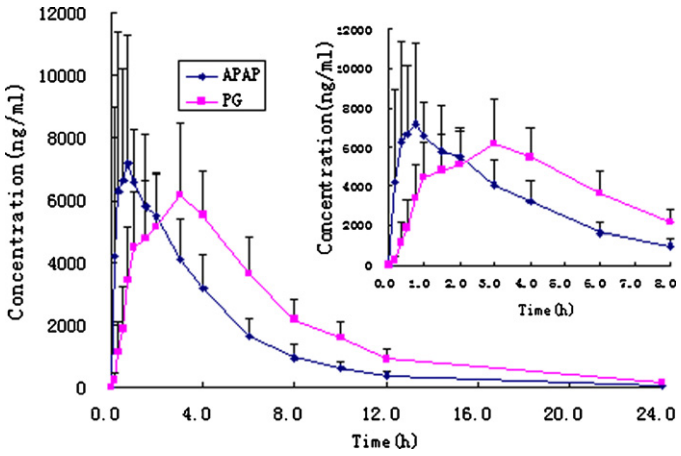

Fig. 4. The average concentration–time curves of APAP and PG in plasma from Chinese healthy subjects after oral administration of 500 mg paracetamol tablet (circles, n = 12), (mean ± SD).

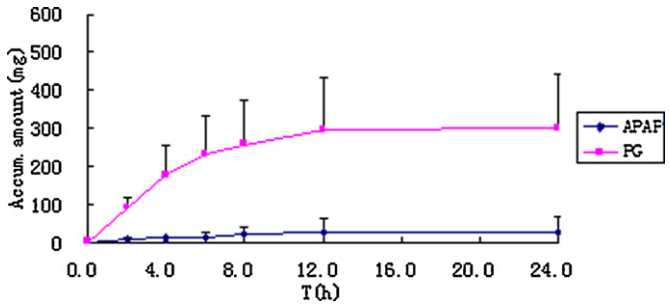

Fig. 5. The cumulative amount–time curves of APAP and PG in urine from Chinese healthy subjects after oral administration of 500 mg paracetamol tablet (circles, $n = 12$), (mean ± SD).

were obtained directly from experiment observations. The mean of C_{max} was 7162.5 ng/mL for APAP and 6174.6 ng/mL for PG, respectively; the median of T_{max} in 12 volunteers was found to be 0.75 h for APAP and 3.0 h for PG; the AUC_{0-t} and $AUC_{0-\infty}$ calculated by the linear trapezoidal method were found to be (30,444.7 ± 8697.8) (mean ± SD, $n = 12$, the same below) and (32,101.4 ± 9236.7) ng·h/mL for APAP, and (37,729.8 ± 9169.7) and (41,906.5 ± 9766.7) ng·h/mL for PG; the elimination half-life ($t_{1/2}$) was estimated as (2.98 ± 0.70) h for APAP and (3.17 ± 0.62) h for PG.

4. Conclusions

To the best of our knowledge, we have developed for the first time fully validated LC–MS/MS method with ESI interface was developed and validated for the simultaneously determination of APAP and PG in human plasma and urine. The method has significant advantage over other techniques used for measuring the two compounds paracetamol and paracetamol glucuronide in biological fluids, which provides the higher sensitivity using a simple protein precipitation procedure which did not involve reconstitution or drying step to achieve the desired sensitivity. Further we can still go lower by adding drying and reconstitution step to the current method. The major advantages of this method are the simple preparation, good specificity, the rapidity of separation, and the efficiency of analyzing two analytes simultaneously. This method was successfully applied to pharmacokinetic study for paracetamol and its glucuronide conjugate.

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