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Short communication

Simultaneous determination of paracetamol, pseudoephedrine, dextrophan and chlorpheniramine in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

For the first time, a highly sensitive and simple LC–MS/MS method after one-step precipitation was developed and validated for the simultaneous determination of paracetamol (PA), pseudoephedrine (PE), dextrophan (DT) and chlorpheniramine (CP) in human plasma using diphenhydramine as internal standard (IS). The analytes and IS were separated on a YMC-ODS-AQ C_{18} Column (100 mm × 2.0 mm, 3 μm) by a gradient program with mobile phase consisting of 0.3% (v/v) acetic acid and methanol at a flow rate of 0.30 mL/min. Detection was performed on a triple quadrupole tandem mass spectrometer via electrospray ionization in the positive ion mode. The method was validated and linear over the concentration range of 10–5000 ng/mL for PA, 2–1000 ng/mL for PE, 0.05–25 ng/mL for DT and 0.1–50 ng/mL for CP. The accuracies as determined from quality control samples were in range of −8.37% to 3.13% for all analytes. Intra-day and inter-day precision for all analytes were less than 11.54% and 14.35%, respectively. This validated method was successfully applied to a randomized, two-period cross-over bioequivalence study in 20 healthy Chinese volunteers receiving multicomponent formulations containing 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate.

1. Introduction

Paracetamol (N-acetyl-p-amino-phenol, PA) is a safe and effective analgesic and antipyretic agent although its anti-inflammatory is weak [1,2]. Pseudoephedrine ([*S*-(*R**,*R**)]-α-[1-(methylamino)ethyl]-benzenmethanol, PE) is a direct- and indirect-acting sympathomimetic agent. It is a stereoisomer of ephedrine and has similar action, but causes less pressor activity and central nervous system effect [3]. Dextrorphan (*O*-desmethyl dextromethorphan, DT), is a major metabolite of dextromethorphan ((+)-3-methoxy-17-methyl-(9α,13α,14α)-morphinan, DM), an over-the-counter antitussive which acts through depression of the medullary centers of the brain to decrease the involuntary urge to cough [4,5]. Chlorpheniramine (2-pyridinepropanamine, γ-(4-chlorophenyl)-*N*,*N*-dimethyl, (*Z*)-2-butenedioate, CP) is a potent antihistaminic that causes a moderate degree of sedation [6]. PA, PE, DM (or DT) and CP are commonly combined in one formulation for symptomatic treatment of coughs and the common cold.

A variety of methods have been employed for the detection of the individual component in plasma, including HPLC, GC–MS, LC–MS or LC–MS/MS [7–14]. However, these reported methods required time-consuming and laborious extraction procedures, relatively large sample volume, long chromatographic analysis time and also showed low sensitivity which were not adequate for pharmacokinetic and bioequivalence studies. Recently, a LC–MS/MS method was reported for the simultaneous determination of CP and PE in human plasma by liquid–liquid extraction with a lower limit of quantitation (LLOQ) of 0.2 and 2.0 ng/mL for CP and PE, respectively [15]. The method achieved sufficient sensitivity in short run time, but suffered from time-consuming extraction procedure and large sample volume requirement. Another method was also reported to determine PA and CP in human plasma simultaneously, but the extraction recovery of PA was only approximately 20% [16]. As the polarity and plasma concentration of PA, PE, DT and CP are significantly different, no previous method with a simultaneous determination of the four component had been described. Therefore, a highly sensitive and simple LC–MS/MS method after one-step precipitation was developed and validated for the simultaneous determination of PA, PE, DT and CP in human plasma. This developed method offered higher sensitivity, more simple sample procedure, smaller sample volume requirement and shorter run time. This method was successfully applied to a

Abbreviations: PA, paracetamol; PE, pseudoephedrine; DT, dextrophan; CP, chlorpheniramine; DPD, diphenhydramine.

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bioequivalence study in 20 healthy Chinese volunteers receiving multicomponent formulations containing 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate.

2. Experimental

2.1. Materials and reagents

PA (98.4% purity), PE (99.7% purity) and CP (99.9% purity) were generously supplied by Zhejiang Apeloa Kangyu Pharmaceutical Co. Ltd. (Hengdian, China). DT was obtained from Hoffmann-La. Roche, Inc. (New Jersey, USA). Diphenhydramine (DPD), purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) was used as the internal standard (IS). Methanol and acetic acid were of HPLC grade and purchased from Merck Company Inc. (Darmstadt, Germany). Blank human plasma was obtained from the Blood Center of Zhejiang Province (Hangzhou, China). Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). Test formulation, compound pseudoephedrine hydrochloride dispersible tablets (lot 20090902) were manufactured by Zhejiang Apeloa Kangyu Pharmaceutical Co. Ltd. Reference formulation, paracetamol, pseudoephedrine hydrochloride, dextromethorphan hydrobromide and chlorphenamine maleate tablets (TYLENOL®, lot 070330581) were purchased from Shanghai Johnson & Johnson Pharmaceuticals, Ltd. (Shanghai, China). Each formulation contained 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate.

2.2. Instrumentation

All experiments were performed on a Thermo Scientific (San Jose, USA) LC–MS/MS system including a Accela® autosampler, a Accela® pump and a TSQ Quantum Access® triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. Data acquisition was performed with Xcalibur® 2.5.6 software. Peak integration and calibration were performed with LCquan® 2.0.6 software.

2.3. LC–MS/MS conditions

The chromatographic separation was achieved on a YMC-ODS-AQ C_{18} Column (100 mm × 2.0 mm, 3 μm, Kyoto, Japan), protected by a Hypersil Gold guard C_{18} cartridges (10 mm × 2.1 mm, 3 μm, Thermo Scientific, San Jose, USA). The mobile phase consisted of 0.3% (v/v) acetic acid (A) and methanol (B). Gradient elution at a constant flow rate of 0.3 mL/min was performed as follows: started at 100% A for 3.0 min, linear decrease to 2% A in the next 1.0 min, hold for 1.0 min, then changed back to 100% A immediately, and reequilibrate for 1.0 min before the next injection. The total run time was 6.0 min and the injection volume was 10 μL. Moreover, the column and sample temperature was maintained at 30 °C and 4 °C, respectively.

Mass spectrometer was operated in the positive mode. The capillary voltage was 4200 kV and the temperature of capillary was set at 320 °C. Nitrogen was used as the sheath (12 L/min) and auxiliary gas (2.1 L/min). Argon was used as the collision gas at a pressure of 0.2 Pa. The mass spectrometer was operated in a 5-channel selected reaction monitoring (SRM) mode. The precursor–product ion pairs used for SRM of PA, PE, DT, CP and DPD were m/z 152.0 → 110.0 (collision energy: 16 V), m/z 166.2 → 148.2 (10 V), m/z 258.1 → 157.0 (37 V), m/z 275.0 → 229.9 (24 V) and m/z 256.0 → 167.0 (14 V), respectively, with a scan time of 0.05 s per ion pair. Both Q1 and Q3 were set at 0.7 unit mass resolution. An on-line motorized six-port divert valve was used to introduce the LC eluent flow to the mass spectrometer over the period of 3.6–5.6 min for data acquisition, the eluent flow from other time was diverted to waste.

2.4. Preparation of stock and working solutions

Standard stock solutions of PA, PE, DT, CP and IS were prepared separately by dissolving accurately weighed reference standards in methanol to result in a final concentration of 1 mg/mL for each analyte. Various volumes of PA, PE, DT and CP stock solutions were mixed and diluted with methanol to prepare working solutions in the nominal concentration of 500 μg/mL for PA, 100 μg/mL for PE, 2.5 μg/mL for DT and 5 μg/mL for DT. IS working solution was prepared by diluting the 1 mg/ml stock solution with methanol to the nominal concentration of 2 ng/mL. These working solutions were kept at −20 °C before use.

2.5. Preparation of standard and quality control samples

An aliquot of the working solutions was dried and reconstituted in blank human plasma to obtain the nominal plasma concentration of 5000 ng/mL for PA, 1000 ng/mL for PE, 25 ng/mL for DT and 50 ng/mL for CP. The plasma was then successively diluted with blank human plasma to the other standard concentrations. The nominal plasma concentrations of calibration standards were 10, 50, 100, 500, 1000, 2000, 5000 ng/mL for PA; 2, 10, 20, 100, 200, 400, 1000 ng/mL for PE; 0.05, 0.25, 0.5, 2.5, 5, 10, 25 ng/mL for DT; 0.1, 0.5, 1,5, 10, 20, 50 ng/mL for CP. The quality control (QC) samples were prepared in the same way as the calibration standards to obtain plasma concentrations of 20, 400, 2000 ng/mL for PA; 4, 80, 800 ng/mL for PE; 0.1, 2, 20 ng/mL for DT; 0.2, 4, 40 ng/mL for CP. The spiked plasma samples (standard and QC samples) were pretreated and detected in each analytical batch along with the unknown samples. All of the plasma samples were stored at −20 °C until use.

2.6. Sample preparation

A 50 μL of plasma sample was transferred to a microcentrifuge tube, and then 250 μL of IS working solution (2 ng/mL DPD diluting with methanol) was added. The analytes and IS were extracted from plasma by vortex-mixing for 20 s. After centrifugation at 36700 × *g* (Allegra™ 64R centrifuge, Beckman Coulter, USA) for 10 min at 10 °C, the supernatant was transferred to vials on the rack of the autosampler and a 10 μL aliquot was injected into the LC–MS/MS system for analysis.

2.7. Assay validation

The method was validated in terms of selectivity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guidelines for validation of bioanalytical method [17]. Chromatogram comparisons of blank and spiked human plasma were used to evaluate the selectivity of the method. Calibration curves were constructed from the peak area ratios of each analyte to internal standard versus plasma concentrations through the linear least squares regression calculation with a weighting factor of $1/X$. The LLOQ was determined as the lowest concentration that could be quantified with an acceptable precision and accuracy within ±20% and fulfil the requirement of a signal-to-noise ratio ≥10. Both precision and accuracy of the method were determined by analyzing six replicates of QC samples at low, medium and high concentrations. Intra-day precision was calculated as the relative standard deviation (RSD) resulting from the same day. Inter-assay precision was assessed by the RSD of the mean concentration on three consecutive days. The accuracy was determined by the percent of mean deviation from nominal concentration, bias% = (measured concentration − nominal concentration)/nominal concentration × 100%. The recovery of each analyte from human plasma matrix was

evaluated by comparing the mean peak areas obtained from processed QC samples of low, medium and high concentrations with standard solutions of equivalent concentration. Short-term stability of the analytes in human plasma was determined by assessing QC samples after 2 h at room temperature. Freeze–thaw stability was checked after three cycles and long-term stability was determined by assessing QC samples stored at −20 °C for 23 d and 58 d. QC samples were prepared and injected, and reinjected after the samples of that batch were maintained in the autosampler at 4 °C for 24 h. The samples were concluded to be stable if the assay values were within the acceptable limits of ±15% deviation from the nominal concentration.

2.8. Application of the method

The method was applied to a randomized, two-period, crossover bioequivalence study in Chinese volunteers. The study protocol was approved by the Independence Ethics Committee of the 2nd affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China. Twenty healthy male Chinese volunteers were given written informed consent to participate in the study. After a 12 h overnight fast, the volunteers received a single oral dose of the test or reference products with 200 mL water. No food was allowed until 4 h after dose administration. Following drug administration, venous blood samples (1.0 mL) were collected into tubes containing EDTA anticoagulant (BD, NJ, USA) before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 24, 48 and 72 h after dosing. Blood samples were centrifuged at 1640 × *g* (Allegra™ 6R centrifuge, Beckman Coulter, USA) for 10 min and plasma was separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. LC–MS/MS method optimization

Quantification was performed using selected reaction monitoring (SRM) in the positive ion mode. Fig. 1 displays the product ion spectra of $[M+H]^+$ ions from PA, PE, DT, CP and IS. For PA, the product mass spectrum was recorded from the precursor ion m/z 152.0 $[M+H]^+$ and the most abundant fragment was monitored at m/z 110.0 $[M+H-CO{=}CH_2]^+$. PE gave an intense product ion at m/z 148.2, formed by losing a molecule water. For DT, the most abundant fragments obtained from the precursor ion *m/z* 258.1 were m/z 157.0 and m/z 198.99. But the fragment ions of m/z 157.0 is more stable than m/z 198.99, so m/z 157.0 was selected as quantify ion. CP showed an intense ion at m/z 229.9 corresponding to the loss of dimethylamine $[NH(CH_3)_2]$ from $[M+H]^+$ ion. DPD showed a major fragment ion at m/z 167.0 corresponding to the loss of $[HOCH_2CH_2N(CH_3)_2]$. These fragment ions were the most sensitive ions detected and are specific for the analytes and IS.

The chromatographic conditions were optimized to improve peak responses and peak symmetry because of different polarity

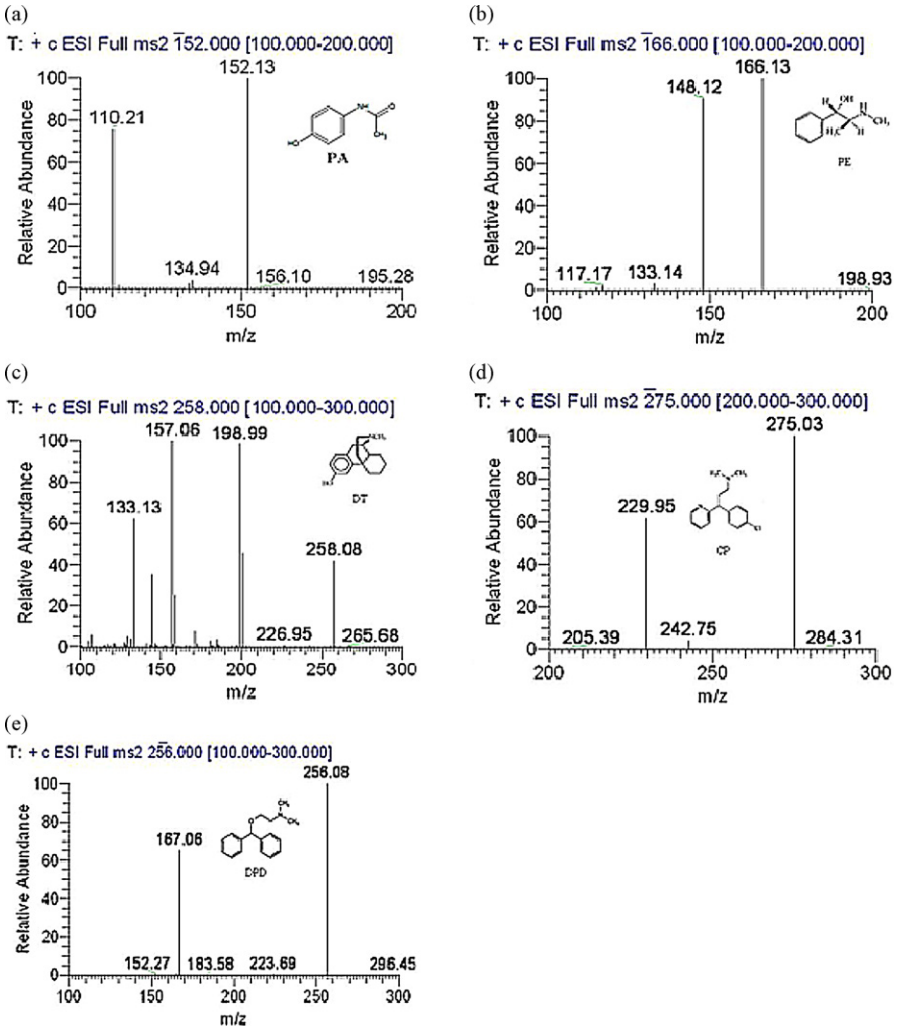

Fig. 1. Product scan mass spectra of (a) PA, (b) PE, (c) DT, (d) CP and (e) DPD.

Table 1
Precision, accuracy and extraction recovery for PA, PE, DT and CP in plasma QC samples.

Components	Nominal concentration (ng/mL)	Intra-day ($n = 6$)			Inter-day ($n = 18$)			Extract recovery (%) ($n = 6$)
		Measured concentration (ng/mL)	RSD (%)	Bias (%)	Measured concentration (ng/mL)	RSD (%)	Bias (%)	
PA	20	19.9 ± 1.34	6.75	−0.51	20.3 ± 2.03	9.97	1.71	101.12
	400	390.9 ± 10.96	2.80	−2.27	400.3 ± 17.29	4.32	0.09	109.74
	4000	4094.3 ± 100.5	2.45	2.36	4123.8 ± 192.3	4.66	3.09	111.96
PE	4	4.12 ± 0.31	7.63	3.13	4.11 ± 0.29	7.04	2.72	99.32
	80	78.59 ± 4.79	6.10	−1.76	79.8 ± 4.32	5.41	−0.27	93.90
	800	750.54 ± 41.48	5.53	−6.18	758.9 ± 42.24	5.56	−5.13	101.32
DT	0.1	0.098 ± 0.0113	11.54	−2.00	0.101 ± 0.015	14.35	1.39	99.39
	2.0	1.83 ± 0.039	2.15	−8.37	1.88 ± 0.13	7.14	−6.12	99.88
	20	19.37 ± 0.79	4.10	−3.15	20.15 ± 1.04	5.15	0.74	100.96
CP	0.2	0.203 ± 0.013	6.55	1.58	0.200 ± 0.022	11.08	0.28	92.10
	4	3.83 ± 0.27	7.03	−4.35	3.82 ± 0.22	5.72	−4.49	105.28
	40	39.94 ± 1.31	3.29	−0.15	38.8 ± 1.70	4.38	−3.05	106.27

of each analyte. It was found that the gradient program beginning with more aqueous phase could improve the peak shape of PA, while it had no effect on the other three analytes and IS. Therefore a gradient program beginning with 100% aqueous phase was used to achieve good resolution and symmetrical peak shapes of the analytes and IS as well as a short run time. The YMC-ODS-AQ C_{18} Column was chosen based on its good tolerance for 100% aqueous phase. LC–MS/MS chromatograms showed that the analytes and IS were separated with no interference from each other. Furthermore, proteins precipitation produced no matrix effects on the chromatograms.

3.2. Separation and specificity

The specificity of the method was investigated by analyzing human plasma of six different sources. No endogenous interference peaks were found at the retention times of all analytes and IS. The total run time was 6.0 min and the retention times of all analytes were less than 5.12 min. Typical SRM chromatograms of a blank plasma sample, a blank plasma sample spiked with PA, PE, DT, CP and IS, and a volunteer sample are shown in Fig. 2.

3.3. Linearity and sensitivity

The assay was linear over the concentration range of 10–5000 ng/mL for PA, 2–1000 ng/mL for PE, 0.05–25 ng/mL for DT and 0.1–50 ng/mL for CP in human plasma, respectively. The correlation coefficients for the calibration regression curve were 0.998 or greater. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations and coefficient of variation (CV) were less than 15% for all concentrations.

The current assay had an LLOQ of 10 ng/mL for PA, 2 ng/mL for PE, 0.05 ng/mL for DT and 0.1 ng/mL for CP, respectively. These limits are sufficient for clinical pharmacokinetic studies following oral administration of therapeutic dose.

3.4. Accuracy and precision

A summary of inter- and intra-day precisions and accuracies at QC concentrations are shown in Table 1. The intra- and inter-day precisions were less than 11.54% and 14.35% for all analytes. The accuracies were in range of −8.37% to 3.13% for all analytes. The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

3.5. Recovery

To calculate the absolute recovery of the protein precipitation procedure, six replicates of spiked plasma QC samples of low, medium and high concentrations were extracted under the conditions noted above. The integrated peak area response of each analyte was compared with those obtained from the standard solutions of equivalent concentration subjected to the same extraction

Table 2
Stability of PA, PE, DT and CP in human plasma under different storage conditions ($n = 6$).

Components	Nominal concentration (ng/mL)	Room temperature for 2 h	Autosampler at 4 °C for 24 h	Three freeze–thaw cycles			Storage at −20 °C	
				1	2	3	23 d	58 d
PA	20	2.17	−2.45	0.78	1.72	12.98	12.55	−8.29
	400	9.12	6.57	−7.39	−2.48	−1.23	0.17	4.98
	4000	8.40	8.69	2.22	−3.32	2.44	−0.18	5.63
PE	4	2.61	−0.27	2.06	3.38	−6.40	1.15	−12.52
	80	10.27	0.62	5.66	−0.16	−2.22	−0.12	6.66
	800	6.52	2.12	−3.57	−9.12	−1.03	−3.74	1.20
DT	0.1	10.83	−2.17	12.00	9.17	8.00	7.17	7.50
	2.0	6.75	−1.16	3.48	−1.53	−3.80	−7.84	−7.03
	20	10.83	4.43	−2.75	−5.91	−2.23	−5.35	0.06
CP	0.2	3.58	6.75	−3.08	−13.75	5.92	4.58	6.00
	4	−3.35	−8.34	−1.82	−1.54	−10.53	−1.82	0.91
	40	−5.02	−10.12	−3.21	−8.20	−7.86	−6.64	5.92

All values are represented as the percent of mean deviation from nominal concentration, bias% = (measured concentration − nominal concentration)/nominal concentration × 100%.

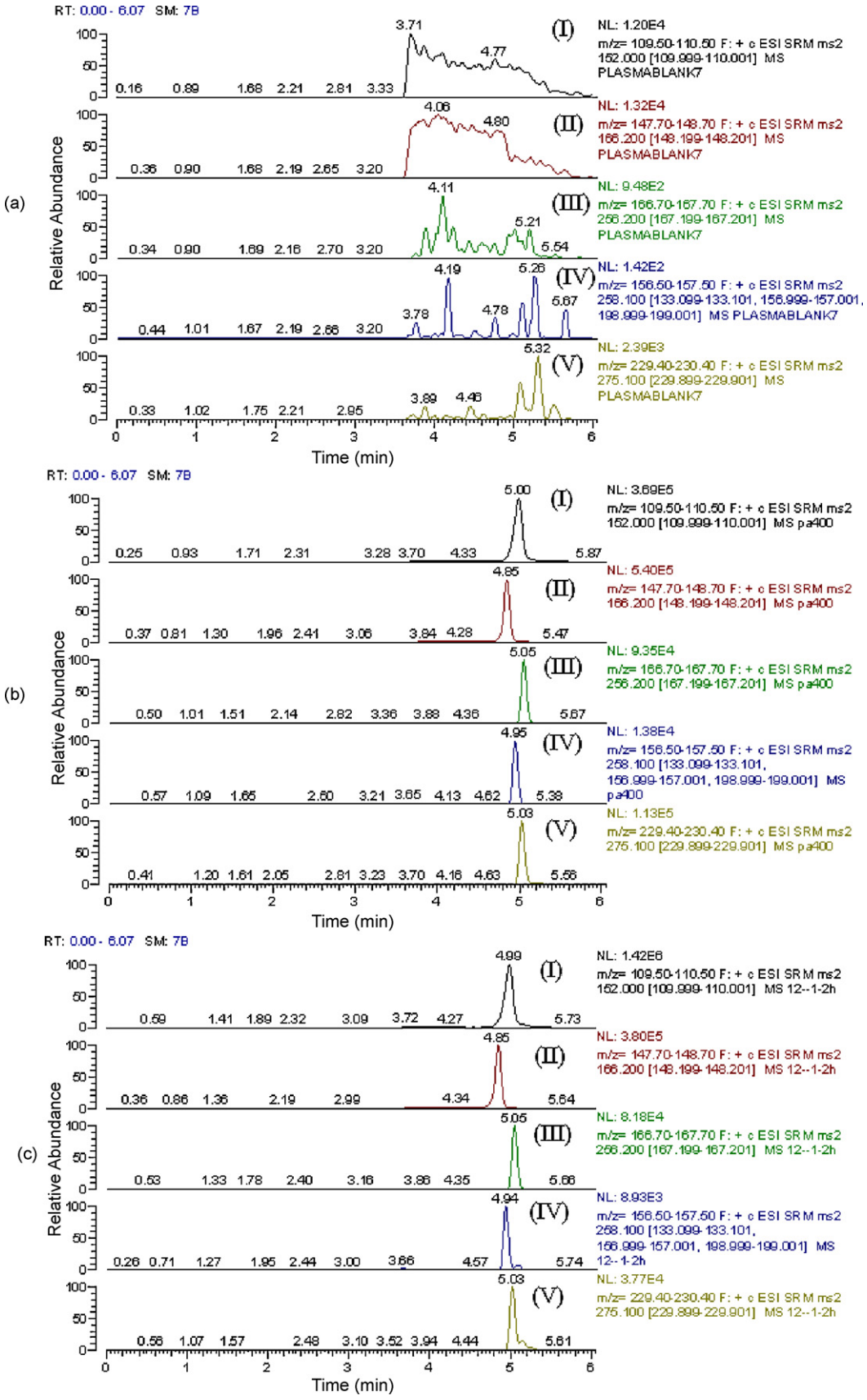

Fig. 2. Representative SRM chromatograms of PA(I), PE(II), DPD(III), DT(IV) and CP(V) in human plasma. (a) A blank plasma sample; (b) a blank plasma sample spiked with 400 ng/mL of PA, 80 ng/mL of PE, 2 ng/mL DT, 4 ng/mL CP and 2 ng/mL of IS; (c) a volunteer plasma sample 2 h after oral dose of 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate.

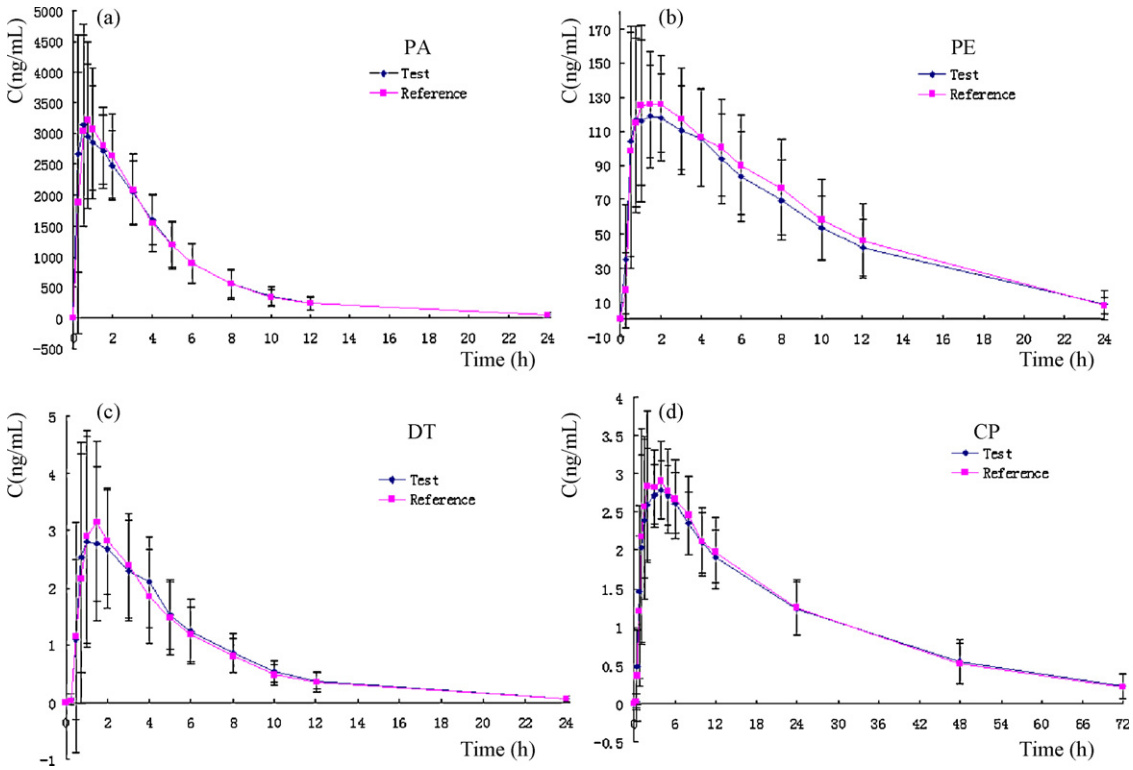

Fig. 3. Mean plasma concentration–time profiles of (a) PA, (b) PE, (c) DT and (d) CP after an oral administration of the test and reference multicomponent formulations containing 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate ($n = 20$, $\bar{x} \pm s$).

procedure as plasma samples. Table 1 also summarizes the extraction recoveries of all analytes. It was found that the recoveries of all analytes were in range of 92.10–111.96%. Thus, extraction efficiency was within the acceptance criteria.

3.6. Stability

The four components of PA, PE, DT and CP were found to be stable under following conditions: in plasma at room temperature for 2 h, in the autosampler at 4 °C for 24 h, three freeze–thaw cycles, or in plasma at −20 °C for 23 d and 58 d. All bias values between the measured concentration and the nominal concentration were in range of −13.75% to 12.98%, which is shown in Table 2. These results suggested that all analytes were stable under various storage conditions.

3.7. Application of the method

The method was successfully applied to a randomized, two-period, cross-over bioequivalence study in 20 healthy Chinese volunteers receiving multicomponent formulations containing 325 mg of paracetamol, 30 mg of pseudoephedrine hydrochloride, 15 mg of dextromethorphan hydrobromide and 2 mg of chlorphenamine maleate. The mean plasma concentration–time curves of PA, PE, DT and CP are shown in Fig. 3. The curves showed that there were no significant differences among test and reference formulations. Therefore, the two formulations are bioequivalent and can be prescribed interchangeably.

4. Conclusions

To our knowledge, this is the first fully validated LC–MS/MS method for the simultaneous quantification of PA, PE, DT and CP in human plasma. The method was proved to be sensitive, accurate, precise and reproducible. Sample preparation showed high recovery for the quantitative determination of all four analytes in human plasma. The method is very sensitive with a LLOQ of 10 ng/mL for PA, 2 ng/mL for PE, 0.05 ng/mL for DT and 0.1 ng/mL for CP, respectively. Protein precipitation instead of solid-phase or liquid–liquid extraction simplifies sample pretreatment procedure, and the high specificity of LC–MS/MS reduces analytical time. This method offers significant advantages in terms of improved sensitivity and selectivity, shorter run time (6.0 min) and smaller sample volume requirements (50 μL). The simple, inexpensive protein precipitation and high sample turnover rate makes this method a suitable and valuable tool in the investigation of the clinical pharmacokinetics and bioequivalence.

Acknowledgments

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