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Simultaneous quantitation of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid and sensitive method based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous quantitation of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine in human plasma has been developed and validated. After sample preparation by liquid–liquid extraction, the analytes and internal standard (diphenhydramine) were analyzed by reversed-phase HPLC on a Venusil Mp-C_{18} column (50 mm × 4.6 mm, 5 μm) using formic acid:10 mM ammonium acetate:methanol (1:40:60, v/v/v) as mobile phase in a run time of 2.6 min. Detection was carried out by electrospray positive ionization mass spectrometry in the multiple-reaction monitoring mode. The method was linear for all analytes over the following concentration (ng/ml) ranges: paracetamol 5.0–2000; caffeine 10–4000; pseudoephedrine 0.25–100; chlorpheniramine 0.05–20; cloperastine 0.10–40. Intra- and inter-day precisions (as relative standard deviation) were all ≤11.3% with accuracy (as relative error) of ±5.0%. The method was successfully applied to a study of the pharmacokinetics of the five analytes after administration of a combination oral dose to healthy Chinese volunteers.

1. Introduction

The common cold is generally a mild, self-limiting illness that improves with time. However, various cold medications are available to treat the symptoms until the immune system can eliminate the virus [1]. Combination medications are commonly used and often include an analgesic like paracetamol, an antihistamine like chlorpheniramine and a decongestant like pseudoephedrine. Caffeine is also commonly included as a mild stimulant and vasodilator to counteract the sedative effects of the antihistamine and any systemic vasoconstriction due to the decongestant. Some antihistamines like cloperastine have antitussive properties and can be included instead of the more traditional cough suppressants like dextromethorphan. Recently, a new oral dosage form containing paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine has been developed and shown to be highly effective and generally well accepted in treating the symptoms of colds and allergies [2,3]. Such a combination not only poses an analytical challenge but raises questions about the effects of co-administration on the pharmacokinetics of the individual agents.

Human pharmacokinetic studies involving simultaneous multicomponent quantitation require the high specificity of high performance liquid chromatography (HPLC) with fluorescence or mass spectrometric detection. When other factors are considered such as the widely different polarities of the drugs, the wide range of concentrations encountered in plasma after an oral dose and the desire for an analytical method that involves simple sample preparation and a rapid run time, method selection devolves onto liquid chromatography–tandem mass spectrometry (LC–MS/MS). Previously this technique has been applied to pharmacokinetic studies of combination cough, cold and allergy medications containing chlorpheniramine with paracetamol [4], chlorpheniramine with pseudoephedrine [5], pseudoephedrine with cetirizine [6], pseudoephedrine with fexofenadine [7] and chlorpheniramine, paracetamol and caffeine with amantadine [8]. However, these methods possess a number of disadvantages such as low sensitivity for paracetamol (12.2 ng/ml [9]), caffeine (48.8 ng/ml [9]), pseudoephedrine (2 ng/ml [5,7] and 5 ng/ml [6]) and chlorpheniramine (0.2 ng/ml [4,5]) and the need for a large sample volume and/or relatively long analytical run time. Moreover, there have been no reports of pharmacokinetic studies of cloperastine nor of analytical methods for its quantitation in combination with paracetamol, caffeine, pseudoephedrine and chlorpheniramine (Fig. 1) in biological matrices. Here we report a rapid and sensitive LC–MS/MS method for the simultaneous quantitation of these five drugs in

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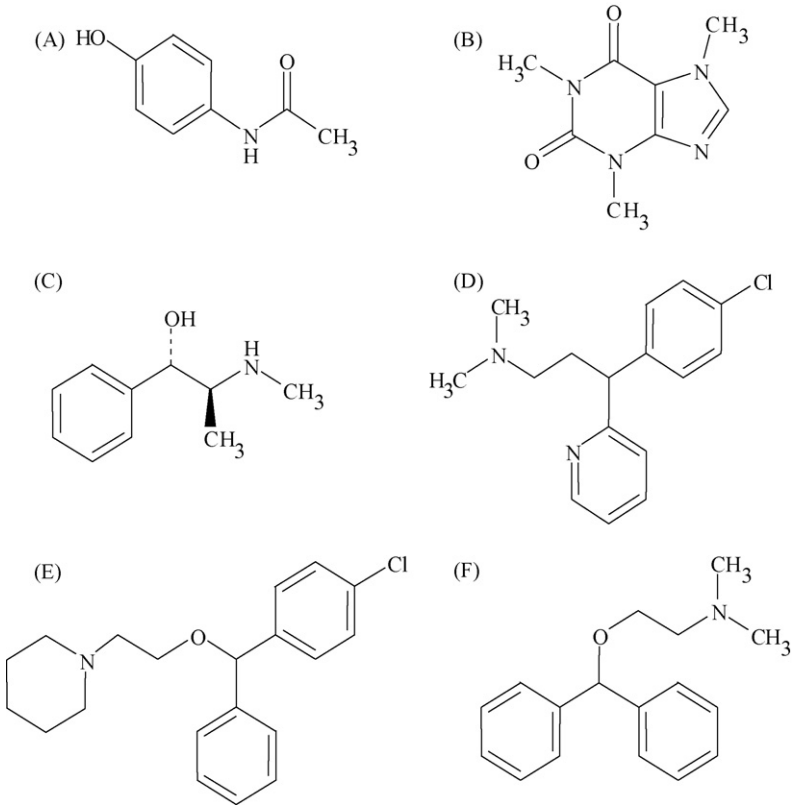

Fig. 1. Chemical structures of (A) paracetamol, (B) caffeine, (C) pseudoephedrine, (D) chlorpheniramine, (E) cloperastine, and (F) diphenhydramine.

human plasma and its application to a pharmacokinetic study in healthy volunteers after a single oral dose of a combination capsule formulation.

2. Experimental

2.1. Chemicals and reagents

Paracetamol, caffeine, pseudoephedrine hydrochloride, chlorpheniramine maleate, cloperastine hydrochloride (purity > 99.0% for all drugs) and diphenhydramine hydrochloride (internal standard, I.S., purity > 99.0%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Capsules containing a mixture of the five drugs were supplied by YangTian Pharmaceutical Co., Ltd. (Sichuan, PR China). Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Heparinized blank (drug-free) human plasma was obtained from the Changchun Blood Donor Service (Changchun, PR China). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals were HPLC grade.

2.2. LC–MS/MS conditions

The LC–MS/MS system consisted of an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) binary pump, an autosampler connected to a Venusil Mp-C_{18} column (50 mm × 4.6 mm, 5 μm; Agela Technologies Inc., Delaware, USA) and an Applied Biosystems Sciex Q-trap™ mass spectrometer (Concord, Ontario, Canada) using electrospray ionization (ESI). The mobile phase was formic acid:10 mM ammonium acetate:methanol (1:40:60, v/v/v) at a flow-rate of 1.0 ml/min. An approximately 1:1 split of the column eluant was included prior to entry into the mass spectrometer. The detector was operated in the positive ion mode at unit resolution using multiple-reaction monitoring (MRM) of transitions of the protonated molecular ions. These transitions (m/z) with associated declustering potentials (V) and collision energies (eV) were: paracetamol 152.0 → 110.1, 80, 25; caffeine 195.1 → 138.1, 35, 20; pseudoephedrine 166.0 → 148.0, 24, 15; chlorpheniramine 275.1 → 230.0, 24, 17; cloperastine 330.3 → 201.1, 20, 19; diphenhydramine 256.3 → 167.1 50, 25. MS parameters set during syringe pump infusion of a solution containing analytes and I.S. in mobile phase were as follows: curtain gas, gas 1 and gas 2 (all nitrogen) 20, 65 and 65 units, respectively; dwell time 100 ms per channel; source temperature 550 °C; ion spray voltage 1400 V. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.3.2 software.

2.3. Preparation of standard and quality control samples

All solutions were prepared in methanol:water (50:50, v/v) Independently prepared stock solutions of paracetamol, caffeine, pseudoephedrine HCl, chlorpheniramine maleate and cloperastine HCl (all 1.0 mg/ml) were used to prepare mixed standard and QC solutions (Table 1). A 1.0 mg/ml stock solution of diphenhydramine HCl was used to prepare a 50 ng/ml working I.S. solution. All solutions were stored at 4 °C until required.

2.4. Sample preparation

An aliquot of plasma (100 μl) was placed in a 10 ml glass tube followed by 100 μl I.S. solution, 100 μl 0.1 M Na_2CO_3, 100 μl methanol:water (50:50, v/v) or a standard or QC solution, and extracted with 3 ml diethyl ether:dichloromethane (60:40, v/v) for 10 min. After centrifugation at 2000 × g for 10 min, the organic phase was transferred to another tube and evaporated to dryness

Table 1
Concentrations of combined calibration standards and QC solutions.

	Paracetamol (ng/ml)	Caffeine (ng/ml)	Pseudoephedrine HCl (ng/ml)	Chlorpheniramine maleate (ng/ml)	Cloperastine HCl (ng/ml)
QC 3	10	20	0.50	0.10	0.20
QC 2	100	200	5.0	1.0	2.0
QC 1	1600	3200	80	16	32
LLOQ	5.0	10	0.25	0.05	0.10
C 6	10	20	0.50	0.10	0.20
C 5	30	60	1.5	0.30	0.60
C 4	100	200	5.0	1.0	2.0
C 3	300	600	15	3.0	6.0
C 2	1000	2000	50	10	20
C 1	2000	4000	100	20	40
Concentration ranges	5.0–2000	10–4000	0.25–100	0.05–20	0.10–40

at 40 °C under a gentle stream of nitrogen. Residues were reconstituted in 180 μl aliquots of mobile phase and 8 μl injected into the LC–MS/MS system.

2.5. Method validation

Method validation was performed according to FDA Guidance for Industry, Bioanalytical Method Validation [10]. Specificity was assessed by comparing chromatograms of spiked plasma samples using plasma from six different individuals. Linearity was evaluated by assay of three independent calibration curves on 3 different days by weighted linear regression ($1/x^2$) of analyte-I.S. peak area ratios. Accuracy (as relative error (RE)) and precision (as relative standard deviation (RSD)) were determined by assay of six replicates of low, medium and high QC samples on 3 different days. LLOQ was determined as the concentration below which the inter-day RSD exceeded 20%. Recovery was determined by comparing the mean peak areas for triplicate analysis of low, medium, and high QC samples with those of blank plasma extracts reconstituted with the corresponding QC solutions. Matrix effects were evaluated at the assay concentration of the internal standard and at low, medium, and high QC levels of the five analytes by comparison of the analytical responses for post-extraction spiked blank plasma samples with those of solutions containing the five analytes and internal standard at corresponding concentrations ($n = 3$ in each case). Stability of analytes in plasma was assessed on storage at −20 °C for 30 days,

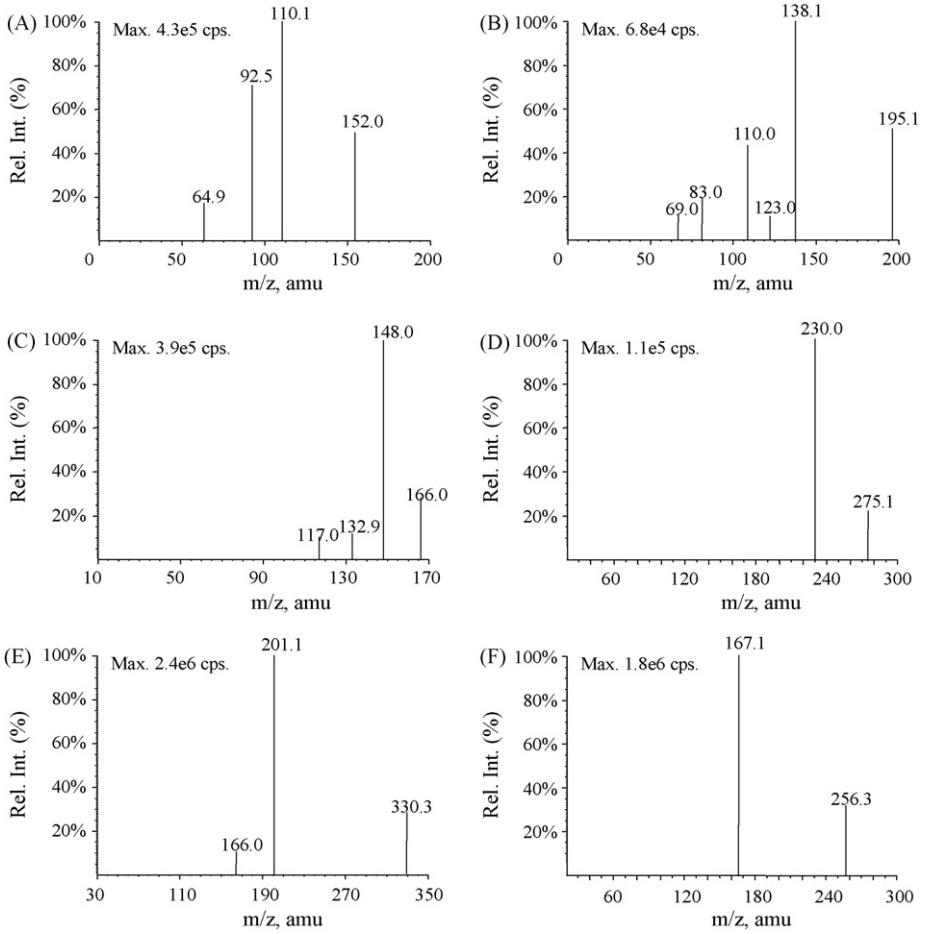

Fig. 2. Full-scan product ion mass spectra of $[M+H]^+$ ions for (A) paracetamol, (B) caffeine, (C) pseudoephedrine, (D) chlorpheniramine, (E) cloperastine, and (F) diphenhydramine (I.S.).

Table 2
Accuracy (RE) and precision (RSD) for the determination of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine in human plasma (data are based on assay of 6 replicates on 3 different days).

	Concentration (ng/ml)		RSD (%)		RE (%)
	Nominal conc.	Mean found conc.	Intra-day	Inter-day	
Paracetamol	5.00	4.96	5.2	2.3	−0.8
	10.0	9.87	4.5	7.4	−1.3
	100	105	3.9	7.7	5.0
	1600	1580	4.9	5.5	−1.3
Caffeine	10.0	10.1	3.3	3.2	1.0
	20.0	20.0	4.0	2.7	0.0
	200	196	5.9	6.9	−2.0
	3200	3170	4.4	3.1	−0.9
Pseudoephedrine HCl	0.250	0.238	6.1	1.9	−4.8
	0.500	0.503	2.6	11.3	0.6
	5.00	5.14	4.3	6.2	2.8
	80.0	80.0	4.1	4.7	0.0
Chlorpheniramine maleate	0.050	0.0489	4.3	5.0	−2.2
	0.100	0.0994	4.9	3.9	−0.6
	1.00	0.995	4.5	1.2	−0.5
	16.0	16.0	9.8	3.8	0.0
Cloperastine HCl	0.100	0.101	4.4	2.7	1.0
	0.200	0.201	4.8	1.3	0.5
	2.00	1.98	3.0	7.6	−1.0
	32.0	32.1	3.2	6.4	0.3

at room temperature for 12 h and after three freeze–thaw cycles. Stability of prepared samples in mobile phase at room temperature for over 24 h was also assessed.

2.6. Pharmacokinetic study

A group of healthy male volunteers ($n = 22$) was enrolled in the study. The clinical protocol was approved by the Ethics Committee of the First Hospital of Jilin University, PR China. All volunteers read the protocol and gave written informed consent before entering the study. They were not allowed to consume alcohol or take any other medication during the study. After a 12 h fast, subjects received a single oral dose of two capsules containing 300 mg paracetamol, 25 mg caffeine, 30 mg pseudoephedrine HCl, 2.5 mg chlorpheniramine maleate and 12 mg cloperastine HCl. Blood samples were collected into heparinized glass tubes before administration and at 0.17, 0.33, 0.5, 0.75, 1, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24 36, 48 and 72 h post-dose. Plasma was separated immediately by centrifugation at $3000 \times g$ for 10 min and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. MS/MS conditions

Full-scan product ion mass spectra of each analyte are shown in Fig. 2. The spectra are similar to those shown in previous reports for paracetamol [4,9], chlorpheniramine [4,5], caffeine [9], pseudoephedrine [5] and diphenhydramine [5]. The most abundant product ion of each analyte was selected for MRM monitoring. Under optimum conditions, paracetamol and pseudoephedrine had at least 10 times higher ion abundances than the other analytes and, in order to determine all analytes simultaneously, it was necessary to decrease the signal intensities of paracetamol and pseudoephedrine by adopting high CEs and non-optimal DPs.

3.2. HPLC conditions

Using reversed-phase HPLC, pseudoephedrine was found to elute considerably earlier than cloperastine which was the last to elute. A high proportion of methanol (60%) in the mobile phase enhanced ionization efficiency for all analytes and provided an acceptable signal for psueudoephedrine, the first analyte to elute from the column. For the aqueous mobile phase component, the use of 10 mM ammonium acetate enhanced response for all compounds and the use of formic acid to reduce pH reduced the retention times and sharpened the peaks of the basic compounds, caffeine, chlorpheniramine and cloperastine. Of a number of C_{18} columns evaluated (Diamonsil, Zorbax Extend, Nucleosil, Venusil Mp-C_{18} and Hypersil), Nucleosil and Hypersil gave rise to serious matrix effects for paracetamol, caffeine and pseudoephedrine while Diamonsil and Zorbax caused increased retention of cloperastine. Venusil Mp-C_{18} provided the best separation time and resolution for all compounds in a cycle time of only 2.6 min.

3.3. Sample preparation

Different methods of sample preparation including solid-phase extraction (SPE), protein precipitation and liquid–liquid extraction (LLE) with various organic solvents (such as diethyl ether, dichloromethane, hexane, isopropanol and ethyl acetate) were evaluated. SPE led to low recovery of caffeine and protein precipitation introduced serious matrix effects for all compounds. LLE using diethyl ether–dichloromethane (60:40, v/v) produced clean extracts with high extraction efficiency. The addition of 0.1 M Na_2CO_3 to plasma prior to extraction enhanced the recovery of caffeine, chlorpheniramine and cloperastine by about 20% and reduced that of paracetamol, the major component in the formulation, by about 30%.

3.4. Assay validation

3.4.1. Specificity and sensitivity

There was no significant interference from endogenous substances in plasma at the retention times of the I.S. and analytes. LLOQs (ng/ml) were paracetamol 5.0, caffeine 10, pseudoephedrine 0.25, chlorpheniramine 0.05, and cloperastine 0.1. Accuracy and precision at the LLOQs were within ±20.0% for all analytes. Fig. 3 shows typical MRM chromatograms of blank plasma, a plasma sample spiked at the LLOQ and a plasma sample from a

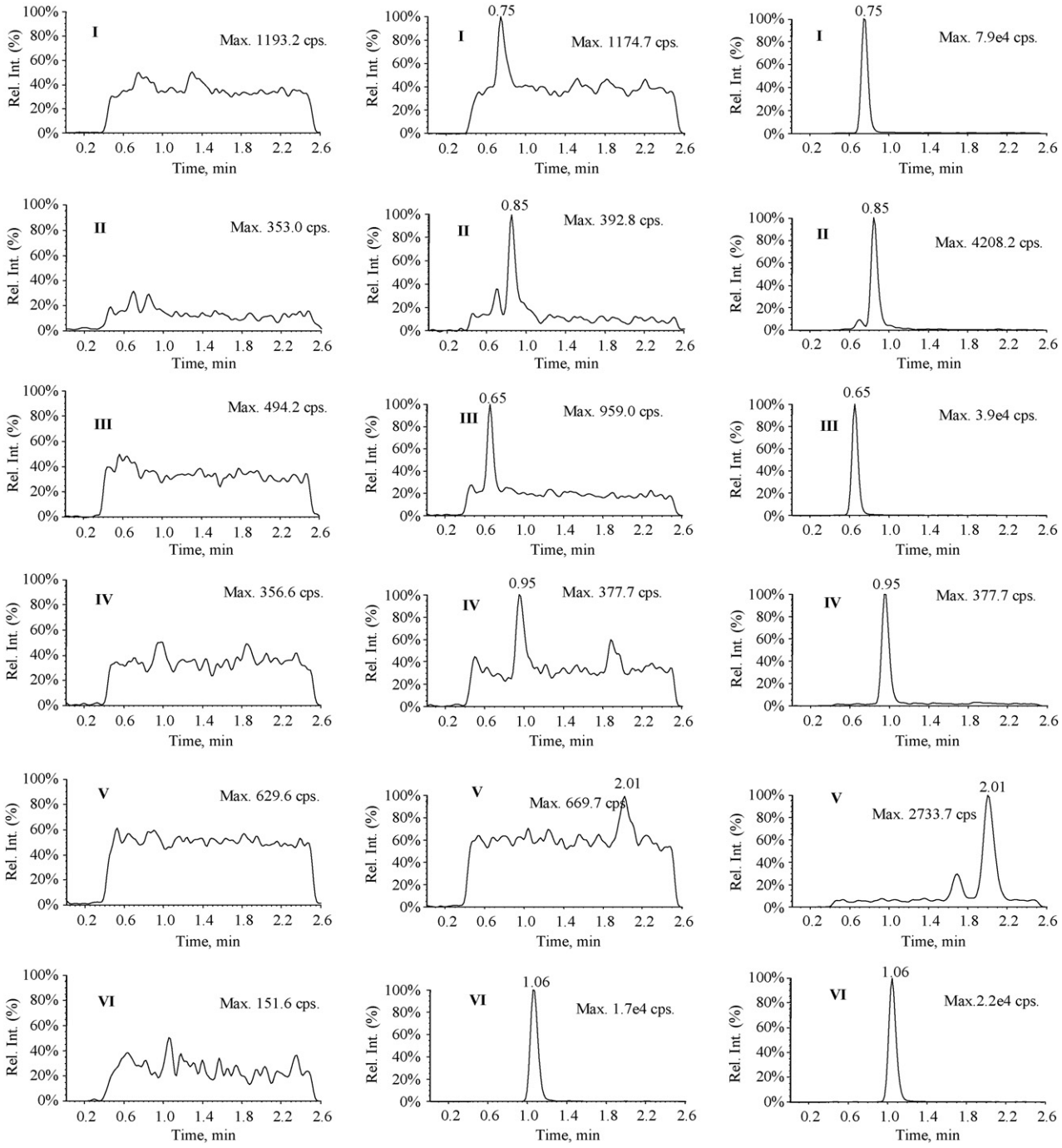

Fig. 3. Representative MRM chromatograms for (I) paracetamol, (II) caffeine, (III) pseudoephedrine, (IV) chlorpheniramine, (V) cloperastine, and (VI) diphenhydramine (I.S.). Left chromatograms are blank plasma; centre chromatograms are at the LLOQs; right chromatograms are samples from a healthy volunteer 1.0 h after a single oral administration of two capsules containing 300 mg paracetamol, 25 mg caffeine, 30 mg pseudoephedrine HCl, 2.5 mg chlorpheniramine maleate and 12 mg cloperastine HCl.

healthy volunteer 1.0 h after oral administration of the capsule formulation.

3.4.2. Linearity, accuracy and precision

All calibration curves were linear within the concentration ranges tested. Typical equations of calibration curves were as follows—paracetamol: $y = 0.00581C + 0.0163$, $r = 0.9976$; caffeine: $y = 0.00172C + -0.001$, $r = 0.9983$; pseudoephedrine: $y = 0.0925C + 0.0118$, $r = 0.9965$; chlorpheniramine: $y = 0.214C + 0.00869$, $r = 0.9985$; cloperastine: $y = 0.116C + 0.0015$, $r = 0.9988$. Intra- and inter-day precisions of the five analytes were $\leq$11.3% at each QC level with accuracies of $\pm$5.0%. These values indicate the method is suitably accurate and precise (Table 2).

3.4.3. Recovery, matrix effects and stability

The recoveries for each analyte are shown in Table 3. Mean recovery for the internal standard was 92.6 ± 7.9%.

In terms of matrix effects, actual concentrations as percentages of nominal concentrations (ng/ml in brackets) were as follows: paracetamol 80.6 ± 6.9 (10), 83.5 ± 2.9 (100), 84.9 ± 1.2 (1600); caffeine 85.9 ± 4.5 (20), 90.3 ± 1.5 (200), 89.1 ± 1.9 (3200); pseudoephedrine 77.9 ± 4.1 (0.50), 78.2 ± 6.2 (5.0), 76.9 ± 4.9 (80);

Table 3
Absolute recoveries (%) for the five analytes in human plasma (data are mean ± SD, $n = 3$).

	Recovery		
	Low QC	Medium QC	High QC
Paracetamol	30.5 ± 3.0	35.1 ± 3.8	34.6 ± 1.6
Caffeine	70.0 ± 2.7	70.5 ± 1.8	69.0 ± 3.9
Pseudoephedrine	48.1 ± 0.7	44.6 ± 2.2	44.0 ± 2.3
Chlorpheniramine	90.4 ± 8.6	88.5 ± 4.8	91.2 ± 2.6
Cloperastine	79.3 ± 6.1	80.5 ± 2.5	81.5 ± 1.9

chlorpheniramine 91.2 ± 4.9 (0.10), 94.1 ± 5.9 (1.0), 95.0 ± 2.0 (16); cloperastine 95.9 ± 6.8 (0.20), 97.4 ± 1.6 (2.0), 96.0 ± 4.1 (32). The corresponding value for the I.S. was 95.4 ± 2.3%. The results indicate that matrix effects are not a problem for any of the analytes.

As regards stability, all analytes were found to be stable under all the conditions evaluated. In addition, they were stable in stock solutions for at least 24 h at 24 °C and for 20 days at 4 °C.

3.5. Pharmacokinetic study

Plasma concentration–time profiles for the five analytes are illustrated in Fig. 4 and corresponding pharmacokinetic parameters are summarized in Table 4. The results show that the method is applicable to clinical pharmacokinetic studies of the five drugs given in combination. Pharmacokinetic parameters for chlorpheniramine are consistent with previous studies [4,5] when corrected for the different doses. Somewhat surprisingly given their similar structures, the C_{max} for cloperastine was only slightly greater and the AUC less than the corresponding values for chlorpheniramine despite the considerably greater dose of cloperastine.

4. Conclusions

A rapid and sensitive LC–MS/MS method has been developed for the simultaneous quantitation of paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine in human plasma. The method involves simple sample preparation and a short run

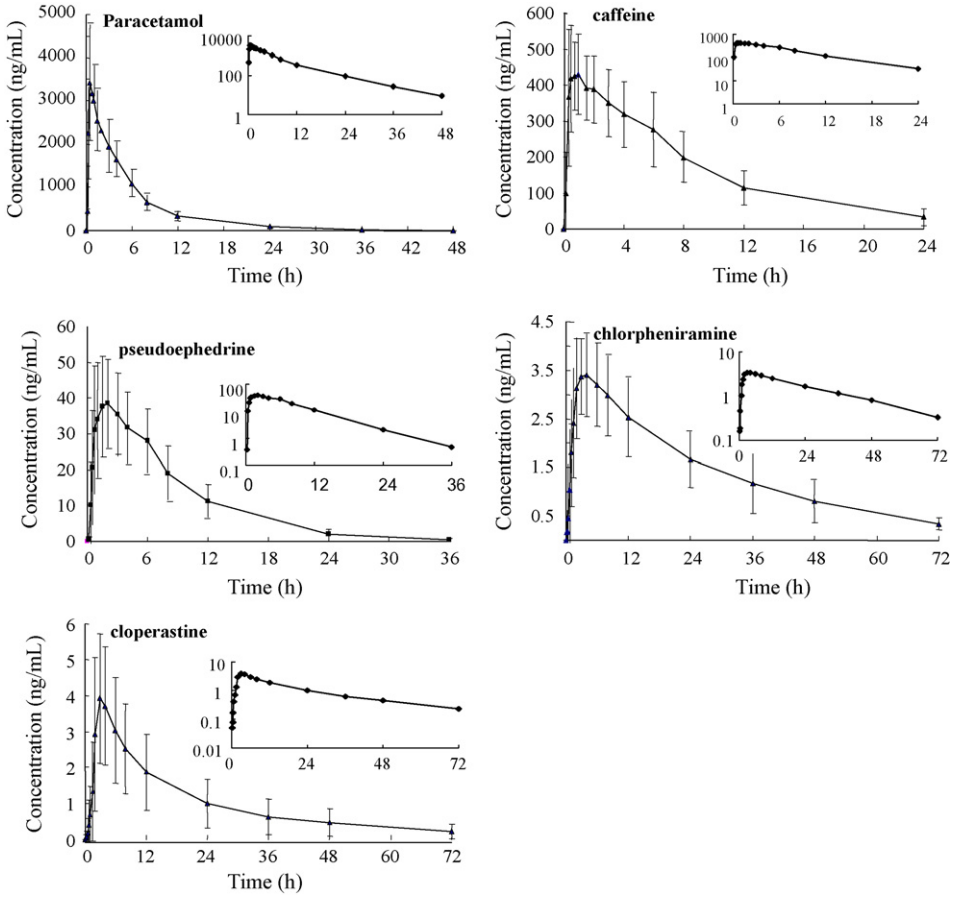

Fig. 4. Mean plasma concentration–time curves for paracetamol, caffeine, pseudoephedrine, chlorpheniramine and cloperastine after oral administration of compound paracetamol, caffeine, pseudoephedrine, chlorpheniramine, cloperastine capsules. Half-logarithmic scale profile is placed at right upper corner of each profile (data are mean ± S.D. for 22 healthy volunteers).

Table 4
Mean pharmacokinetic parameters for five drugs after oral administration of a single oral dose containing 300 mg paracetamol, 25 mg caffeine, 30 mg pseudoephedrine, 2.5 mg chlorpheniramine and 12 mg cloperastine.

Parameters	T_{max} (h)	C_{max} (ng/ml)	$AUC_{0-\infty}$ (ng × h/ml)	$T_{1/2}$ (h)
Paracetamol	0.6 ± 0.2	3770 ± 1040	18800 ± 4220	6.0 ± 1.3
Caffeine	0.9 ± 0.8	508 ± 126	4340 ± 1401	5.9 ± 1.7
Pseudoephedrine	1.3 ± 0.7	45.3 ± 12.3	360 ± 110	4.7 ± 0.7
Chlorpheniramine	3.2 ± 1.5	3.75 ± 0.87	111 ± 34.0	21 ± 3.8
Cloperastine	3.4 ± 1.1	4.45 ± 1.87	81.0 ± 46.9	23.0 ± 7.7

allowing high sample throughput. The method has been successfully applied to a pharmacokinetic study involving administration of a single oral dose of a combined formulation to healthy volunteers.

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