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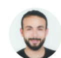

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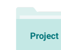

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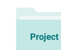

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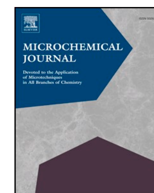
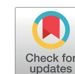


UPLC-MS/MS estimation of paracetamol, pseudoephedrine hydrochloride and brompheniramine maleate in plasma: Application to a pharmacokinetic study on healthy Egyptian volunteers based on ethnic difference



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ABSTRACT

The current study was focused on establishing a novel ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method for the quantitative estimation of the co-formulated drugs; paracetamol (PAR), pseudoephedrine hydrochloride (PSD) and brompheniramine maleate (BRP) in human plasma to Egyptian volunteers. Additionally, the study aimed to recognize whether the co-administration of the target drugs to different ethnic population affects their pharmacokinetics. The drugs extraction involved liquid-liquid extraction technique with the aid of ethyl acetate. Reversed phase UPLC separation was accomplished on Agilent Zorbax SB C18 (50 mm × 2.1 mm, 1.8 µm) column using acetonitrile: 0.1% formic acid (70: 30 *v/v*) as the mobile phase. Positive electrospray ionization and multiple reaction monitoring were employed. The short analysis time (1 min/sample) was promising as it has allowed the analysis of many human plasma samples per day. The developed method displayed linear ranges of 0.05–20.0 µg/mL for PAR, 1.0–500.0 ng/mL for PSD and 0.1–50.0 ng/mL for BRP. A detailed validation of the developed method was performed in compliance with the FDA guidelines where all the validation parameters results were satisfactory. The UPLC-MS/MS method was utilized for studying the pharmacokinetics of the three drugs after the oral administration of their combined dosage form to Egyptian healthy volunteers. The pharmacokinetic study was accomplished after agreement of the ethics committee. The achieved pharmacokinetic results by the newly developed method were; C_{max} (ng/mL) 8001.77, 127.76, 1.92, t_{max} (h) 0.75, 1.5, 4.0 and $t_{½}$ (h) 3.3, 4.65, 16.26 for PAR, PSD and BRP, respectively, these results were compared with those obtained from other reported clinical trials done on other races. It was clear that the pharmacokinetic parameters of PAR and PSD were not affected when the same dose was given to volunteers from different ethnic populations. Additionally, the co-administration of PSD and BRP with PAR has not altered the pharmacokinetics of PAR. The pharmacokinetics of PSD when it was co-administered with PAR and BRP was almost similar to that when it was co-administered with benorylate and chlorpheniramine, however, the C_{max} of PSD was greatly affected when it was co-administered with caffeine, chlorpheniramine and cloperastine.

1. Introduction

Common cold is normally a mild, self-limiting viral infectious disease that recovers with time. Nevertheless till the immune system can overcome this virus; numerous cold medicines could be administered to treat the symptoms [1]. Usually combination medicines are utilized which are often comprised of an analgesic like paracetamol (PAR), a decongestant as pseudoephedrine (PSD) and an antihistamine as brompheniramine (BRP). Comtrex® Maximum Strength tablets is emerging as one of the recently extensively prescribed combination for treating the symptoms of common cold in Egypt.

PAR; N (4 hydroxyphenyl) acetamide (Fig. 1), has antipyretic and analgesic properties which is comparable to non-steroidal anti-inflammatory drugs (NSAIDs), yet it possess no anti-inflammatory effect.

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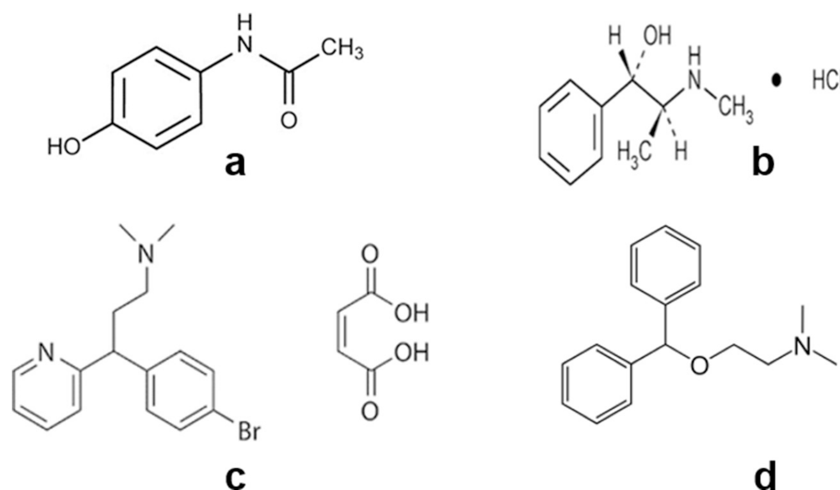

Fig. 1. Chemical structures of: a- Paracetamol, b- Pseudoephedrine HCl, c- Brompheniramine maleate and d- Diphenhydramine (IS).

Furthermore, PAR is considered the optimal choice for patients in whom the use of the NSAIDs is contraindicated as in the cases of peptic ulcers, bronchial asthma and pregnancy [2]. PAR is commonly considered as a weak inhibitor of prostaglandin synthesis by cyclooxygenase-2 (COX-2) [3].

PSD; 2-methylamino-1-phenyl-1-propanol hydrochloride (Fig. 1), is a sympathomimetic alkaloid, it acts by stimulating the α-adrenergic receptors in the respiratory tract mucosa. PSD shrinks the inflamed mucous membrane of the nasal passages, consequently decreasing congestion and edema and increasing the nasal air way patency [4].

BRP; [3-(4-bromophenyl)-3-(pyridin-2-yl)propyl] dimethylamine (Fig. 1), is a first-generation H1 histamine receptors antagonist. Additionally, BRP acts as an anticholinergic agent. Through antagonizing the histamine effect; BRP is able to cure sneezing, itching of the eyes and runny nose [5].

A diversity of analysis methods were utilized for the quantification of the investigated analytes individually or in combination with other analytes in plasma, including colorimetry [6], GC–MS [7–10], HPLC-UV detection [11–15] and LC–MS/MS [16–25]. Regarding the colorimetric methods which were used for the determination of PAR [6], they were considered unspecific as these methods have depended on the unspecific acid hydrolysis of PAR to 4-aminophenol. However, 4-aminophenol could also be produced from the acid hydrolysis of PAR metabolites and the acid-labile PAR conjugates, thus, leading to gross overestimates of the true free PAR concentration. For the utilized GC methods [7–10] although they have demonstrated good specificity, yet, derivatization of the analytes was required to be converted to volatile derivatives. HPLC is typically the method of choice for the estimation of the plasma analyte concentration. However, the utilized HPLC-UV methods [11–15] have revealed some disadvantages which have led to the unsuitability of these methods to be applied to pharmacokinetic studies. The main disadvantage was their low sensitivity. Besides, these methods have presented tedious extraction steps, utilization of large volumes of samples, high consumption of solvents and requirement of long run time. Human plasma analysis following the oral administration of multicomponents is best achieved through LC-MS/MS due to its great specificity and sensitivity [16–25].

In spite that BRP and chlorpheniramine (CHL) are structurally similar and are commonly used as histamine H1-receptor antagonists, some LC-MS/MS methods [17–19] were reported for the determination of CHL simultaneously with PAR and PSD, however, up till now, there is neither a reported LC method for the quantification of BRP concentration in human plasma nor any analysis method for the simultaneous estimation of PAR, PSD and BRP in plasma samples could be found in the literature. Such a multicomponent medication is not only considered as a challenge for the analytical chemists, but also raises questions regarding the effect of co-administration on the pharmacokinetic parameters of each drug. Consequently, in the present work we established and validated [26] a novel, robust and sensitive bioanalytical method possessing the competence of estimating PAR, PSD and BRP plasma concentrations. The method demonstrated several advantages over the reported methods that did not consider the PAR and PSD combination with BRP [17–19] as will be discussed later. The method was applicable for studying the pharmacokinetics of the three analytes following the oral administration of Comtrex® Maximum Strength tablets (500 mg of PAR, 30 mg of PSD and 2 mg of BRP) to Egyptain healthy volunteers. Finally, as different ethnic population with different patient characteristics might lead to variation in the pharmacokinetics parameters, thus, the achieved pharmacokinetic results were compared with other reported clinical trials [16–18,27,28].

2. Materials and methods

2.1. Reference materials and reagents

Reference materials: Paracetamol whose purity was certified as 99.90% ± 0.49 and brompheniramine maleate with a purity certified to be 99.99% ± 0.38 were kindly purchased from GlaxoSmithKline (Cairo, Egypt). Pseudoephedrine HCl with certified purity 100.01% ± 0.31 was kindly purchased from EPICO Pharmaceutical Company (Giza, Egypt). Diphenhydramine (Fig. 1); internal standard (IS) was kindly obtained from Sigma Pharmaceutical Industries (Steinheim, Germany) and its purity was certified as 100.05% ± 0.20.

Market Samples: Comtrex® Maximum Strength coated tablet (Batch No: A623987) is manufactured by GlaxoSmithKline Egypt for Novartis Pharma Egypt, under license from Novartis Consumer Health (Switzerland), with a labeled composition of 500 mg PAR, 30 mg PSD and 2 mg BRP and was obtained from local market.

Human blank plasma was purchased from VACSERA (Giza, Egypt).

Solvents and materials: Methanol, formic acid, acetonitirile, ethyl acetate and sodium hydroxide (HPLC grade) were bought from Sigma Pharmaceutical Industries (Steinheim, Germany).

2.2. Instruments and conditions

Agilent 1200 HPLC system (Agilent Technologies, USA) with an Agilent Zorbax SB-C18 (50 × 2.1 mm, 1.8 µm) column and coupled to XEVO-TQD triple quadrupole mass spectrometer (Waters, USA). MassLynx software (USA), eppendorf concentrator 5301 (USA), Stuart “BioCote” vortex (United Kingdom) and Hettich EBA 85 centrifuge

(Germany). To calculate the Pharmacokinetics parameters a validated, excel software was used.

The mobile phase was acetonitrile: 0.1% formic acid (70: 30, *v/v*) utilizing 0.3 mL/min flow rate and 7.5 µL injection volume.

Positive ESI was employed. MRM was used to monitor the following transitions; PAR *m/z* 151.88 → 110.02, PSD *m/z* 166.10 → 148.10, BRP *m/z* 321.03 → 276.06, and IS *m/z* 256.12 → 167.04. The mass spectrometric conditions were optimized as follows: Desolvation gas flow 500 L/h, source temperature 150 °C, desolvation temperature was 400 °C. The cone voltage was 25 V, 12 V, 20 V and 10 V and the utilized collision energy was 20 V, 10 V, 25 V and 10 V for PAR, PSD, BRP and IS, respectively, utilizing nitrogen as the collision gas.

2.3. Procedures

2.3.1. Stock and working solutions

Stock solutions with the following concentrations; 1000 µg/mL, 1000 µg/mL and 100 µg/mL of PAR, PSD and BRP, respectively, were prepared separately in methanol.

The stock solutions were diluted with methanol into 10-mL volumetric flasks for the preparation of the first working solutions (WS 1) with the following; concentrations of 200 µg/mL, 100 µg/mL and 1 µg/mL for PAR, PSD and BRP, respectively.

The (WS 1) of PSD and BRP were further diluted in 10-mL volumetric flasks with methanol to prepare the second working solutions (WS 2) with the final concentrations of 500 ng/mL and 100 ng/mL for PSD and BRP, respectively.

The prepared (WS 1) of PAR and (WS 2) of PSD and BRP were then successively diluted using methanol as a solvent to prepare a series of solutions with concentration of 0.5–200 µg/mL, 10–5000 ng/mL and 1–500 ng/mL for PAR, PSD and BRP, respectively. The stock solutions were kept at 8 °C.

2.3.2. Calibration standards and quality control samples

Different blank human plasma samples each of 450 µL volumes were spiked with 50 µL of different concentrations of each PAR, PSD and BRP from the previously prepared working solutions and 200 ng/mL IS (final concentration). The ranges of the drugs final concentrations in the plasma samples were 0.05–20 µg/mL, 1–500 ng/mL and 0.1–50 ng/mL for PAR, PSD and BRP, respectively. The plasma calibration curve was constructed from a blank sample, a zero sample, and non-zero samples that cover the predicted concentration ranges, and the lower limit of quantification (LLOQ).

Besides, preparation of quality control samples (QCs) were performed for LLOQ with final concentration of 0.05 µg/mL, 1 ng/mL and 0.1 ng/mL, low quality control (LQC) possessing the final concentration of 0.15 µg/mL, 3 ng/mL and 0.3 ng/mL, medium quality control (MQC) with final concentration of 8 µg/mL, 200 ng/mL and 20 ng/mL and high quality control (HQC) with final concentration of 16 µg/mL, 400 ng/mL and 40 ng/mL for each of PAR, PSD and BRP, respectively.

2.3.3. Extraction procedure

The above mentioned spiked plasma with drugs and IS was treated with 100 µL sodium hydroxide (1 M) and the solution was mixed using a vortex for 30 s. For liquid-liquid extraction (LLE); 4 mL ethyl acetate was added, the solution was then vortexed for 60 s and centrifuged at 4000 rpm for 10 min. The upper organic layer was collected and evaporated to dryness at 40 °C and the dry residue was re-dissolved in methanol then vortex-mixed.

2.4. Validation procedures

Method validation was conducted in accordance to FDA guidelines for the bioanalytical method [26].

2.4.1. Selectivity

Six dissimilar batches of blank human plasma were arbitrarily chosen and were used to evaluate the selectivity of the method. Preparation and analysis of blank plasma samples was carried out as described previously. The obtained chromatograms from the blank plasma were compared to chromatograms of the plasma samples which were spiked with the LLOQ of the three drugs.

2.4.2. Linearity and range

Plasma calibration curves were established by constructing the peak area ratios of each individual drug/IS against the equivalent concentration. For acceptable linearity range, the correlation coefficient (r) must not be lower than 0.99 whereas the back calculated concentrations must be with ± 15% from the nominal values with the exception of the LLOQ where variation was permitted to up to ± 20%.

2.4.3. Precision and accuracy

Six replicates of LLOQ, LQC, MQC and HQC were analyzed on both the same day and different days in order to assess the intra-day and inter-day precision and accuracy. Precision was evaluated by the percentage relative standard deviation (RSD%) while accuracy was judged through calculating the recovery%. For a precise and accurate method, the variation should not exceed 15% with the exception of the LLOQ where deviation should not exceed 20%.

2.4.4. Recovery

The recovery was determined through comparing the peak areas of PAR, PSD and BRP acquired from pre-extracted QC samples to their peak areas acquired from post-extracted samples at the same concentrations.

2.4.5. Matrix effects

The matrix effects on the suppression or enhancement of the ions signals which is due to plasma components was examined through comparing the peak areas of the drugs obtained from post-extracted QC samples to their peak areas from neat samples (pure stock solutions) having similar concentrations.

2.4.6. Stability studies

The stability studies were conducted utilizing six replicates of both LQC and HQC samples of each drug.

- *Short term stability* was inspected through allowing the QC samples to thaw at room temperature and keeping them for 6 h before being analyzed.
- *Freeze-thaw stability* for three cycles was performed where the frozen samples were left to thaw for about 2 h at room temperature, then freeze overnight at −86 °C in every cycle.
- *Post-preparative stability* was investigated through leaving the processed QC samples in the autosampler at 25 °C for one day followed by their analysis.
- *Long term stability* was studied where the QC samples were stored at −86 °C until their analysis after 30 days.

The stability was evaluated under all previously described conditions by the comparison of the mean recovery% of PAR, PSD and BRP in the investigated samples to those of fresh ones where the RE% and RSD% were calculated for each analyte.

2.5. Application to a pharmacokinetic study

After complete validation of the developed LC-MS/MS method; it was applied for the estimation of PAR, PSD and BRP in the plasma of four healthy fasting Egyptian volunteers. The volunteers were acquainted with the objectives and possible threats of the presented work where the protocol of work was accepted by the October University for

Modern Sciences and Arts ethics committee (Reference number AC8/EC8/2016F). The pharmacokinetic study was accomplished through collecting blood samples after administering an oral dose of Comtrex® Maximum Strength coated tablet (500 mg PAR, 30 mg PSD and 2 mg BRP) at zero time, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 48 and 72 h. Blood samples were instantaneously relocated to heparinized tubes followed by centrifugation (3200 rpm) for 10 min. Then, the separated plasma was relocated to Eppendorf tubes and stored at −86 °C till the analysis day. Plasma samples were handled as previously explained under the experimental procedures to determine the concentrations of the drugs. Then the plasma concentration–time curves were drawn and the pharmacokinetic data was calculated. The pharmacokinetic parameters were calculated using a validated Excel software [29].

3. Results and discussion

3.1. Sample preparation

The sample preparation method is crucial for developing of a bioanalytical method as it leads to enhanced selectivity and sensitivity. Protein precipitation, solid phase extraction (SPE) and LLE are usually utilized for extracting the analytes from human plasma. However, SPE is regarded as an expensive technique, especially in a high throughput analysis encompassing many samples. In our study we have tried both protein precipitation and LLE utilizing different solvents. Protein precipitation has led to certain matrix effects for all drugs. On the other hand, it was found that the best recovery of PAR, PSD and BRP in addition to the minimum matrix effect were obtained using 4 mL of ethyl acetate as an extracting solvent in alkaline medium (1 M NaOH). It may be attributed to the hydrophobicity of the studied drugs (relatively high log P) which enhances their migration ability to the organic layer.

3.2. Method development

LC when coupled with MS/MS detection provides a fundamental technique for pharmacokinetic studies as it offers the sensitivity and selectivity that are demanded from an analytical method. Additionally, LC-MS/MS produces insignificant interference from the endogenous plasma constituents as only the ions that are resulting from the target analytes are observed provided that the MRM mode is utilized. Accordingly, this technique was selected for the present method development. Upon investigating both the positive and negative ionization modes; it was apparent that the positive mode has resulted in higher signal intensities which could be explained by the capability of the target analytes and the IS to accept protons.

In the Q1 full scan mass spectra; PAR, PSD, BRP and IS displayed the main protonated $[M + H]^+$ parent ions at m/z 151.88, 166.10, 321.03 and 256.12, respectively, (Fig. 2). While the product ion mass spectra displayed the following predominant ions of m/z 110.02 for PAR which corresponds to the ion $[NH_3–C_6H_4–OH]^+$ obtained after a $CO{=}CH_2$ neutral loss from the parent ion, m/z 148.10 for PSD which is interpreted as loss of a molecule of H_2O from the parent ion, m/z 276.06 for BRP which corresponds to loss of $[NH(CH_3)_2]$ from the parent ion and m/z 167.04 for the IS (Fig. 2). Furthermore, the source temperature was optimized to 150 °C and the desolvation temperature to 400 °C.

To optimize the chromatographic separation, the impact of various chromatographic conditions (mobile phase, column, injection volume and flow rate) were investigated thoroughly to accomplish the ideal separation and retention for the studied drugs. The use of acetonitrile: 0.1% formic acid (70: 30, v/v) has resulted in the best response and elution of the target drugs in a short time. Formic acid has contributed in attaining a good response for positive mode mass detection. Regarding the column; Zorbax C18 (2.1 mm × 50.0 mm, 1.8 µm) has resulted in the best peak shape and intensity. Additionally, the highest chromatographic performance was attained when using 0.3 mL/min as flow rate and 7 µL as injection volume (Fig. 3).

Our method is a clear advance on current methods [17–19] that were performed by LC-MS/MS technique for the determination of PAR, PSD and chlorpheniramine (CHL). As demonstrated in Table 1 the application of the UPLC with the smallest column size (50.0 × 2.1 mm, 1.8 µm) in our proposed method has led to the shortest run time approximately 1 min which has offered a fast reliable analysis of the studied drugs and has allowed for the analysis of many human plasma samples per day. Furthermore, the small particle size has led to obtain sharp intense peak shape. Besides, our method has utilized the simplest mobile phase which was composed of acetonitrile as the only organic modifier and 0.1% formic acid with isocratic elution. Thus, compared to Q. Liao et al. [18] we have avoided the utilization of different organic modifiers or the utilization of a gradient program as suggested by H. Lou et al. [19], thus, the optimization of the chromatographic conditions in our method was much easier than the reported methods which necessitated more efforts to optimize the gradient elution's programming for three drugs' separation. The usage of the low flow rate 0.3 mL/min with a total run time of 1 min has resulted in the least solvent consumption in comparison to the three reported methods, where our method has led to about 60% reduction in solvent consumption per run when compared to Q. Liao et al. [18] and approximately 85% reduction if compared to H. Li et al. [17] and H. Lou et al. [19]. Using less solvent is an important privilege introduced by our method from both the economic and environmental points of view. In developing countries like Egypt, solvent cost is actually a key decision factor in method development where the less the consumables, the less the operational costs will be. Moreover, as most developing countries are now more environmentally alert, the prospect of disposing less solvent is given great consideration. Finally, regarding the sensitivity, H. Li et al. [17] method has shown the highest sensitivity, however, over a narrow linearity range. Yet, our method has demonstrated the sensitivity required to quantify PAR, PSD and BRP with the large variation in their concentration in the complex matrix of human plasma.

3.3. Method validation

3.3.1. Selectivity

As shown in Fig. 3 which demonstrates the chromatograms of blank plasma, plasma spiked with PAR, PSD, BRP and IS and plasma of a volunteer 4 h after administrating one Comtrex® Maximum Strength coated tablet (500 mg PAR, 30 mg PSD and 2 mg BRP); there was no foreign peaks observed in the chromatograms of blank plasma at the retention time of the drugs or IS which proves that there was no noteworthy intrusion of endogenous plasma components.

3.3.2. Linearity and range

The constructed plasma calibration curves showed linearity in the following ranges of 0.05–20 µg/mL for PAR, 1–500 ng/mL for PSD and 0.1–50 ng/mL for BRP. In all the calibration curves; the correlation coefficients were greater than 0.990.

$y = 0.0960\ x - 0.0046$, $r = 0.9996$ for PAR

$y = 0.0033\ x + 0.0079$, $r = 0.9997$ for PSD

$y = 0.0102\ x + 0.006$, $r = 0.9985$ for BRP

y = Peak area ratio of each drug to that of the IS, x = Drugs' concentrations and r = Correlation coefficient.

The back-calculated concentrations for the chosen points on the calibration curve were all acceptable as they were within ± 15% of the nominal concentrations. The LLOQ was 0.05 µg/mL, 1 ng/mL and 0.1 ng/mL for PAR, PSD and BRP, respectively. The back calculated concentrations at the LLOQ were satisfactory as the results were below 20% for the three drugs.

3.3.3. Precision and accuracy

The calculated precision and accuracy were acceptable as their values were found to be in range of ± 15% for all QC samples and ± 20%

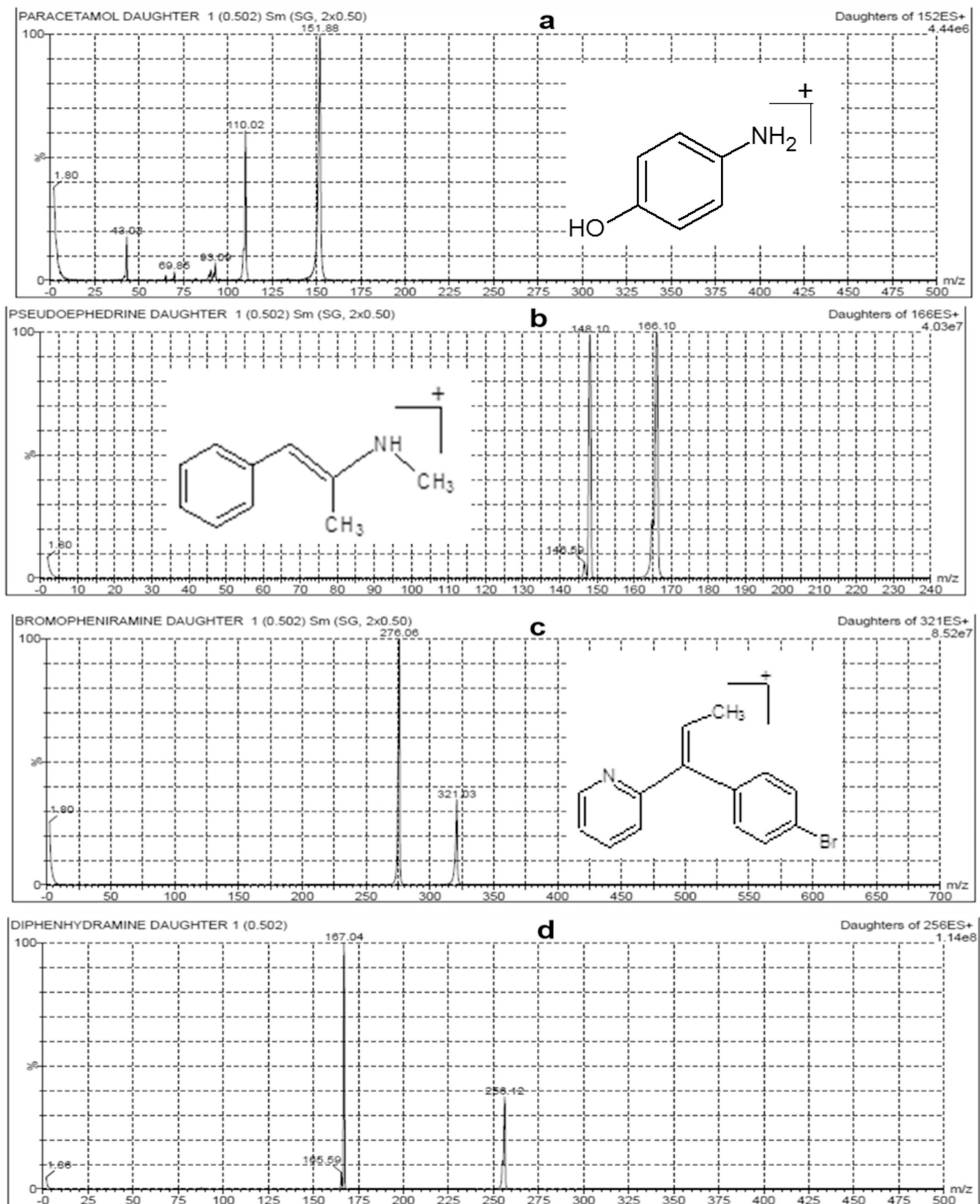

Fig. 2. Product ion spectra of $[M + H]^+$ of: a- Paracetamol, b- Pseudoephedrine HCl, c- Brompheniramine maleate and d- Diphenhydramine (IS).

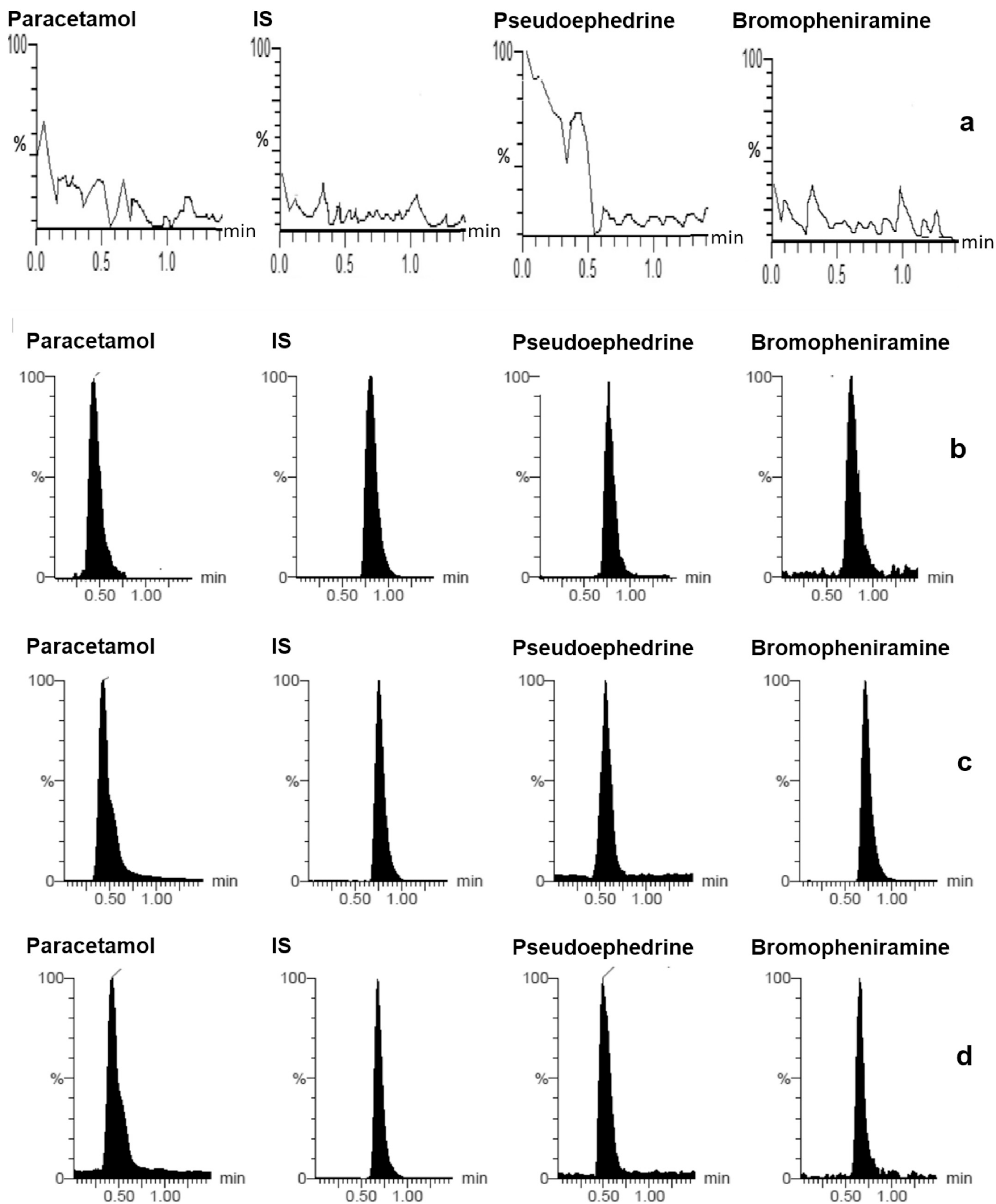

Fig. 3. Mass chromatograms of: a- Blank plasma, b- Plasma spiked with LLOQ of the drugs, c- Plasma spiked with HQC of the drugs and d- Plasma from volunteers 4 h after administration of Cometrex® Maximum Strength coated tablets.

Table 1
Comparison between the chromatographic conditions utilized for the determination of paracetamol, pseudoephedrine, chlorpheniramine and brompheniramine in human plasma by different reported LC-MS/MS methods and our proposed method.

	H. Li et al. [17]	Q. Liao et al. [18]	H. Lou et al. [19]	Proposed method
Column	Venusil Mp-C18 (50 × 4.6 mm, 5 µm)	Aquasil-C18 (150 × 2.1 mm, 5 µm)	YMC-ODS-AQ C18 (100 × 2.0 mm, 3 µm)	Agilent Zorbax SB-C18 (50 × 2.1 mm, 1.8 µm)
Mobile phase	Formic acid: 10 mM ammonium acetate: methanol (1:40:60, v/v/v)	Methanol: acetonitrile: 1% formic acid (60:20:20, v/v/v)	0.3% acetic acid and methanol (Gradient program)	Acetonitrile: 0.1% formic acid (70: 30, v/v)
Run time	2.6 min	2.5 min	6 min	1 min
Flow rate	1 mL/min	0.3 mL/min	0.3 mL/min	0.3 mL/min
Linearity range (ng/ mL)	PAR 5-2000, PSD 0.25-100, CHL 0.05-20	PAR 20-10000, PSD 1-500, CHL 0.1-50	PAR 10-5000, PSD 2-1000, CHL 0.1-50	PAR 50-20000, PSD 1-500, BRP 0.1-50

Table 2
Intra-day and inter-day precision and accuracy for the determination of paracetamol, pseudoephedrine and brompheniramine in human plasma.

Studied drug	QC level	Intra-day, n = 6		*Inter-day, n = 6 × 3	
		Accuracy%	RSD%	Accuracy%	RSD%
Paracetamol	LLOQ (50 ng/mL)	94.18	3.595	91.59	5.789
	LQC (150 ng/mL)	106.90	0.852	97.06	7.672
	MQC (8000 ng/mL)	98.11	1.514	97.42	1.796
	HQC (16,000 ng/mL)	93.13	2.320	93.69	1.305
Pseudoephedrine	LLOQ (1 ng/mL)	95.68	2.675	93.38	5.673
	LQC (3 ng/mL)	97.24	3.596	96.51	0.957
	MQC (200 ng/mL)	96.26	1.016	99.98	3.977
	HQC (400 ng/mL)	97.13	1.435	96.55	2.545
Brompheniramine	LLOQ (0.1 ng/mL)	93.18	2.657	94.48	2.097
	LQC (0.3 ng/mL)	97.49	1.804	97.14	2.246
	MQC (20 ng/mL)	97.94	3.941	98.06	0.886
	HQC (40 ng/mL)	99.81	2.354	98.73	1.013

* n = 3 days, 6 replicates per day, RSD is relative standard deviation.

Table 3
Recovery data and matrix effect for the determination of paracetamol, pseudoephedrine and brompheniramine in human plasma.

Parameter	QC level	Paracetamol		Pseudoephedrine		Brompheniramine	
		Recovery%	RSD%	Recovery%	RSD%	Recovery%	RSD%
Recovery data	LQC	91.07	0.169	93.23	1.912	93.14	0.982
	MQC	96.54	1.235	91.39	0.994	91.35	1.587
	HQC	94.30	0.890	91.68	1.549	92.47	2.041
Matrix effect	LQC	97.22	1.327	94.81	0.665	92.23	0.591
	HQC	101.87	1.342	100.51	3.931	102.01	3.431

*Mean percentage recovery and RSD (relative standard deviation) were calculated using six lots of plasma samples.

for LLOQ. The intra-day precision (RSD%) varied from 0.852 to 3.595%, 1.0163.596% and 1.804–3.941% for PAR, PSD and BRP, respectively. However, the intra-day accuracy varied from 93.13 to 106.90%, 95.68–97.24% and 93.18–99.81% for PAR, PSD and BRP, respectively. The inter-day precision (RSD%) varied from 1.305 to 7.672%, 0.957–5.673% and 0.886–2.246% for PAR, PSD and BRP, respectively. While, the inter-day accuracy the recovery% varied from 91.59 to 97.42%, 93.38–99.98% and 94.48–98.73% for PAR, PSD and BRP, respectively, as shown in Table 2.

3.3.4. Recovery

The recoveries of PAR, PSD and BRP were calculated at the LQC, MQC and HQC levels in six replicates. Generally the recovery defines the effectiveness of the separation of analytes from the plasma samples. Accordingly, the results that are demonstrated in Table 3 verify the proficiency of the current described extraction protocol of our proposed method as the recoveries of the three drugs were satisfactory and reliable. The mean recovery was more than 85% for all QC samples. It varied from 91.07 to 96.54% for PAR, 91.39–93.23% for PSD and 91.35–93.14% for BRP.

Table 4
Results of stability tests for determination of paracetamol, pseudoephedrine and brompheniramine in QC plasma samples by the proposed LC-MS\MS method.

Stability condition	QC level	Paracetamol			Pseudoephedrine			Brompheniramine		
		Recovery%	RE%	RSD%	Recovery%	RE%	RSD%	Recovery%	RE%	RSD%
Freeze-thaw stability	LQC	95.74	−4.26	0.845	100.86	0.86	0.394	99.57	−0.43	0.817
	HQC	104.19	4.19	0.517	100.64	0.64	0.540	99.17	−0.83	0.581
Postoperative stability	LQC	99.00	−1.00	0.974	103.09	3.09	0.650	101.78	1.78	0.422
	HQC	102.63	2.63	0.599	103.38	3.38	0.956	101.85	1.85	1.309
Short term stability	LQC	96.25	−3.75	1.566	100.89	0.89	0.971	97.56	−2.44	0.982
	HQC	93.29	−6.71	0.989	99.98	−0.02	1.232	101.82	1.82	1.247
Long term stability	LQC	91.48	−8.52	1.033	95.94	−4.06	0.949	94.18	−5.82	0.308
	HQC	94.33	−5.67	1.076	93.68	−6.32	0.576	94.81	−5.19	1.163

3.3.5. Matrix effect

The matrix effect indicates the capability of the analyte ionization in the ion source without interference from the co-eluting matrix components. The matrix effect was examined for PAR, PSD and BRP on the LQC and HQC levels. The data presented in Table 3 has revealed that the ionization of the drugs was not affected by the matrix effect.

3.3.6. Stability

As per the FDA guidelines for bioanalytical method validation [26]; stability experiments should reflect what actually occurs during the handling and analysis of the plasma samples. The results demonstrated in Table 4 were within the permitted limits and has confirmed a good stability of the studied drugs.

3.4. Application to a pharmacokinetic study and comparison to reported clinical trials

The developed UPLC-MS/MS method was utilized to estimate the concentrations of PAR, PSD and BRP from healthy Egyptian volunteers who were subjected to the oral administration of Cometrex® Maximum Strength coated tablets (500 mg PAR, 30 mg PSD and 2 mg BRP). All the volunteers were under fasting conditions to exclude any probable interaction from food/drinks intake. The study was performed after the approval of the ethics committee of the October University for Modern Sciences and Arts (MSA). The investigated pharmacokinetic parameters has included; C_{max} (ng/mL), t_{max} (h), $AUC_{0-\infty}$ (ng.h/mL), $t_{1/2}$ (h) and elimination rate constant; Kel (h^{-1}). The mean plasma concentration-time curves of PAR, PSD and BRP are demonstrated in Fig. 4 while the pharmacokinetic results are shown in Table 5.

The obtained results were comparable to previously reported pharmacokinetic studies. Regarding the obtained pharmacokinetic parameters of PAR (500 mg in the administered tablet) by this developed UPLC-MS/MS method; the t_{max} was 0.75 ± 0.25, $t_{1/2}$ was 3.3 ± 0.5, C_{max} was 8001.77 ± 2900.03 and the $[AUC]_{0-\infty}$ was 35,091 ± 7580. These results are quite similar to the results obtained by Tan et al. [16] who have given an oral dosage of 500 mg paracetamol tablet to Chinese healthy volunteers where t_{max} was 0.75, $t_{1/2}$ was 2.98 ± 0.7, C_{max} was 7162.5 and the $[AUC]_{0-\infty}$ was 32,101.4 ± 9236.7. Additionally, Portolés et al. [27] have given Termalgin tablets (500 mg paracetamol) to Spanish volunteers as reference formulation and the calculated pharmacokinetic parameters were very close to ours; the t_{max} was 0.73, $t_{1/2}$ was 2.12, C_{max} was 7440 and the $[AUC]_{0-\infty}$ was 14,340–51,560. Thus, it is clear that the pharmacokinetic parameters of PAR were not affected when the same dose was given to volunteers from different ethnic populations. Additionally, the co-administration of PSD and BRP with PAR has not altered its pharmacokinetics.

Similarly for PSD (30 mg in the administered tablet) the calculated parameters by our proposed method were found to be as follows the t_{max} 1.5 ± 0.5, $t_{1/2}$ 4.65 ± 1.2, C_{max} 127.76 ± 30.54 and the $[AUC]_{0-\infty}$ 865.98 ± 303.07. These results were in good agreement with Liao et al. [18] where in their study healthy Chinese volunteers have received a tablet with the composition of 300 mg benorylate, 30 mg PSD and 2 mg CHL providing the following results t_{max} 1.6 ± 0.7, $t_{1/2}$ 4.6 ± 1.0, C_{max} 140.90 ± 55.90 and the $[AUC]_{0-\infty}$ 1033.60 ± 391.50. In another study which was performed by Li et al. [17] that has involved also healthy Chinese volunteers in which the volunteers have taken single oral dose capsules with the composition of 300 mg PAR, 25 mg caffeine, 30 mg PSD, 2.5 mg CHL and 12 mg cloperastine, the calculated parameters were found to be t_{max} 1.3 ± 0.7, $t_{1/2}$ 4.7 ± 0.7, C_{max} 45.30 ± 12.30 and the $[AUC]_{0-\infty}$ 360 ± 110. It is very clear that both our proposed method and Li et al. [17] method were similar in the t_{max} and $t_{1/2}$, however, the C_{max} and $[AUC]_{0-\infty}$ were different which might be attributed to the co-administered drugs in Li et al. [17] study. So we can conclude that, the pharmacokinetics of PSD when it was co-administered with PAR and BRP was almost similar to that when it was co-administered with benorylate and CHL, however, the C_{max} of PSD was greatly affected when it was co-administered with caffeine, CHL and cloperastine.

For BRP (2 mg in the administered tablet) the calculated parameters by our proposed method were found to be as follows the t_{max} 4.0 ± 1.4, $t_{1/2}$ 16.26 ± 4.3, C_{max} 1.92 ± 0.26 and the $[AUC]_{0-\infty}$ 19.07 ± 5.95. Only one clinical trial was found in the literature which was performed by Simons et al. [28] in Canada where BRP syrup was administered in a single dose of 0.13 mg/kg to healthy volunteers. The calculated parameters were found to be t_{max} 3.1 ± 1.1, $t_{1/2}$ 24.9 ± 9.3 and C_{max} 11.60 ± 3.00. As demonstrated both the t_{max} and $t_{1/2}$ are comparable between our method and Simons et al. method [28]. However, keeping in consideration that the mean BRP ingested dose in this study [28] was about 9.8 ± 1.7 mg, while, BRP dose in our study is 2 mg, thus, the C_{max} obtained by Simons et al. [28] data was different from our data. Regarding the concentration of BRM at 24 h and 72 h it is approximate value as it is below the LLOQ which is 0.1 ng/mL, thus, the concentration at 24 h and 72 h was detected but could not be accurately quantified (as indicated by a footnote under Fig. 4).

4. Conclusion

A novel, high throughput UPLC-MS/MS method was successfully established for the quantification of paracetamol, pseudoephedrine and brompheniramine simultaneously in human plasma. In contrast to other reported methods, herein, our method has involved a very short run time (1 min/run), thus, leading to fast reliable analysis of many plasma samples per day and very little solvent consumption which is considered of high impact from both the economic and environmental points of view. Additionally, the utilized mobile phase was very simple, composed of only one organic modifier and was run in isocratic elution.

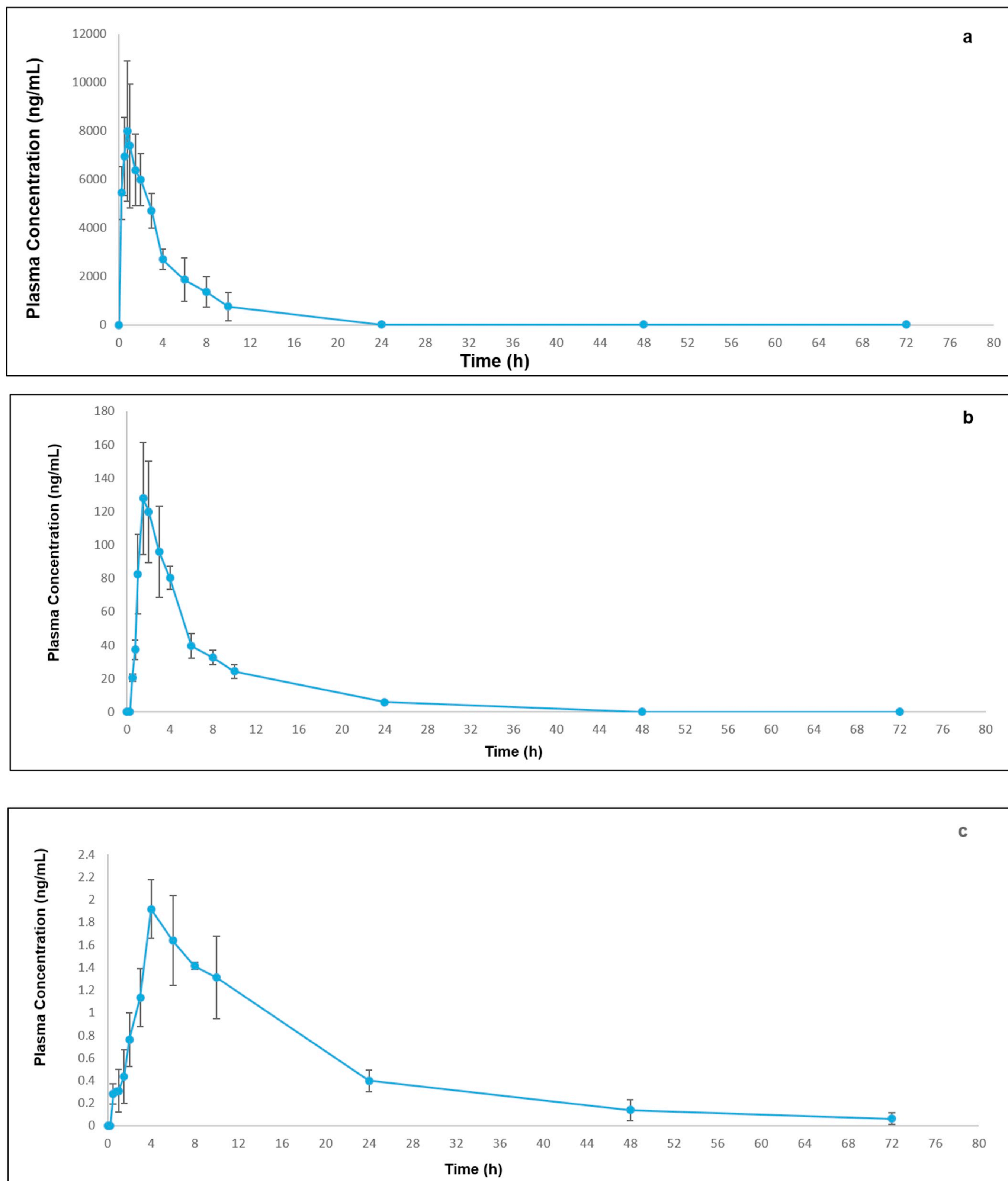

Fig. 4. Mean plasma concentration–time profile of a- Paracetamol, b- Pseudoephedrine HCl and c- Brompheniramine maleate in human plasma following oral dosing of one Cometrex® Maximum Strength coated tablets.
The 24 h and 72 h concentrations of BRP are detected but could not be accurately quantified as the concentration is below the linearity range.

Table 5
Pharmacokinetic parameters for paracetamol, pseudoephedrine and brompheniramine following oral administration of one Comtrex® Maximum Strength coated tablet nominally containing 500 mg of paracetamol, 30 mg of pseudoephedrine and 2 mg brompheniramine to Egyptian healthy volunteers.

Parameters	Paracetamol	Pseudoephedrine	Brompheniramine
C_{max} (ng/mL)	8001.77 ± 2900.03	127.76 ± 30.54	1.92 ± 0.26
t_{max} (h)	0.75 ± 0.25	1.5 ± 0.5	4.0 ± 1.4
$t_{½}$ (h)	3.3 ± 0.5	4.65 ± 1.2	16.26 ± 4.3
$[AUC]_{0-\infty}$ (ng.h/mL)	35,091 ± 7580	865.98 ± 303.07	19.07 ± 5.95
Kel (h^{-1})	0.161 ± 0.038	0.176 ± 0.065	0.0594 ± 0.013

C_{max} = Plasma maximum concentration, T_{max} = Time of the maximum plasma concentration, $t_{1/2}$ = Plasma concentration half time, $AUC_{0-\infty}$ = Area under the plasma concentration curve extrapolated to infinite time, Kel = Elimination rate constant. The study was performed on 4 healthy volunteers (n = 4).

The method was verified to be selective, accurate, precise, stable and presented no matrix interference as implied from the full validation which was performed as per FDA guidelines. Finally, the method was effectually used to estimate plasma concentrations of the studied drugs in Egyptian healthy volunteers where the pharmacokinetic parameters (C_{max}, t_{max}, $AUC_{0-\infty}$, $t_{1/2}$ and Kel) were calculated for the three drugs and were compared with previously reported clinical trials. So far no method is available for the simultaneous quantification of these three studied drugs in human plasma with pharmacokinetics evaluation on Egyptian human volunteers.

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