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Simultaneous determination of triprolidine and pseudoephedrine in human plasma by liquid chromatography–ion trap mass spectrometry

Ashok K. Shakya[a,*], Tawfiq A. Arafat[b], Ahmad N. Abuawwad[b], Munther Melhim[b], Jafar Al-Ghani[b], Mahmoud J. Yacoub[b]

[a] Department of Medicinal and Pharmaceutical Chemistry, Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, PO Box 263, Amman 19328, Jordan
[b] Jordan Center for Pharmaceutical Research, PO Box 950435, Amman 11195, Jordan

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ABSTRACT

A highly efficient, selective and specific method for simultaneous quantitation of triprolidine and pseudoephedrine in human plasma by liquid chromatography–ion trap-tandem mass spectrometry coupled with electro spray ionization (LC–ESI-ion trap-tandem MS) has been validated and successfully applied to a clinical pharmacokinetic study. Both targeted compounds together with the internal standard (gabapentin) were extracted from the plasma by direct protein precipitation. Chromatographic separation was achieved on a C_{18} ACE® column (50.0 mm × 2.1 mm, 5 μm, Advance Chromatography Technologies, Aberdeen, UK), using an isocratic mobile phase, consisting of water, methanol and formic acid (55:45:0.5, v/v/v), at a flow-rate of 0.3 mL/min. The transition monitored (positive mode) was *m/z* 279.1 → *m/z* 208.1 for triprolidine, *m/z* 165.9 → *m/z* 148.0 for pseudoephedrine and *m/z* 172.0 → *m/z* 154.0 for gabapentin (IS). This method had a chromatographic run time of 5.0 min and a linear calibration curves ranged from 0.2 to 20.0 ng/mL for triprolidine and 5.0–500.0 ng/mL for pseudoephedrine. The within- and between-batch accuracy and precision (expressed as coefficient of variation, %C.V.) evaluated at four quality control levels were within 94.3–106.3% and 1.0–9.6% respectively. The mean recoveries of triprolidine, pseudoephedrine and gabapentin were 93.6, 76.3 and 82.0% respectively. Stability of triprolidine and pseudoephedrine was assessed under different storage conditions. The validated method was successfully employed for the bioequivalence study of triprolidine and pseudoephedrine formulation in twenty six volunteers under fasting conditions.

1. Introduction

Triprolidine (Fig. 1a) and pseudoephedrine (Fig. 1b) are chemical compounds used in combination for the treatment of allergic rhinitis [1].

Actifed® is a drug product, composed of triprolidine (2.5 mg) and pseudoephedrine (60.0 mg per tablet) is used as antihistaminic drug, it antagonizes central and peripheral H-1 receptors, drying nasal and sinus passages (non-selective antihistamine) [2–4]. Pseudoephedrine stimulates smooth muscle alpha-adrenergic receptors, producing vasoconstriction and reducing nasal congestion (sympathomimetic) [5]. Actifed® was used as a reference drug product in a clinical pharmacokinetic study.

Several analytical methods have been described for the determination of active ingredients (triprolidine and pseudoephedrine) alone in human plasma and formulation. Pseudoephedrine has been determined in plasma or in formulation using several techniques like LC–MS/MS [6,7], simultaneously with other drugs by LC–ion trap-MS [8,9], HPLC-UV [10] HPLC-RF [11], micellar electrokinetic chromatography [12]. The limit of quantitation of pseudoephedrine ranged from 1.25 to 10.0 ng/mL in these methods. Triprolidine has also been determined in plasma and formulation by different methods involving HPLC, GC and high performance thin layer chromatography [13–17]. The limit of quantitation ranged from 1.0 to 5.0 ng/mL in plasma. These drugs were also determined in formulation by HPLC-UV method [15]. To the best of our knowledge, there has been no reported analytical method in the literature taking in consideration simultaneous determination of both drugs in human plasma; herein it is necessary to establish a new analytical method for the simultaneous determination of triprolidine and pseudoephedrine.

The importance of LC–MS technique in the bioanalytical methods came from its high sensitivity and selectivity together with a short run time in-order to analyze a large number of plasma samples especially if it contains more than one targeted drug products intended for clinical study. Direct precipitation procedure has been published to quantify pseudoephedrine alone in plasma samples

* Corresponding author. Tel.: +962 788 259894/79 5653403.
E-mail addresses: ashokshakya@hotmail.com, ashok2811@yahoo.com (A.K. Shakya), tawfiqarafat@yahoo.com (T.A. Arafat).

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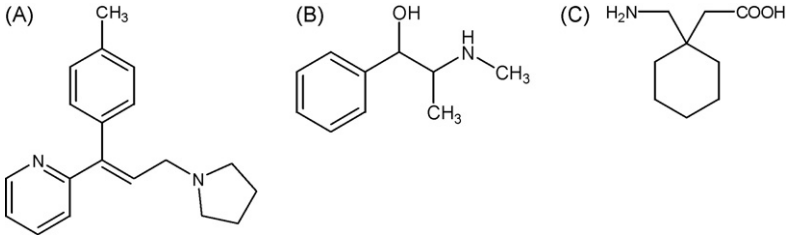

Fig. 1. Chemical structure of (a) triprolidine, (b) pseudoephedrine and (c) gabapentin (IS).

[8]. Protein precipitation procedure is quick, simple and economical as compared to multiple steps sample processing procedures, like liquid–liquid extraction [18] or solid phase extraction [19]. Triprolidine has also been extracted by several techniques or analyzed by direct injection of plasma sample [20–23], but till date no published paper has been documented for the analysis of triprolidine from direct precipitation of plasma samples. To the best of our knowledge triprolidine in human plasma has never been quantified by LC–MS technique. To find a convenient, rapid, economical, sensitive and selective method for simultaneous determination of triprolidine and pseudoephedrine in human plasma, we have developed and validated LC–ESI-MS method.

2. Experimental

2.1. Chemicals

Triprolidine HCl (purity 99.75 %) drug substance, pseudoephedrine (99.90%), and gabapentin (99.71 %) (IS) were obtained from United Pharmaceuticals (Amman, Jordan). LC–MS quality deionized water and methanol (Lichrosolv®) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. The blank plasma was collected from Blood Bank, Islamic Hospital, Amman, Jordan. Plasma was obtained by centrifugation of blood treated with sodium heparin.

2.2. Standard solutions

Stock solutions (1.0 mg/mL) of triprolidine, pseudoephedrine and gabapentin (IS) were prepared in methanol, and solutions were stored refrigerator (4–8 °C). These solutions were further diluted in 70% methanol to give appropriate working solutions used to prepare the calibration and quality control samples.

2.3. LC–MS–MS instrument and conditions

For Chromatographic analysis, Thermo-Finnigan SPECTRA System® was used, the instrument equipped with a constant binary solvent delivery pump (P2000), on-line vacuum degasser (SCM 1000), an injector (Rheodyne 7125, holding 100 μl loop) linked up with an auto-sampler (AS3000), supported with a tray cooling system and column oven. Data acquisition, instrument control, quantitation were carried out by a Finnigan™ Xcalibur® 1.4 Data Management software (Thermo-Finnigan, San Jose, CA, USA).

Separation of the targeted compounds was made on a C-18 ACE® Column (50.0 mm × 2.1 mm i.d., 5 μm, Advance Chromatography Technologies, Aberdeen, UK), at 25 ± 1 °C, the analytical column was preserved by a Phenomenex C-18 guard column (4.0 mm × 2.0 mm i.d., 1.5 μm, Phenomenex, USA). The mobile phase consisted of water–methanol–formic acid (55:45:0.5, v/v/v) and delivered with a constant flow-rate of 0.3 mL/min throughout the analyses.

A quadruple ion trap mass spectrometer (Finnigan™ LCQ Advantage Max, Finnigan Thermo electron corporation, USA) equipped with an ESI source (Finnigan™), protected by a built-in waste/detector switcher valve, was used for the analysis of targeted compounds. The optimum parameters were obtained by an automatic tuning while a built-in infusion pump is continuously supplying the ESI source with 1.0 μg/mL of triprolidine and 5.0 μg/mL of pseudoephedrine separately in methanol, aided by a normal HPLC flow via a T-connecter in the infusion mode. Enhanced signal was attained by a sheath gas (Nitrogen) with a flow of 34 units (units refer to arbitrary values set by the LCQ software) and 350 °C heated capillary temperature. The spray voltage was set at 4 kV. Collection time for the ion trap was set at 200 ms and no cross-talk was found between transitions.

A positive scan mode spectrum showed a strong ion mass signal for a mono-protonated molecule $[MH]^+$ of triprolidine, at m/z 279.1, for pseudoephedrine m/z 165.9, and for gabapentin (IS) found at m/z 172.0, these masses were detected in the selected ion monitoring scan mode (SIM), and in a subsequent stage these parent ion molecules were fragmented by a helium collision gas in the ion trap under 56, 46 V of collision energy for triprolidine and pseudoephedrine respectively, to produce significant ion daughter fragments. The collision energy for gabapentine (IS) was 46 V. The mass spectra resulting from these fragmentation processes were acquired in the selected reaction monitoring (SRM) scan mode at m/z 208.1, 148.0, 154.0 for triprolidine, pseudoephedrine and IS respectively. These product ions were monitored and selected for quantification of both target drugs depending on the analytical signal area ratio of triprolidine over IS, and pseudoephedrine over IS.

2.4. Sample processing

A 200 μL volume of plasma was transferred to an Eppendorf tube, 200 μL of 5% w/v trichloroacetic acid (containing 0.16 μg/mL IS) was added to the sample while gentle vortexing. The mixture was vortexed for 30 s using a Vibrax Type VX-Z, VXR Basic Vortexer (IKA-Werke GmBH & Co. Staufen, Germany) and then centrifuged using Multitude Sigma1-15 (Sigma, Germany) for 5 min at 14,000 rpm. The supernatant was transferred to an auto-sampler micro-vial and 2 μL was injected into the analytical column.

2.5. Bioanalytical method validation

The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, matrix effect and stability according to USFDA guideline [24].

2.5.1. Standard calibration curves and quality control samples

Standard curves were prepared in human plasma to yield final concentrations of 0.2, 0.4, 0.8, 2.0, 4.0, 12.0 and 20.0 ng/mL of triprolidine and 5.0, 10.0, 20.0, 50.0, 100.0, 300.0 and 500.0 ng/mL of pseudoephedrine. Similarly, quality control samples were prepared in pooled plasma at concentration of 0.2 (LLOQ), 0.6 (low), 10.0 (mid) and 16.0 (high) ng/mL for triprolidine and 5.0 (LLOQ), 15.0 (low), 250.0 (mid) and 400.0 (high) ng/mL for pseu-

doephedrine. Working solution of gabapentin (IS, 0.16 μg/mL) was prepared daily in 5% w/v trichloroacetic acid by diluting stock solution.

The lowest concentration for both calibration curves was considered to be low limit of quantitation (LLOQ). All the calibration plasma samples were divided into aliquots and stored in deep freezer at $-70 \pm 5\,^{\circ}C$ until analysis. Calibration curves were constructed from a blank sample (a plasma sample processed without an IS), a zero sample (a plasma processed with IS) and seven non-zero samples covering the total range including lower limit of quantification (LLOQ).

Validation runs were conducted on three separate days, each validation run consisted of a set of spiked standard samples of seven concentrations over the concentration range ($n = 5$, at each concentration), LLOQ, QC samples at three concentrations, low, medium and high ($n = 10$, each concentration), blank and zero samples. Calibration samples were analyzed from low to high concentration at the beginning of each validation run and the other samples were distributed randomly through the run, except the blank plasma samples which were placed after the high calibration sample. Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle is able to avoid any carry forward of injected sample in the subsequent runs. The stability and the freeze–thaw samples were analyzed on the day three along with other validation samples. Linearity was assessed by a weighted ($1/x$) least-squares regression analysis. The calibration curve had to have a correlation coefficient (r) of 0.999 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%. At least 67% of non-zero standard should meet the above criteria including LLOQ and upper limit of quantitation [24].

2.5.2. Accuracy and precision

Within-batch accuracy and precision evaluations were determined by analyzing ten sets of quality control samples in a batch. The between-batch precision and accuracy were determined by analyzing ten sets of quality control samples on three different days. The quality control samples were randomized daily, processes and analyzed in position either (a) immediately following the standard curve, (b) in the middle of batch or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% for LLOQ and 15% for the other concentrations.

2.5.3. Recovery and matrix effect

Recovery of triprolidine and pseudoephedrine from the precipitation procedure was determined by a comparison of peak area of drugs in processed spiked plasma samples as described in Section 2.4 (low, medium and high quality controls) with the peak of drugs in unprocessed samples prepared by spiking supernatant drug free plasma samples with the same amount of triprolidine and pseudoephedrine at the step immediately prior to chromatography.

Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turns affects the amount of charged ion in the gas phase, which ultimately reaches the detector. Matrix effect was checked with five different lots of plasma. Five samples each of LQC, MQC and HQC were prepared by directly spiking the analytes into reconstitution solution (mobile phase) with or without the presence of residue extracted from the different lots of plasma, ion suppression or enhancement was assessed by comparing the mean analyte peak area obtained from these sets of testing samples. It is considered there is no matrix effect if the deviation of the mean test responses were within 15% of freshly prepared or comparison samples. Matrix effect was calculated [25,26] as per the following equation:

$$\text{Matrix effect} = \left[\left(\frac{\text{analyte peak area of extracted plasma residue}}{\text{analyte peak area of neat solution}}\right) \times 100\right] - 100$$

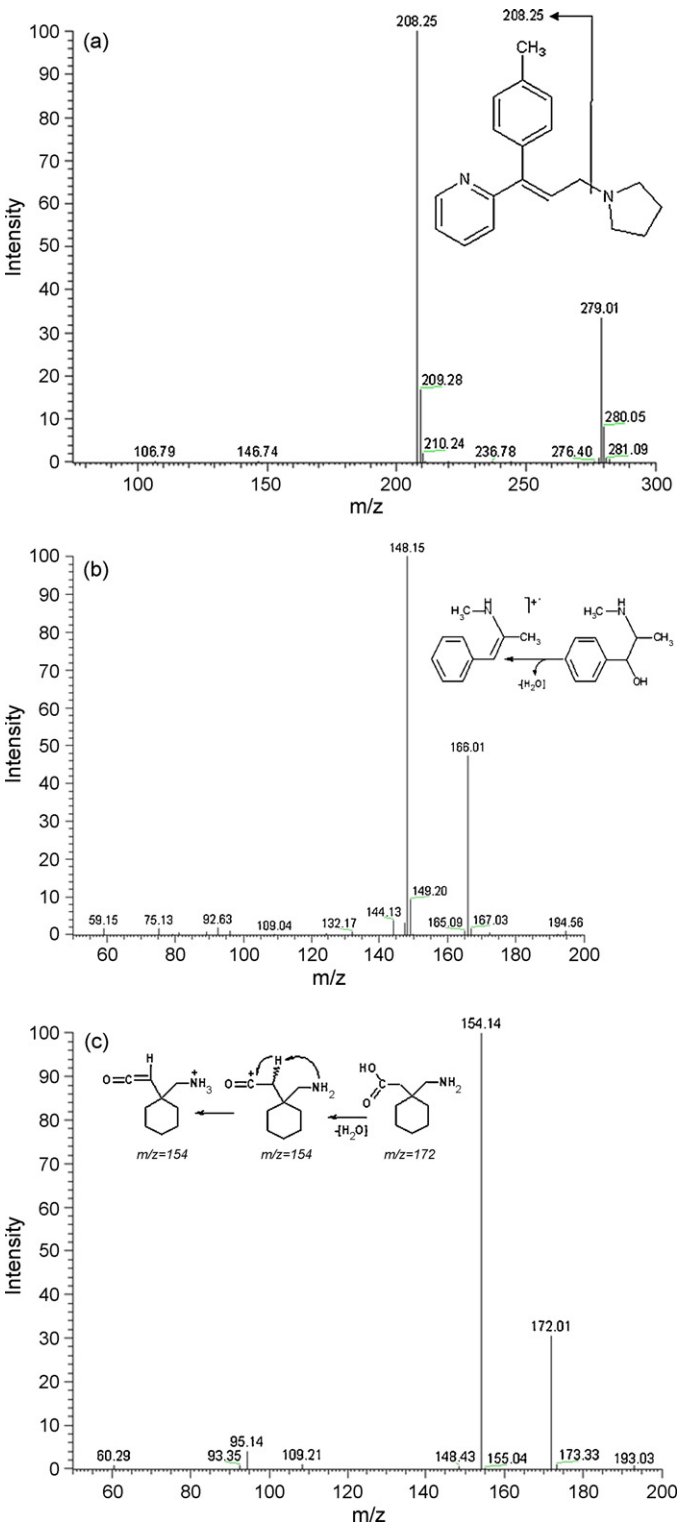

Fig. 2. Full-scan spectra showing product ion of $[M+H]^+$ of: (a) triprolidine; (b) pseudoephedrine and (c) gabapentin (internal standard).

Table 1
Mean peak area and analyte-to-IS peak area ratio of triprolidine (0.2 ng/mL) and pseudoephedrine (5 ng/mL) in six different lots of human plasma (heparin).

Plasma lot	Triprolidine mean peak area	IS mean peak area	Analyte/IS ratio	Pseudoephedrine mean peak area	IS mean peak area	Analyte/IS ratio
Lot 1	202,552 (4.8%)	5,639,726 (4.7%)	0.0359 (0.7%)	732,051 (6.3%)	5,542,782 (4.9%)	0.1320 (1.8%)
Lot 2	206,484 (4.2%, 1.9%)	5,672,873 (3.1%, 0.6%)	0.0364 (1.5%, 1.3%)	746,413 (4.43%, 1.9%)	5,626,836 (3.8%, 1.5%)	0.1326 (1.5%, 0.5%)
Lot 3	207,663 (3.9%, 2.5%)	5,735,990 (3.7%, 1.7%)	0.0362 (1.3%, 0.5%)	735,041 (3.9%, 0.4%)	5,521,257 (4.8%, −0.4)	0.1332 (1.3%, 0.9%)
Lot 4	203,859 (4.9%, 0.6%)	5,612,395 (4.0%, −0.5%)	0.0363 (1.5%, 0.3%)	732,009 (4.29%, −0.01%)	5,535,769 (3.7%, −0.1%)	0.1322 (1.3%, 0.2)
Lot 5	199,451 (6.5%, −1.5%)	5,518,381 (4.6%, −2.2%)	0.0361 (1.9%, 0.5%)	712,071 (2.5%, −2.7%)	5,201,342 (4.3%, −6.2%)	0.1361 (1.2%, 3.1%)
Lot 6	198,624 (5.4%, −1.9%)	5,525,228 (5.7%, −2.0%)	0.0360 (1.0%, 0.4%)	740,640 (3.7%, 1.2%)	5,464,512 (4.1%, −1.4%)	0.1356 (3.5%, 2.7%)

Values in parenthesis are %CV, $n = 6$; and % difference from Lot 1.

2.5.4. Specificity

Specificity is the ability of an analytical method to differentiate and quantify the analyte in presence of other components in the sample. The specificity of the method was evaluated by screening six different lots of blank plasma. These lots were spiked with known concentration of analytes (LLOQ). The spiked samples were analyzed after protein precipitation to confirm lack of interference and absence of lot-to-lot variation.

2.5.5. Stability

The bench top stability was examined by keeping replicates of the low, mid and high quality control samples at room temperature for approximately 12 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h. Auto-sampler stability of triprolidine and pseudoephedrine was tested by analysis of processed and reconstituted low, mid and high quality control samples, which were stored in the auto-sampler tray for 24 h. Stability of triprolidine and pseudoephedrine was tested after storage at approximately −70 °C for 30 days. For each concentration and storage condition, three replicates were analyzed in one analytical batch. The concentrations of triprolidine and pseudoephedrine after each storage period were related to the initial concentration as determined for the samples.

2.5.6. Stock solution stability

The stability of stock solution was tested and established at room temperature for 2, 24 h and under refrigeration (4–8 °C) for 30 days.

2.6. Clinical application

The developed and validated LC/MS method was applied to investigate a bioequivalence study of Actifed®, the reference product (Batch No. 0424077 expiry date 06/2010, Pfizer, Walton-on-the Hill, Surrey, US) versus Unifed® the test product (Batch No. 3685, expiry date 06/2009, United Pharmaceuticals, Jordan) in 26 Jordanian male volunteers (age 18–40 years, mean 28.88 ± 7.73). Each tablet containing 2.5 mg of triprolidine and 60 mg of pseudoephedrine (*complete data on file*, *JCPR*, *Amman*).

3. Result and discussion

3.1. Internal standard

A stable analyte has to be used as an IS to deal with sample matrix effects. Since such internal standard is not available commercially, an alternative approach has been used. Internal standard chosen should match the chromatographic properties, recovery and ionization properties of the analyte [27]. Gabapentin was found to match these criteria and also serve our purpose of method development, therefore it was chosen as an internal standard. Gabapentin is having –COOH and $–NH_2$ group, it is easily protonated after neutral loss under experimental conditions (Fig. 2c). Gabapentin was selected because of its same recovery as compared to the drugs. The result indicates that the IS did not alter or deteriorate the performance of the proposed method, also the intensity of triprolidine and pseudoephedrine molecular ion peaks in mass spectrometry analysis remained unaffected as compared to others.

3.2. Separation and specificity

LC–MS detection mode exhibits a high selectivity, and no interferences were observed. Triprolidine, pseudoephedrine and IS gave protonated molecules $[M+H]^+$ in the positive MS mode, the major ions observed in the ESI spectrum were at m/z 279.1 for triprolidine, m/z 165.9 for pseudoephedrine, and m/z 172.0 for gabapentin (IS). Fig. 2 shows mass spectrums for the most intense molecular ion of the analytes with their product ions fragments. A significant product ions fragments were observed in the SRM spectra that are $m/z = 208.1$, 148.0, 154.0 for triprolidine, pseudoephedrine and IS respectively.

The chromatographic conditions, were optimized through several trials to achieve symmetric peaks shapes for the analytes and the IS, as well as short run time. It was found that a mixture of water–methanol–formic acid (55:45:0.5, v/v/v), could achieve this purpose and was finally adopted as the mobile phase.

The development of the current method was focused on the short run time to assure high throughput, with minimum matrix effects as well as good peak shapes. The retention times of triprolidine, pseudoephedrine and IS were ∼2.75, 1.65 and 1.8 min respectively. The specificity of the method was examined by analyzing six different blank human plasma precipitated ($n = 6$). The degree of interference was assessed by inspection of SRM chromatograms. No significant interfering peaks from the serum were found at the retention time and in the ion channel of either the analyte or the IS. The peak area of triprolidine (0.2 ng/ml), pseudoephedrine (5 ng/ml), along with analyte/IS peak area ratio from each lot of plasma, is shown in Table 1. It was observed that there was no significant enhancement or suppression of MS response of the analyte signals. It was interesting to note that the result of triprolidine, pseudoephedrine and its internal standard were very similar in all six lots of plasma. The coefficient of variation (%CV) observed for the analytes and IS peak area among six replicate of samples in each of these six lots of plasma was less than 6.5%.

A small unidentified peak was observed in one of the blank chromatogram of triprolidine, which did not have a significant impact on the analyte quantification. The interference observed at the retention time of analyte was less than 1% of the area of LLOQ. The product ion chromatogram extracted from the plasma and volunteer sample are depicted in Fig. 3a which shows an LC/MS chromatogram of blank plasma indicating no endogenous peaks at retention time of triprolidine, pseudoephedrine and gabapentin; (b) human plasma spiked with LLOQ of both drugs (0.2 ng/mL of triprolidine and 5.0 ng/ml of pseudoephedrine) and IS; (c) an extracted volunteer plasma sample after 1.0 h following oral administration of Actifed®. Both drugs were unambiguously identified and were quantitated (back-calculated) as 5.22 and

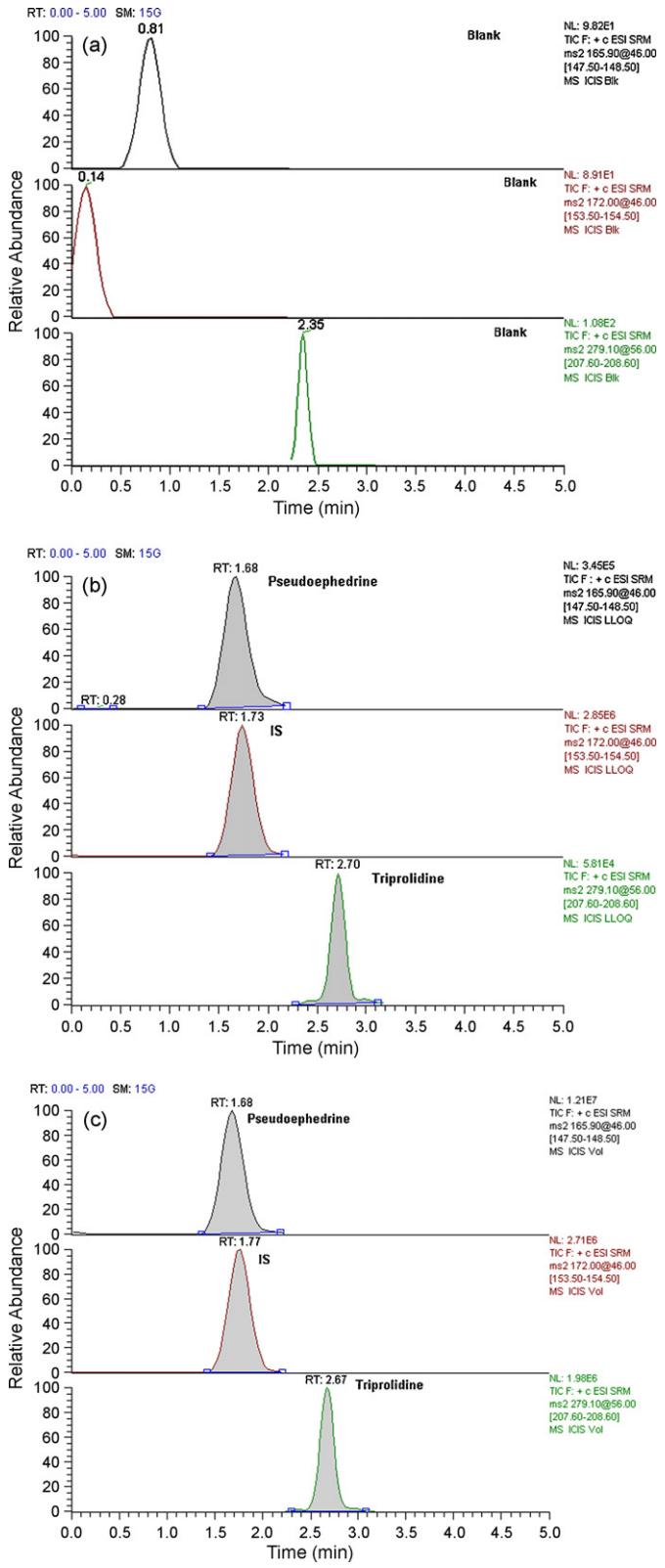

Fig. 3. LC–MS–MS chromatograms of: (a) human plasma blank, (b) LLOQ for both drugs and (c) volunteer sample.

Table 2
Statistical evaluation of the analysis results for triprolidine and pseudoephedrine in standard curves ($n = 5$).

Concentration added (ng/mL)	Precision (%)	Bias (%)
Triprolidine		
0.2	13.6	3.8
0.4	6.4	4.6
0.8	5.1	4.4
2.0	5.5	3.1
4.0	4.9	1.3
12.0	4.4	2.2
20.0	4.7	2.4
Pseudoephedrine		
5.0	7.3	2.6
10.0	4.1	4.5
20.0	1.9	−1.8
50.0	3.4	5.4
100.0	4.8	0.0
300.0	1.4	−1.3
500.0	1.1	1.0

223.67 ng/mL for triprolidine and pseudoephedrine respectively. There was no response found in blank plasma after high calibration sample injection, which indicates no carryover of the analyte in subsequent runs.

3.3. Linearity and limit of quantitation

The peak area ratios of both drugs to IS in human plasma were linear with respect to the analyte concentration over the calibration range (0.2–20 ng/mL for triprolidine, 5.0–500 ng/mL for pseudoephedrine). The heteroscedasticity of the data was determined with unweighted and weighted regression of assay data across the whole concentration range. The regression parameters of the calibration curve were generated for unweighted and weighted ($1/x$) and the respective $\sum|\%RE|$ were calculated. The weighting factor $1/x$ calculated from the peak area ratio gave smallest $\sum|\%RE|$ than unweighted factor. The calibration curves were calculated by weighted least-squares linear regression analysis ($1/x$) of the analytes versus areas ratio of the target drugs to that of the IS concentrations. The mean linear regression equation ($y = mx + c$) of calibration curve for the triprolidine and pseudoephedrine were $y = 0.0696\,x + 0.0218$ and $y = 0.02088x + 0.00839$ respectively. The correlation coefficient (r) was above 0.999 for both drugs over the concentration range used. The limit of quantitation was 0.2 and 5.0 ng/mL ($n = 5$) for triprolidine and pseudoephedrine respectively. At these concentrations the signal to noise ratio is approximately 10:1 and 500:1 for both drugs respectively. The precision, characterized by the relative standard deviation were 13.6 and 7.3 % for triprolidine and pseudoephedrine respectively (Table 2).

Table 3
Accuracy and precision of LC–MS–MS (SRM mode) assay method for pseudoephedrine and triprolidine.

Concentration added (ng/mL)	Within-batch ($n = 10$)		Between-batch ($n = 30$)	
	Precision (%)	Bias (%)	Precision (%)	Bias (%)
Triprolidine				
0.2 (LLOQ)	4.0	2.0	4.5	1.2
0.6 (low)	1.9	0.8	6.6	2.8
10.0 (medium)	2.6	0.7	9.6	2.9
16.0 (high)	4.0	1.5	3.5	6.3
Pseudoephedrine				
5.0 (LLOQ)	2.4	1.9	3.8	2.4
15.0 (low)	6.5	−5.7	5.6	−3.6
250.0 (medium)	3.9	0.4	6.3	−1.2
400.0 (high)	4.2	0.2	1.0	1.0

Table 4A
Extraction recovery of triprolidine, pseudoephedrine and gabapentin (IS) from plasma.

Analyte	Nominal concentration (ng/mL)	Recovery (mean ± SD) (%)	CV (%)	Matrix effect (%)
Triprolidine ($n = 5$)	0.6	90.2 ± 1.9	2.1	1.1
	10.0	99.2 ± 2.4	2.4	−0.4
	16.0	91.4 ± 5.5	6.0	−1.6
Pseudoephedrine ($n = 5$)	15.0	74.8 ± 3.8	5.1	−1.8
	250.0	80.5 ± 4.3	5.3	−2.6
	400.0	73.6 ± 1.4	1.9	−1.2
Gabapentin(IS) ($n = 5$)	160.0	82.0 ± 3.3	4.0	−0.8

3.4. Precision and accuracy

Precision is stated as the relative standard deviation and accuracy is reported as the percentage difference from the nominal value. Table 3 summarizes the mean values of accuracy and precision for both within and between days assays. Both precision and accuracy were within the acceptable ranges for bio-analytical purpose. Within-day precision ranged from 1.9 to 4.0% for triprolidine and 3.9 to 6.5% for pseudoephedrine. Between days precision ranged from 3.5 to 9.6% for triprolidine and 1.0–6.3% for pseudoephedrine. Within and between day relative errors (bias, %) were less than 2.0 and 6.3% for triprolidine, whereas for pseudoephedrine these were less than −5.7 and −3.6%. The precision for triprolidine and pseudoephedrine was ranged from 2.7 to 8.4%. The accuracy was ranged from 95.5 to 104.5%. The overall precision and accuracy were 4.4–6.1% and 98.7–102.7%. The result indicates good precision and accuracy during the analysis of study samples as well.

3.5. Extraction recovery and matrix effect

Recovery results indicate that the maximum recovery was achieved with triprolidine (93.6%) followed by pseudoephedrine (76.3%). The extraction recovery of gabapentin was (82.0%) (Table 4A). The extraction recovery was found to be satisfactory as it was consistent, precise and reproducible. Thus protein precipitation procedure used in this method proved to be efficient and simple enough to extract three drugs (including IS) simultaneously from human plasma.

The endogenous components are mainly the cause of ion suppression or enhancement effects during electro-spray ionization. The extent of this effect is mainly dependent on sample extraction procedure and also compound dependent [28]. The results indicated that the matrix components did not alter or deteriorate the performance of proposed method. Quality control samples at each level along with the set of calibration standards were analyzed, and the % bias of the samples analyzed was found within ±15% for each QC level for triprolidine and pseudoephedrine (Table 4B). Hence, this clearly proves that the elution of endogenous peaks during the run has no effect on the estimation of triprolidine and pseudoephedrine. Therefore, the method of extraction of triprolidine and pseudoephedrine from plasma was rugged enough and gave consistent and accurate result when applied to real volunteer samples.

3.6. Stability

Table 5 summarizes the result of stability study carried out under various conditions. Both the analytes were found to be stable at ambient temperature (20–30 °C) for at least 8 h in human plasma. Stability of plasma samples was performed as described earlier in the text.

The freeze–thaw stability results showed that triprolidine and pseudoephedrine were stable for at least three cycles. Stability results indicated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples. Processed samples were stable when kept in auto-injector (at 5 ± 1 °C) for up to 24 h without any changed in the concentration. QC samples were stable for at least 30 days if kept frozen at approximately −70 °C. Stock solution of triprolidine, pseudoephedrine and gabapentin were prepared in methanol, these solutions were stable for at least 3 months when stored in refrigerator. Results indicates reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria of ±15%. These findings indicates that storage of plasma samples for both drugs at −70 °C is adequate, and no stability-related problems would be

Table 4B
Matrix effect ($n = 5$) for triprolidine and pseudoephedrine.

Triprolidine										
Sr. No	Plasma Lot no.	LQC (0.6 ng/ml)			MQC (10.0 ng/ml)			HQC (16.0 ng/ml)		
		Mean concentration	% CV	% Bias	Mean concentration	% CV	% Bias	Mean concentration	% CV	% Bias
1	Lot-1	0.613	1.27	2.17	10.20	1.57	2.02	15.86	4.11	−0.88
2	Lot-2	0.606	1.54	1.00	9.96	5.88	−0.38	16.32	3.67	2.00
3	Lot-3	0.608	0.88	1.33	9.35	5.54	−6.48	15.50	4.44	−3.13
4	Lot-4	0.610	1.17	1.67	9.97	4.42	−0.30	14.88	0.76	−7.00
5	Lot-5	0.594	2.06	−0.96	10.29	1.82	2.90	16.12	3.07	0.75
Pseudoephedrine										
Sr. No	Plasma Lot No.	LQC (15 ng/ml)			MQC (250 ng/ml)			HQC (400 ng/ml)		
		Mean concentration	% CV	% Bias	Mean concentration	% CV	% Bias	Mean concentration	% CV	% Bias
1	Lot-1	14.41	3.27	−3.93	247.5	1.71	−1.00	387.2	3.24	−3.20
2	Lot-2	15.29	2.57	1.93	242.5	3.99	−3.00	394.0	4.09	−1.50
3	Lot-3	14.26	3.57	−4.93	241.4	4.84	−3.44	386.2	3.96	−3.45
4	Lot-4	14.55	4.31	−3.00	242.4	2.59	−3.04	396.2	1.89	−0.95
5	Lot-5	15.16	2.52	1.07	245.0	4.8	−2.00	411.8	3.89	2.95

Table 5
Stability of the triprolidine and pseudoephedrine quality control samples.

Sample concentration Triprolidine	Precision (%)	Bias (%)	Sample concentration Pseudoephedrine	Precision (%)	Bias (%)
Short term stability for 12h ($n=6$) in plasma					
0.6 (low)	3.9	8.2	15.0 (low)	8.2	0.1
10.0 (medium)	6.7	−2.9	250.0 (medium)	0.5	−7.0
16.0 (high)	2.0	−8.9	400.0 (high)	3.3	2.9
Three freeze and thaw cycles ($n=6$)					
0.6 (low)	3.1	−2.6	15.0 (low)	6.8	−1.8
10.0 (medium)	7.9	6.4	250.0 (medium)	0.7	1.8
16.0 (high)	1.9	1.6	400.0 (high)	2.1	5.2
Auto-sampler stability ($5\pm1\,^{\circ}C$) for 24h ($n=6$)					
0.6 (low)	3.3	0.2	15.0 (low)	5.8	3.0
10.0 (medium)	7.2	−3.3	250.0 (medium)	0.6	5.1
16.0 (high)	3.4	−6.4	400.0 (high)	4.8	4.3
30-days stability at $-70\,^{\circ}C$ ($n=6$)					
0.6 (low)	1.3	0.1	15.0 (low)	0.6	−2.0
10.0 (medium)	1.8	−2.3	250.0 (medium)	4.0	1.6
16.0 (high)	5.6	−0.5	400.0 (high)	4.0	4.0

expected during the routine sample analysis for pharmacokinetic, bioavailability or bioequivalence studies.

3.7. Application and clinical study

The validated method has been successfully used to estimate triprolidine and pseudoephedrine in human plasma samples after oral administration of single dose of Actifed® product versus test drug. The analyses were accomplished in accordance to the FDA bio-analytical method validation guidance. The mean plasma profile of 26 subjects for triprolidine and pseudoephedrine for both products are presented in Fig. 4 (*data on file*).

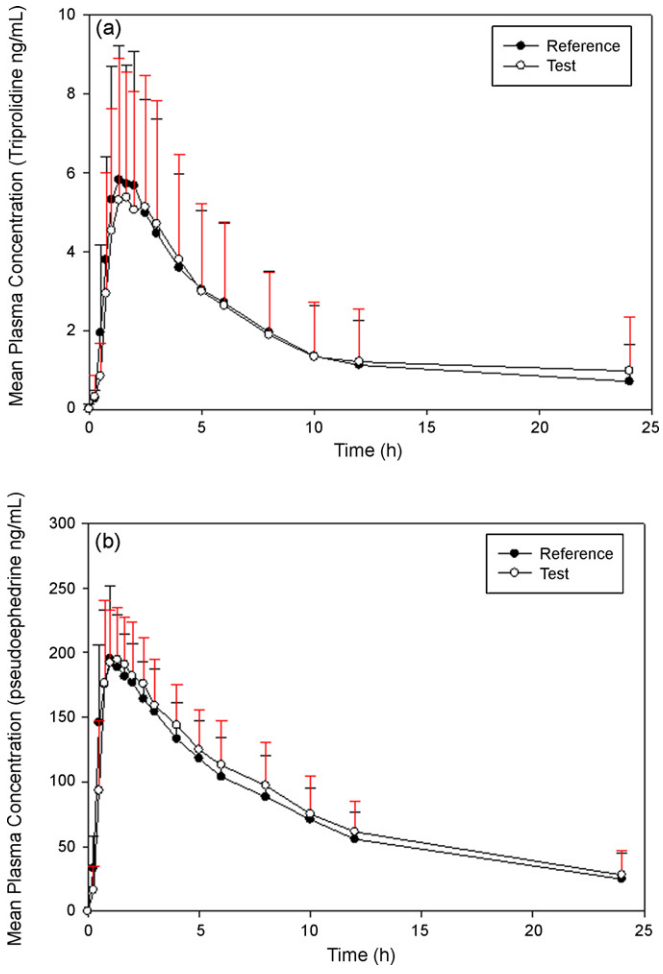

Fig. 4. Mean plasma concentration–time curve of (a) triprolidine and (b) pseudoephedrine tablet (reference and test) ($n=26$).

4. Conclusion

The developed LC–ESI-MS method is highly specific due to inherent selectivity of tandem mass spectrometry. The method demonstrates high throughput capability because of short run time required for analysis. The validated method presents a simple, rapid and cost effective sample treatment procedure with quantitative and reproducible recoveries of triprolidine and pseudoephedrine. No interference from endogenous plasma components or other sources were found and no 'cross-talk' effect was observed in plasma samples. The on-column loading of triprolidine (0.4 pg) and pseudoephedrine (10 pg) was very low as compared to other reported procedures for the determination of drugs in plasma [6–10,20]. Both the analytes were found to be stable in human plasma for 30 days when stored at $-70\,^{\circ}C$. A simple and convenient sample processing procedure makes this method more feasible for bioanalysis of triprolidine and pseudoephedrine in human plasma. Method described here is simple, selective, sensitive and fully validated as per guideline [24]. This method has shown acceptable precision, accuracy and adequate sensitivity for use in clinical studies. Also, the established LLOQ is sufficiently low to conduct the bioequivalence study of triprolidine and pseudoephedrine. The current method has been applied for the bioequivalence study of the Actifed® versus test formulation in healthy volunteers. This validated method allows quantification of triprolidine in the range of 0.2–20 ng/mL, and pseudoephedrine in the range of 5.0–500.0 ng/mL range.

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