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# Investigation of *ex vivo* stability of fesoterodine in human plasma and its simultaneous determination together with its active metabolite 5-HMT by LC–ESI-MS/MS: Application to a bioequivalence study

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ABSTRACT

Fesoterodine is a non-selective muscarinic-receptor antagonist, used in the treatment of overactive bladder syndrome. A highly sensitive, selective and rapid method has been developed for the simultaneous determination of fesoterodine and its active metabolite, 5-hydroxymethyl tolterodine (5-HMT) in human plasma by liquid chromatography–tandem mass spectrometry (LC–ESI-MS/MS). Due to rapid conversion of parent drug to 5-HMT, *ex vivo* stability of fesoterodine in human plasma was extensively studied to optimize the extraction protocol. The analytes and their deuterated analogs were quantitatively extracted from 100 μL human plasma by liquid–liquid extraction in methyl *tert*-butyl ether: *n*-hexane. The chromatographic separation of analytes was achieved on a Kromasil C18 (100 mm × 4.6 mm, 5 μm) column under isocratic conditions. The method was validated over a dynamic concentration range of 0.01–10 ng/mL for both the analytes. Ion-suppression effects were investigated by post-column infusion of analytes. The precision (% CV) values for the calculated slopes of calibration curves, which would reflect the relative matrix effect, were less than 1.5% for both the analytes. The intra-batch and inter-batch precision (% CV) across quality control levels varied from 1.82 to 3.73% and the mean extraction recovery was >96% for both the analytes. The method was successfully applied to a bioequivalence study of 8 mg fesoterodine tablet formulation (test and reference) in 12 healthy Indian subjects under fasted and fed condition. The assay reproducibility estimated by reanalysis of incurred samples showed a change of ±12.0%.

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## 1. Introduction

Overactive bladder is a debilitating and chronic disorder defined by collection of symptoms, in particular, urinary urgency with or without urgency urinary incontinence, usually accompanied by increased micturition frequency and nocturia [1,2]. This ailment affects both men and women equally, with approximately 12% overall prevalence rate (7–10% in individuals below 39 years and up to 21% in those with age ≥ 60 years) in USA, Canada and Europe [3,4]. Antimuscarinic agents like oxybutynin, propiverine, solifenacin, trospium chloride, tolterodine, darifenacin and fesoterodine are the first-line pharmacotherapy for overactive bladder treatment [5]. These muscarinic receptor antagonists are thought to mediate their clinical effects by blocking muscarinic receptors on the detrusor muscle, thereby inhibiting bladder contraction [6]. Fesoterodine (FESO) is relatively a new non-selective type antimuscarinic agent approved by US FDA in October 2008 for oral administration in the treatment of overactive bladder to relieve the symptoms of urinary urge incontinence, urgency, and frequency [7]. FESO functions as a prodrug and gets rapidly and extensively hydrolyzed by nonspecific esterases to its primary active metabolite 5-hydroxymethyl tolterodine (5-HMT), such that it is undetectable in blood after oral administration [3,8]. The antimuscarinic activity of FESO is solely due to 5-HMT, which is also the active metabolite of tolterodine. 5-HMT is formed *via* biotransformation of both fesoterodine and tolterodine, however, by different metabolizing enzymes [9]. Tolterodine is converted to 5-HMT by cytochrome P450 (CYP) 2D6 enzyme system, while conversion of FESO to 5-HMT bypasses the CYP system. Nevertheless, CYP3A4 and CYP2D6 are involved in further metabolism of 5-HMT in the liver to form three inactive metabolites namely carboxy (SPM 5590), carboxy-N-deisopropyl (SPM 7789), and N-deisopropyl

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metabolites (SPM 7790) [2]. FESO is available commercially in two active doses, 4 and 8 mg extended release fesoterodine fumarate tablet formulation under the brand name Toviaz™, Pfizer Canada Inc. The bioavailability of the active metabolite is 52% and has low plasma protein binding (~50%), mainly to albumin and α-1-acid glycoprotein. The peak plasma concentration of 5-HMT is reached in approximately 5 h after oral administration of the parent drug [10].

Literature reveals very few reports on the analysis of FESO and its active metabolite 5-HMT. The research group of Sangoi is actively involved in the analysis of FESO in commercial tablet formulations [11,12] and in development of stability-indicating methods [13,14]. A sensitive and rapid LC–MS/MS method was developed for the quantitative analysis of FESO in tablet formulation for quality control applications [11]. Similarly, a second order derivative UV spectrophotometric method was developed for determination of FESO in extended-release tablets and the results were validated by comparing with liquid chromatography, capillary electrophoresis and LC–MS/MS [12]. Further, two stability-indicating methods based on LC-UV and LC–MS/MS detection [13] and capillary electrophoresis [14] were validated under different stress conditions and applied in quality control analysis. Bioanalytical methods developed to study the pharmacokinetics of FESO are based on determination of 5-HMT concentration in plasma and urine [15,16]. In both these methods, 5-HMT was determined by LC–MS/MS with a lower limit of quantification of 0.02 ng/mL (in plasma) and 1.0 ng/mL (in urine) respectively. The chromatographic separation was achieved in a run time of 6 min. In another LC–MS/MS method [17], the concentration of 5-HMT and its metabolites, SPM 5509, SPM 7789 and SPM 7790 were determined in both these matrices. The calibration range was established from 0.04 to 40 ng/mL for 5-HMT and 0.1 to 100 ng/mL for each metabolite in plasma.

To the best of our knowledge there are no reports on the simultaneous determination of fesoterodine and its active metabolite, 5-HMT in biological samples. In the present work a reliable, sensitive and rapid LC–ESI-MS/MS method is developed and fully validated for the simultaneous determination of FESO and 5-HMT in samples prepared from human plasma as per US FDA guidelines. Due to rapid conversion of parent drug to its active metabolite, the stability of FESO in plasma was systematically investigated and based on which an optimized extraction protocol was defined using sodium metabisulphite as the stabilizing agent in whole blood. The effect of matrix on analyte quantification was evaluated by post-column infusion technique and by calculating the slopes of calibration lines from different plasma sources [18]. The developed method was applied to support a bioequivalence study of FESO in healthy Indian subjects. Further, the assay reproducibility for 5-HMT is successfully demonstrated through incurred sample reanalysis.

## 2. Experimental

### 2.1. Chemicals and materials

Fesoterodine fumarate (99.4%), fesoterodine-d14 fumarate (IS, 98.2%), 5-hydroxymethyl tolterodine (99.2%) and 5-hydroxymethyl tolterodine-d14 (IS, 98.4%) were obtained from Toronto Research Chemicals (North York, ON, Canada). Methanol and acetonitrile were procured from Avantor Performance Materials Inc. (Center Valley, PA, USA). Ammonium acetate, ammonium formate, ascorbic acid, sodium fluoride, sodium metabisulphite, pyridostigmine bromide (PB), phenylmethylsulfonyl fluoride (PMSF), potassium oxalate monohydrate, acetic acid, formic acid and hydrochloric acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade methyl *tert*-butyl ether (MTBE), ethyl acetate, dichloromethane, diethyl ether, *n*-hexane and sodium hydroxide were purchased from Merck Specialties Pvt. Ltd. (Mumbai, Maharashtra, India). Water used in the analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, Karnataka, India). Blank human blood was obtained from in-house facility.

### 2.2. Liquid chromatography and mass spectrometric conditions

A Shimadzu LC-VP HPLC system (Nakagyo-ku, Kyoto, Japan) with Kromasil 100-5 C18 (100 mm × 4.6 mm, 5 μm) analytical column from Eka Chemicals AB (Bohus, Sweden) was used for separation of analytes. For isocratic separation, the mobile phase consisted of 15 mM ammonium formate (pH 5.5, adjusted with formic acid)-acetonitrile (25:75, v/v) and was delivered at a flow rate of 1.0 mL/min. The column oven temperature was maintained at 30 °C and the auto sampler temperature was kept at 5 °C. The total eluant from the column was split in 50:50 ratio; flow directed to the ISP interface was equivalent to 500 μL/min.

A triple quadrupole mass spectrometer, AB SCIEX API-4000 (Foster city, CA, USA) was used for detection and quantitation of analytes and ISs under positive ionization mode. The precursor → product ion transitions of $m/z$ 412.5 → 223.2 (FESO), $m/z$ 342.3 → 223.3 (5-HMT), $m/z$ 426.4 → 223.2 (FESO-d14) and $m/z$ 356.3 → 223.1 (5-HMT-d14) were monitored in the multiple reaction monitoring (MRM) mode. Optimized mass parameters maintained for the analytes and ISs were, ion spray voltage: 5000 V; turbo heater temperature: 500 °C; collision activation dissociation: 5 psig and curtain gas, nitrogen: 25 psig. Declustering potential, collision energy, entrance potential, and cell exit potential were set at 74, 34, 10 and 12 V for FESO; 82, 42, 10 and 14 V for 5-HMT; 79, 38, 10 and 12 V for FESO-d14; 85, 44, 10 and 14 V for 5-HMT-d14 respectively. The dwell time was kept at 100 ms and Quadrupole 1 and 3 were maintained at unit mass resolution.

### 2.3. Treatment of blood samples

Whole blood samples from subjects were collected in $K_2$EDTA vacutainers containing 0.5 M sodium metabisulphite (10% of total blood volume) to stabilize FESO and were kept in wet ice bath. It was then rapidly centrifuged at 1811 × $g$ at 5 °C to separate the plasma, treated with 0.1 M HCl (5% of plasma volume) to avoid any possible conversion to 5-HMT and stored at −70 °C until use. The same pretreatment was given to the blank human blood and the harvested plasma for preparing calibration and quality control (QC) samples.

### 2.4. Calibration standards and quality control samples

Stock solutions (1000 μg/mL) of FESO and 5-HMT were prepared by dissolving accurately weighed reference standards in methanol. Their working solutions (100 μg/mL) used for spiking were prepared in acetonitrile:water (50:50, v/v). Calibration standards (CS) and QC samples were prepared by spiking pretreated blank plasma with working solutions (98 μL blank plasma was spiked with 2 μL of solution). CS1-8 for both the analytes were made at 0.010, 0.020, 0.50, 1.50, 5.00, 7.50, 9.00 and 10.00 ng/mL concentrations respectively, while QC samples were prepared at 10.00, 7.50, 5.00, 2.50, 0.030 and 0.010 ng/mL concentration. Stock solutions (1000 μg/mL) of the internal standards were prepared by dissolving 10.0 mg of FES-d14 and 5-HMT-d14 respectively in 10.0 mL of methanol. Their combined intermediate solution (1.0 μg/mL of each) was prepared by appropriate dilution of the stock solution in acetonitrile: water (50:50, v/v). Standard stock and working solutions used for spiking were stored at 5 °C, while

calibration curve and quality control samples in plasma were kept at −70 °C until use.

*2.5. Optimized sample preparation procedure*

Immediately after thawing, the plasma samples were kept in wet ice bath (below 5 °C) for further processing. To an aliquot of 100 μL of spiked pre-treated plasma sample, 25 μL of combined working solution (20 ng/mL each) of internal standards was added and vortexed for 10 s. Further, 25 μL of 50 mM ammonium acetate was added to maintain pH 6.0 ± 0.2 and vortex mixed for another 10 s. The analytes and ISs were extracted with 2.0 mL of MTBE: *n*-hexane (75:25, v/v) solvent mixture on a rotary mixer at 32 × *g* for 5 min. Thereafter, the samples were centrifuged at 1811 × *g* for 5.0 min at 5 °C. The supernatant was separated and evaporated to dryness in a thermostatically controlled water-bath maintained at 35 °C under a gentle stream of nitrogen. The dried samples were reconstituted with 100 μL of mobile phase and 5 μL were used for injection in the chromatographic system.

*2.6. Procedures for method validation*

US FDA guidelines were followed for all validation parameters [19]. System suitability experiment was performed by injecting six consecutive injections, using extracted standard mixture of FES & 5-HMT (10 ng/mL) and ISs (20 ng/mL for FES-d14 and 5-HMT-d14) at the start of each batch during method validation. System performance was studied by injecting one extracted lower limit of quantitation (LLOQ) sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover of analytes was experimentally determined by sequentially injecting the mobile phase solution → LLOQ sample → extracted blank plasma → upper limit of quantitation (ULOQ) sample → extracted blank plasma → LLOQ sample → extracted blank plasma.

The selectivity of the method toward endogenous plasma matrix components was assessed in 10 different batches which included 6 normal $K_2$ EDTA, 2 haemolysed and 2 lipemic blank plasma. Interference of commonly used medications by human volunteers was checked for paracetamol, chlorpheniramine, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (1000 μg/mL) were prepared by dissolving requisite amount in methanol. Their working solutions (10 μg/mL) were prepared and 5 μL was injected to check for any possible interference at the retention time of analytes.

The linearity of the method was ascertained by measuring the area ratio response (analyte/IS) for five calibration curves containing eight non-zero concentrations. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration curve having analyte response at least ten times more than that of drug free (blank) extracted plasma was accepted as the LLOQ.

For determining the intra-batch accuracy and precision, six replicates of QC samples along with calibration curve standards were analyzed on the same day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision (% CV) at each concentration level from the nominal concentration should not be greater than 15% and the accuracy should be within ±15%. Reinjection reproducibility was also checked by re-injecting one entire validation batch.

Ion suppression/enhancement effects on the MRM LC–MS/MS sensitivity were evaluated by the post column analyte infusion experiment [20]. A standard solution containing FESO, 5-HMT (at ULOQ level) and ISs was infused post column *via* a ‘*T*’ connector into the mobile phase. Aliquots of 5 μL of extracted control (blank) plasma were then injected into the column and MRM chromatograms were acquired for analytes and IS.

Extraction recovery for the analytes and ISs was calculated by comparing the mean area response of pre-spiked samples (spiked before extraction) to that of extracts with post-spiked samples (spiked after extraction) at three QC levels. Absolute matrix effect was assessed by comparing the mean area response of post-spiked samples (spiked after extraction) with mean area of neat standard solutions (in mobile phase). The ‘process efficiency’ (%PE) was calculated by comparing the area response of pre-spiked samples with the area of neat standard solutions [21]. Relative matrix effect was assessed from the precision (% CV) values in the measurement of slopes of calibration curves from ten plasma lots (including haemolysed and lipemic). For a method to be practically free from relative matrix effect the % CV should not be greater than 3–4% [18].

All stability results were evaluated by measuring the area ratio response (analyte/IS) of stability samples against freshly prepared comparison standards at two QC levels. Stock solutions of analytes and ISs were checked for short term and long term stability at 25 °C and 5 °C, respectively. The acceptance criterion was ±10.0% deviation from the nominal value. The autosampler (wet extract), bench top (at 25 °C), freeze–thaw (−20 °C and −70 °C) and long term (−20 °C and −70 °C) stabilities in plasma was also studied at both these levels. Whole blood stability (in presence of sodium metabisulphite) was also determined to ascertain any enzymatic degradation by spiking blood samples with analytes at the LQC and HQC levels for 2.0 h in wet ice bath. The samples were considered stable if the deviation from the mean calculated concentration of freshly prepared quality control samples was within ±15.0%.

Method ruggedness was evaluated on two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns (same make but different batch no.). Dilution reliability was determined by diluting the stock solution prepared as spiked standard at 20.0 ng/mL concentration for FESO and 5-HMT in the screened plasma. The precision and accuracy for dilution integrity standards at 1/5th and 1/10th dilution were determined by analyzing the samples against freshly prepared calibration curve standards.

*2.7. Pharmacokinetic/bioequivalence study and incurred sample reanalysis*

The objective of the study was to determine the single-dose average bioequivalence of 8 mg fesoterodine fumarate extended release tablets manufactured by an Indian Pharmaceutical Company, India with Toviaz (8 mg fesoterodine fumarate extended release tablet) manufactured for Pfizer Labs, NY, USA as test and reference formulation respectively. The primary end point or target variables of the study to determine the bioequivalence of test and reference products were $C_{max}$, $AUC_{0-72}$, and $AUC_{0-inf}$. However, as it was not possible to measure FESO concentration in plasma, the primary end points were analyzed based on 5-HMT using confidence interval approach. The secondary end points of the study included $AUC_{0-72}/AUC_{0-inf}$, $T_{max}$, $t_{1/2}$ and $K_{el}$. The design was an open label, balanced, randomized, two-treatment, two-period, two-sequence, crossover, and single dose bioequivalence study in 12 healthy adult Indian subjects in the age group of 20–42 years (height 161.2–184.6 cm, weight 53.2–86.3 kg, BMI 19.1–24.4 kg/m$^2$) under fasted and fed conditions. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. The study was conducted as per International Conference on Harmonization and USFDA guidelines [22]. The subjects for both the studies were fasted 10 h before administration of the drug formulation. For fed study, the subjects were given high fat and high calorie breakfast (consisting of 250 mL

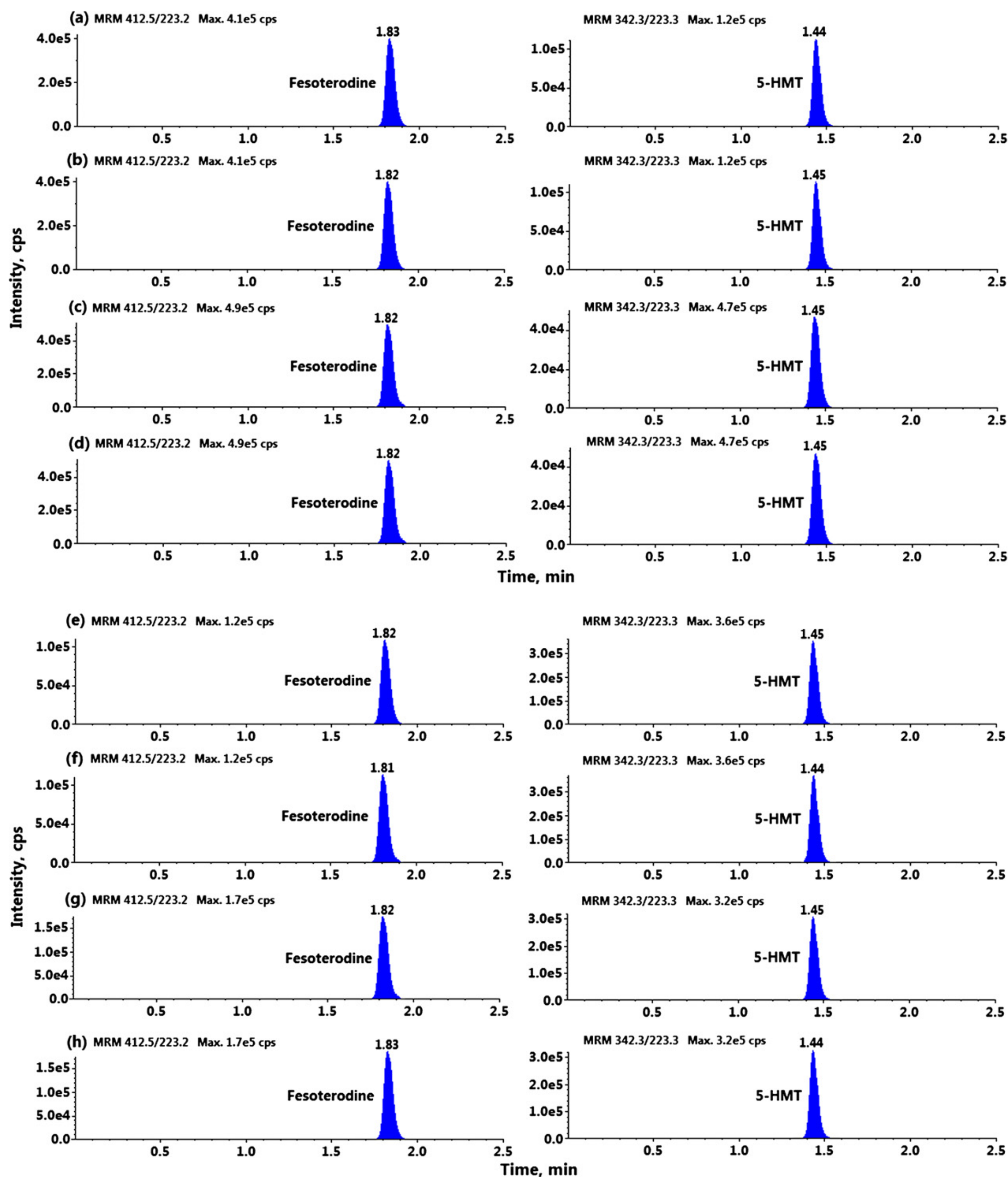


**Fig. 1.** MRM chromatograms showing *ex vivo* stability of fesoterodine in plasma ($m/z$ 412.5 → 223.2) under acidic (0.1 M HCl, a–d) and alkaline (0.01 M NaOH, e–h) conditions. (a and e) normal light at 25 °C, (b and f) yellow light at 25 °C, (c and g) normal light in wet ice bath (below 5 °C) and (d and h) yellow light in wet ice bath. Chromatograms in the right panel represent corresponding conversion to 5-HMT ($m/z$ 342.3 → 223.3).

**Table 1**
Mean extraction recovery trials for fesoterodine at MQC level in presence of different stabilizing agents by LLE in wet ice bath.

| No. | Stabilizing agent in whole blood (10% by volume) with $K_2EDTA$ as anticoagulant | Pretreatment of plasma/extraction pH with methyl *tert*-butyl ether: *n*-hexane (75:25, v/v) | Relative recovery (%) | Absolute matrix effect (%) | Process efficiency (%) |
|---|---|---|---|---|---|
| 1 | Ascorbic acid (2% in water) | 0.1 M HCl (5% of plasma volume)/6.0, adjusted with 50 mM ammonium acetate and acetic acid | 79.2 | 85.4 | 67.6 |
| 2 | Sodium fluoride (0.5 M in water) | | 84.3 | 87.6 | 73.8 |
| 3 | Phenylmethylsulfonyl fluoride (1.0 mM in water) | | 90.7 | 92.1 | 83.5 |
| 4 | Pyridostigmine bromide (10% in water) | | 92.1 | 90.1 | 83.0 |
| 5 | Sodium metabisulphite (0.5 M in water) | | 97.2 | 98.2 | 95.5 |

MQC, medium quality control (5.0 ng/mL); LLE, liquid–liquid extraction.

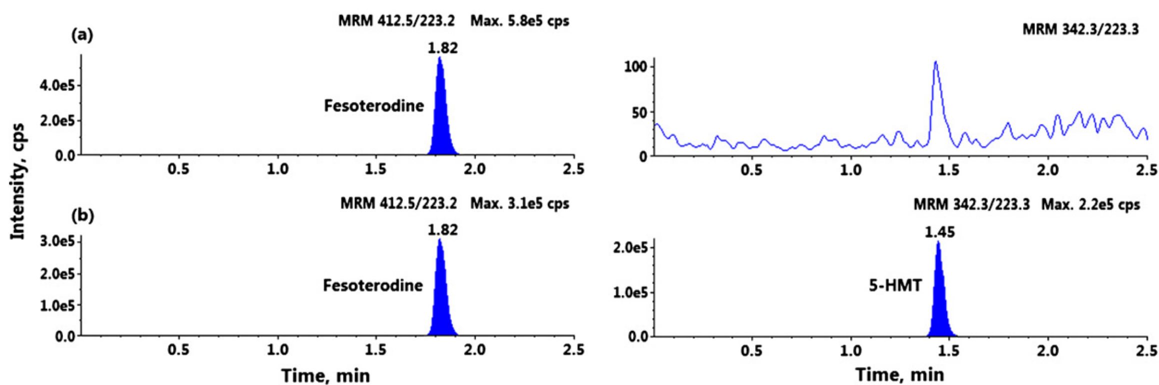


**Fig. 2.** MRM chromatograms showing the extent of conversion of FESO to 5-HMT in (a) presence and (b) absence of sodium metabisulphite in whole blood using the optimized extraction procedure at MQC level. The peak area response for FESO and 5-HMT in the presence and absence of sodium metabisulphite was (a) 2,030,238 and 324, (b) 1,085,384 and 748,642, respectively.

**Fig. 3.** Product ion mass spectra for (a) fesoterodine ($m/z$ 412.5 → 223.2, scan range 100–500 amu), (b) 5-HMT ($m/z$ 342.3 → 223.3, scan range 100–400 amu), (c) fesoterodine-d14 ($m/z$ 426.4 → 223.2, scan range 100–500 amu) and (d) 5-HMT-d14 ($m/z$ 356.3 → 223.1, scan range 100–400 amu) in positive ionization mode.

**Table 2**
Intra-batch and inter-batch precision and accuracy for fesoterodine and 5-HMT.

| QC ID | Nominal conc. (ng/mL) | Intra-batch | | | | Inter-batch | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *n* | Mean conc. observed (ng/mL)[a] | % CV | % accuracy | *n* | Mean conc. observed (ng/mL)[b] | % CV | % accuracy |
| *Fesoterodine* | | | | | | | | | |
| LLOQ QC | 0.010 | 6 | 0.009 | 3.06 | 98.0 | 30 | 0.010 | 3.73 | 101.0 |
| LQC | 0.030 | 6 | 0.029 | 3.68 | 99.7 | 30 | 0.029 | 2.54 | 99.7 |
| LMQC | 2.500 | 6 | 2.406 | 2.36 | 96.3 | 30 | 2.493 | 2.23 | 99.7 |
| MQC | 5.000 | 6 | 5.029 | 1.99 | 100.6 | 30 | 5.047 | 2.36 | 100.9 |
| HQC | 7.500 | 6 | 7.408 | 2.07 | 98.8 | 30 | 7.401 | 3.46 | 98.7 |
| ULOQ QC | 10.00 | 6 | 10.27 | 3.20 | 102.7 | 30 | 9.756 | 1.82 | 97.6 |
| *5-HMT* | | | | | | | | | |
| LLOQ QC | 0.010 | 6 | 0.009 | 3.09 | 97.0 | 30 | 0.010 | 2.94 | 102.0 |
| LQC | 0.030 | 6 | 0.030 | 3.61 | 101.7 | 30 | 0.029 | 3.38 | 98.7 |
| LMQC | 2.500 | 6 | 2.604 | 2.18 | 104.2 | 30 | 2.478 | 2.39 | 99.1 |
| MQC | 5.000 | 6 | 4.960 | 2.77 | 99.2 | 30 | 5.235 | 3.02 | 104.7 |
| HQC | 7.500 | 6 | 7.404 | 2.07 | 98.7 | 30 | 7.623 | 2.04 | 101.6 |
| ULOQ QC | 10.00 | 6 | 9.673 | 3.39 | 96.7 | 30 | 9.668 | 3.57 | 96.7 |

5-HMT, 5-hydroxymethyl tolterodine; CV, coefficient of variation; *n*, total number of observations.
[a] Mean of 6 replicates at each concentration.
[b] Mean of 6 replicates for five precision and accuracy batches.

**Table 3**
Absolute matrix effect, relative recovery and process efficiency for fesoterodine and 5-HMT.

| Analyte | *A* (% CV) | *B* (% CV) | *C* (% CV) | Absolute matrix effect $\left(\frac{B}{A}\right) \times 100$ | Relative recovery $\left(\frac{C}{B}\right) \times 100$ | Process efficiency $\left(\frac{C}{A}\right) \times 100$ |
|---|---|---|---|---|---|---|
| *LQC* | | | | | | |
| Fesoterodine | 12,734 (0.4) | 12,437 (0.8) | 12,056 (0.4) | 97.7 (96.5)[a] | 96.9 (95.4)[a] | 94.7 (92.7)[a] |
| 5-HMT | 11,621 (0.5) | 11,402 (0.6) | 10,987 (0.7) | 98.1 (97.2)[b] | 96.3 (95.5)[b] | 94.5 (92.2)[b] |
| *MQC* | | | | | | |
| Fesoterodine | 2,101,347 (0.8) | 2,063,782 (1.1) | 2,006,151 (0.6) | 98.2 (97.6)[a] | 97.2 (95.1)[a] | 95.5 (92.3)[a] |
| 5-HMT | 1,929,742 (0.5) | 1,892,168 (0.9) | 1,840,782 (0.8) | 98.0 (97.3)[b] | 97.3 (96.2)[b] | 95.4 (93.2)[b] |
| *HQC* | | | | | | |
| Fesoterodine | 3,148,973 (1.2) | 3,079,645 (0.7) | 3,005,814 (0.5) | 97.8 (96.9)[a] | 97.6 (96.7)[a] | 95.5 (93.1)[a] |
| 5-HMT | 2,885,382 (0.9) | 2,830,279 (1.0) | 2,739,538 (0.8) | 98.1 (97.3)[b] | 96.8 (96.1)[b] | 94.9 (92.4)[b] |

5-HMT, 5-hydroxymethyl tolterodine; *A*, Mean area response of six replicate samples prepared in mobile phase (neat samples); *B*, mean area response of five replicate samples prepared by spiking in extracted blank plasma; *C*, mean area response of five replicate samples prepared by spiking before extraction; CV, coefficient of variation.
[a] Values for fesoterodine-d14.
[b] Values for 5-HMT-d14.

**Table 4**
Stability of fesoterodine and 5-HMT under various conditions ($n = 6$).

| Storage conditions | Fesoterodine | | 5-HMT | |
|---|---|---|---|---|
| | Mean stability sample (ng/mL) ± SD | % change | Mean stability sample (ng/mL) ± SD | % change |
| *Bench top stability in wet ice bath; 6 h* | | | | |
| HQC | 7.634 ± 0.157 | 4.72 | 7.349 ± 0.192 | −2.33 |
| LQC | 0.0296 ± 0.0012 | −6.21 | 0.0301 ± 0.0008 | 4.95 |
| *Wet extract stability; 26 h* | | | | |
| HQC | 7.395 ± 0.147 | 5.93 | 7.820 ± 0.225 | −4.56 |
| LQC | 0.0304 ± 0.0005 | −1.42 | 0.0303 ± 0.0011 | 0.82 |
| *Dry extract stability in wet ice bath; 12 h* | | | | |
| HQC | 7.585 ± 0.124 | 7.11 | 7.197 ± 0.209 | 3.83 |
| LQC | 0.0298 ± 0.0009 | −6.44 | 0.0296 ± 0.0006 | 1.99 |
| *Freeze and thaw stability in plasma; 6 cycles, −20 °C* | | | | |
| HQC | 7.482 ± 0.179 | −5.29 | 7.385 ± 0.190 | 2.67 |
| LQC | 0.0302 ± 0.0004 | −1.79 | 0.0298 ± 0.0007 | 4.56 |
| *Freeze and thaw stability in plasma; 6 cycles, −70 °C* | | | | |
| HQC | 7.618 ± 0.190 | −7.03 | 7.280 ± 0.186 | 2.78 |
| LQC | 0.0301 ± 0.0011 | 5.62 | 0.0301 ± 0.0010 | −3.26 |
| *Long term stability in plasma; 122 days, −20 °C* | | | | |
| HQC | 7.703 ± 0.218 | 3.29 | 7.461 ± 0.160 | −5.79 |
| LQC | 0.0295 ± 0.000 7 | −4.81 | 0.0305 ± 0.0012 | 0.91 |
| *Long term stability in plasma; 122 days, −70 °C* | | | | |
| HQC | 7.195 ± 0.206 | −2.98 | 7.672 ± 0.215 | −7.89 |
| LQC | 0.0293 ± 0.0010 | −5.91 | 0.0299 ± 0.0009 | 5.03 |

SD, standard deviation; *n*, number of replicates at each level.

$$\% \text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

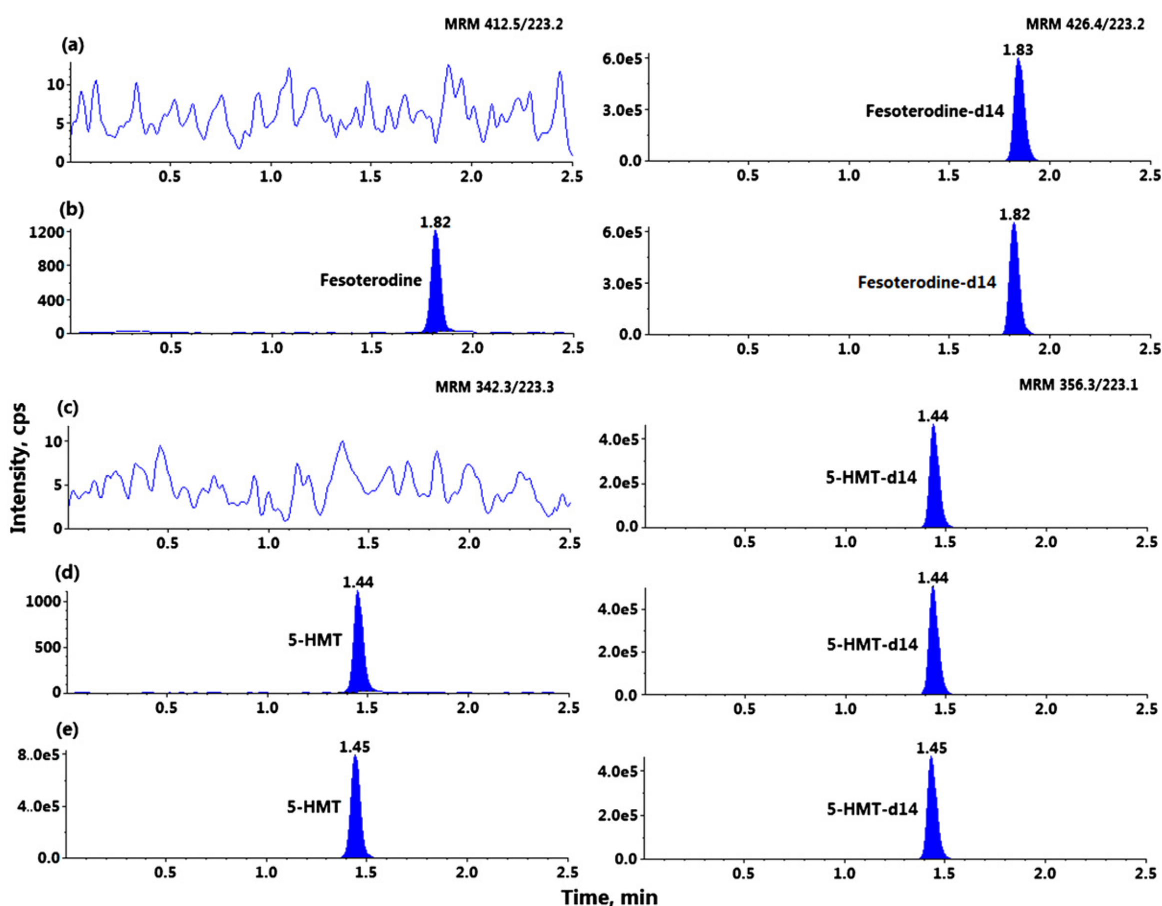


**Fig. 4.** MRM ion-chromatograms of (a) blank plasma and fesoterodine-d14, (b) fesoterodine at LLOQ and fesoterodine-d14, (c) blank plasma and 5-HMT-d14, (d) 5-HMT at LLOQ and 5-HMT-d14, and (e) 5-HMT in real subject sample at $C_{max}$ after administration of 8 mg dose of fesoterodine and IS.

milk with 5 g sugar, 35 g walnuts, two slices of bread with cheese and two cheese cutlets, total 969 calories) 30 min prior to giving the drug formulation. The subjects were orally administered a single dose of test and reference formulations with 240 mL of water after recommended wash out period of one week. Blood samples were collected at 0.00 (pre-dose), 0.17, 0.33, 0.50, 0.67, 0.83, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 10, 12, 15, 20, 24, 36, 48, 60 and 72 h after oral administration of the dose for test and reference formulation and treated as described in section 2.3 until analysis. During study, subjects had a standard diet while water intake was unmonitored. The pharmacokinetic parameters of 5-HMT were estimated by non-compartmental analysis using WinNonlin® software version 5.3 (Pharsight Corporation, Sunnyvale, CA, USA). The statistical analysis for pharmacokinetic parameters of 5-HMT included descriptive statistics, analysis of variance and two one-sided tests for bioequivalence using SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics involved calculation of least square geometric mean for $C_{max}$, $AUC_{0-72}$, and $AUC_{0-inf}$. Analysis of variance was carried out for least square means, the difference between the adjusted formulation means and the standard errors associated with the difference. Further, the 90% confidence interval for the difference of means between the two formulations least square means was calculated for the target variable using log transformed data. Similarly, power and ratio analysis was performed on the log transformed data. To determine whether the test and reference formulations were

**Table 5**
Mean pharmacokinetic parameters of 5-HMT after oral administration of 8 mg fesoterodine fumarate test and reference tablet formulation to 12 healthy Indian subjects under fasted and fed condition.

| Parameter | Fasted | | Fed | |
|---|---|---|---|---|
| | Test Mea ± SD | Reference Mean ± SD | Test Mean ± SD | Reference Mean ± SD |
| $C_{max}$ (ng/mL) | 6.82 ± 0.51 | 6.91 ± 0.63 | 7.12 ± 0.65 | 7.26 ± 0.78 |
| $T_{max}$ (h) | 4.91 ± 0.31 | 4.97 ± 0.42 | 5.06 ± 0.61 | 5.14 ± 0.47 |
| $t_{1/2}$ (h) | 6.87 ± 0.92 | 6.95 ± 1.03 | 7.14 ± 1.09 | 7.26 ± 1.22 |
| $K_{el}$ (1/h) | 0.1105 ± 0.032 | 0.1134 ± 0.026 | 0.1228 ± 0.043 | 0.1257 ± 0.039 |
| $AUC_{0-72}$ (h ng/mL) | 106.73 ± 10.57 | 107.56 ± 11.93 | 109.23 ± 11.88 | 110.76 ± 12.47 |
| $AUC_{0-inf}$ (h ng/mL) | 108.68 ± 11.31 | 109.79 ± 12.43 | 111.46 ± 12.26 | 112.93 ± 13.04 |

$C_{max}$, maximum plasma concentration; $T_{max}$, time point of maximum plasma concentration; $t_{1/2}$, half life of drug elimination during the terminal phase; $K_{el}$, elimination rate constant; $AUC_{0-t}$, area under the plasma concentration–time curve from 0 h to 72 h; $AUC_{0-inf}$, area under the plasma concentration–time curve from 0 h to infinity; SD, standard deviation; 5-HMT, 5-hydroxymethyl tolterodine.

pharmacokinetically equivalent, $C_{max}$, $AUC_{0-72}$, and $AUC_{0-inf}$ and their ratios (test/reference) using log transformed data were assessed; their means and 90% CIs were calculated. The drugs were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90% confidence intervals (CI) for these parameters are within 0.8–1.25.

An incurred sample re-analysis (ISR) was also conducted by selection of 10% of total 672 subject samples near $C_{max}$ and in the elimination phase of the pharmacokinetic profile of 5-HMT. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. As per the acceptance criterion the percent change in the values should not be more than ±20% [23].

## 3. Results and discussion

### 3.1. Method development

The prodrug FESO undergoes rapid hydrolysis by ubiquitous, non-specific esterases to its active metabolite, 5-HMT *in vivo* in all animal species (rats, rabbit, and mouse) and humans. In these species, its remains undetected in plasma and thus 5-HMT is considered as the active principle of FESO and also as a major metabolite. However, dog does not hydrolyze fesoterodine to the same extent as observed in the other species hence; fesoterodine can be detected in dog plasma together with other metabolites [24]. Also, FESO is relatively a new drug having no method for its direct determination (other than for 5-HMT and other metabolites) in plasma and there is no data available on the pharmacokinetics (fast or fed) in Indian population, which is genetically different from Caucasian and Black subjects. Thus, it was thought to develop a method which measures both, the pro-drug and its active metabolite simultaneously. To achieve this, the stability of FESO was extensively evaluated to optimize the extraction conditions for their simultaneous determination in human plasma. Sangoi et al. [13] have developed a stability-indicating LC method by subjecting FESO tablet solution to different stress conditions of acid and basic hydrolysis, oxidation, photolysis and thermal degradation. Their study revealed that the rate of degradation of FESO under ambient temperature was much faster in alkaline conditions (0.01 M NaOH) compared to acidic conditions (2.0 M HCl). Amongst several FESO degradation products identified, those that were formed due to hydrolysis of the ester linkage (corresponding to 5-HMT, *m*/*z* 342), oxidation of the hydroxymethyl group (carboxy metabolite of 5-HMT, *m*/*z* 356) and by the elimination of hydroxymethyl group and subsequent formation of a piperidine ring due to beta-cleavage of diisopropylamine group (*m*/*z* 365) were observed under all stress conditions. Based on these results, different strategies for stabilizing FESO were tried in the present work; these included lowering of temperature and pH, use of antioxidants and inhibitors. Initially, extraction was carried out under acidic (0.01–0.1 M HCl) and alkaline (0.01 M NaOH) conditions to assess the extent of FESO conversion in plasma samples. These included extraction under normal light (laboratory conditions) at room temperature (25 °C), yellow light (589 nm) at 25 °C, in wet-ice bath (below 5 °C) under normal and yellow light respectively. Under alkaline conditions, significant degradation (85–90%) of FESO to 5-HMT was observed under laboratory conditions at room temperature. There was practically no signal corresponding to the parent drug up to CS-4 (1.50 ng/mL). Moreover, for higher concentrations (CS-5 to CS-8) the response was very low (25–35% of the expected area response) as well as inconsistent. Comparatively less (∼15–20%) conversion was seen in presence of 0.1 M HCl at all CSs and QC levels under identical conditions. Fig. 1a–h shows the *ex vivo* stability of FESO (5.0 ng/mL) in plasma under acidic and alkaline conditions respectively. Sample processing under yellow light in acidic medium had little impact, while extraction in wet ice bath afforded minor improvement in extraction recovery of FESO (∼8–10%). Although the combined effect of acidic conditions and lower temperature (in wet ice bath) was useful to an extent, nevertheless, it was not possible to completely circumvent degradation/conversion to 5-HMT. Thus, to overcome this challenge different stabilizing agents such sodium fluoride, phenylmethylsulfonyl fluoride and pyridostigmine bromide used as enzyme inhibitors and antioxidants like ascorbic acid and sodium metabisulphite were tried during blood sample collection and storage to arrest degradation. These stabilizers have been used previously for different classes of analytes with potential for instability in biological fluids [25]. Phenylmethylsulfonyl fluoride and sodium fluoride are effective inhibitors for cholinesterase and carboxylesterase activity, while pyridostigmine bromide is a powerful and reversible acetylcholinesterase inhibitor. Additionally, acidification of plasma samples with 0.1 M HCl helped in preventing conversion of FESO during processing in wet ice bath. Thereafter, liquid–liquid extraction of analytes was tried in ethyl acetate, MTBE, *n*-hexane-ethyl acetate, *n*-hexane-dichloromethane and *n*-hexane-MTBE solvent systems in different proportions. Although there was significant improvement in recovery with much less conversion for almost all the stabilizers, nevertheless, quantitative and consistent results (at all QC levels) were obtained with sodium metabisulphite using MTBE: *n*-hexane (75:25, v/v) as the extraction solvent (Table 1). Encouraging results obtained with the antioxidant sodium metabisulphite compared to other enzyme inhibitors suggests that hydrolysis by esterases may not be the sole cause for rapid conversion/degradation of FESO. Perhaps the use of sodium metabisulphite is supportive at some stage in the drying down of extracts during sample preparation by LLE. Fig. 2 shows the extent of conversion of FESO to 5-HMT in the presence and absence of sodium metabisulphite in whole blood using the optimized extraction procedure at MQC level.

The analytes and ISs were efficiently ionized with electrospray ionization in the positive ion mode. The tuning of MS parameters was carried out to generate sufficient and consistent response for the analytes and ISs by infusing 400 ng/mL solution of each. Full scan Q1 MS spectra for FESO, 5-HMT, FESO-d14 and 5-HMT-d14 predominantly contained protonated precursor $[M+H]^+$ ions at *m*/*z* 412.5, 342.3, 426.4 and 356.3 respectively. The most abundant and stable product ions in Q3 MS spectra for the analytes and ISs were observed at *m*/*z* 223.2, 223.3, 223.2 and 223.1 respectively as reported previously [11,26]. For FESO, the product ion was formed due to the loss of water molecule, diisopropylamine group and cleavage of the C—O ester bond from the protonated precursor ion as shown in Fig. 3a. Similar fragmentation pathway was observed for 5-HMT with the elimination of diisopropylamine group and a water molecule to give a major product ion at *m*/*z* 223.3 (Fig. 3b). A dwell time of 100 ms was adequate and no cross talk was observed between the MRM of analytes and ISs.

Chromatographic conditions were suitably optimized for adequate retention and separation of FESO and 5-HMT. Existing methods have used Symmetry Shield C8 [16,17] and C18 [26] columns for separation and quantitation of 5-HMT along with its metabolites and tolterodine respectively in plasma samples. Sangoi et al. [13] have used Onyx C18 monolithic column for FESO stress degradation studies. Thus, in the present work several reversed-phase columns like Inertsil ODS-3 (50 mm × 4.6 mm, 3 μm), Discovery C18 (50 mm × 4.6 mm, 5 μm), XTerra MS C18 (150 mm × 4.6 mm, 5 μm), Gemini C18 (150 mm × 4.6 mm, 5 μm) and Kromasil C18 (100 × 4.6 mm, 5 μm) column were tested during

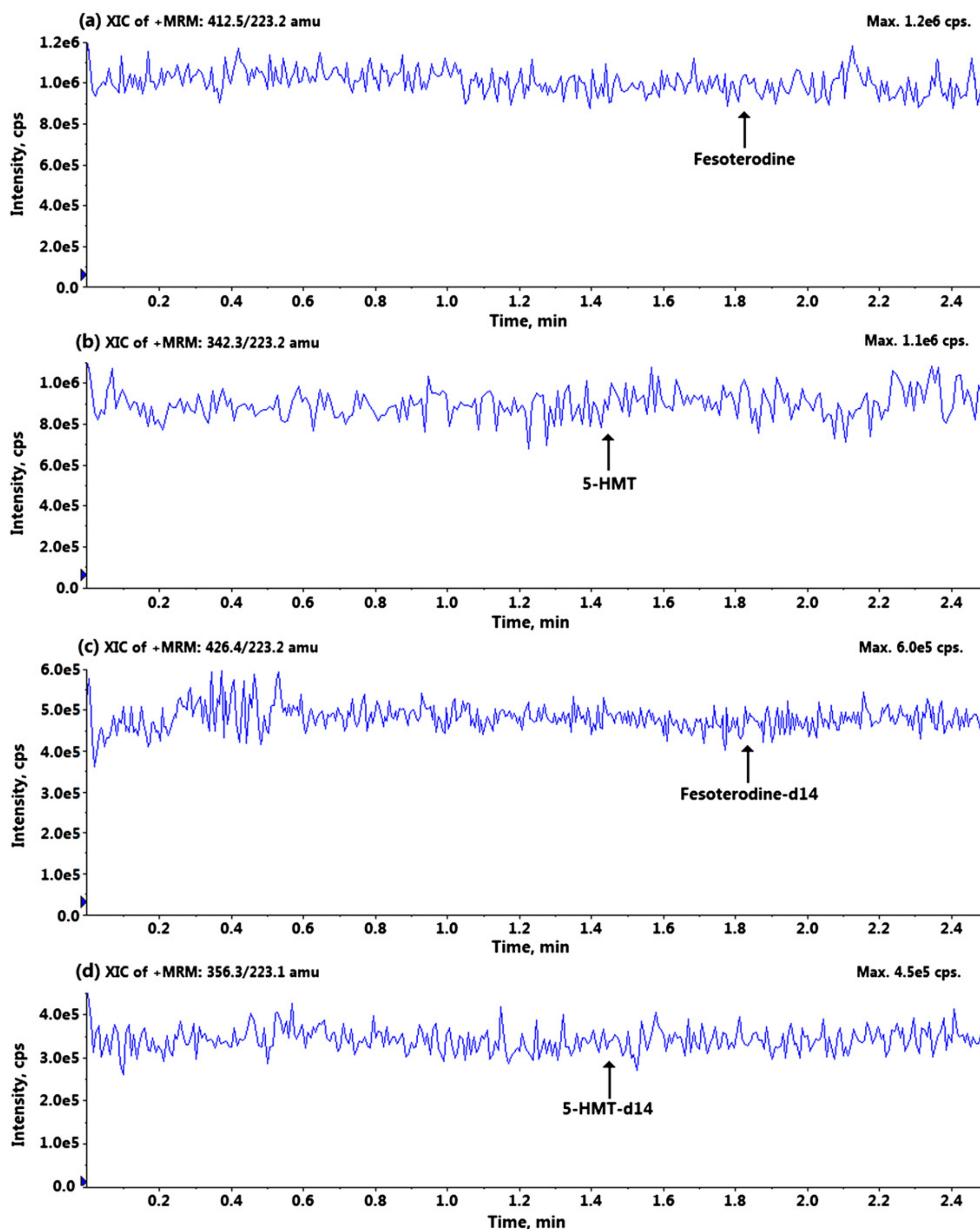


**Fig. 5.** Post column infusion chromatograms for (a) fesoterodine, (b) 5-HMT, (c) fesoterodine-d14 and (d) 5-HMT-d14.

trials for shorter run time, symmetric peak shape and for minimum interference from matrix components. Further, the mobile phase composition of acetonitrile/methanol-ammonium formate buffer pH (3.0–6.0) and flow rate were suitably optimized to get adequate retention and separation of analytes. Although the analytes were baseline separated in all the columns, nonetheless, either the peak shape or the response was not adequate on Inertsil ODS-3, Discovery C18, XTerra MS C18 and Gemini C18 columns. Kromasil C18 column showed excellent peak shape, resolution (*Rs* 2.53) and response for both the analytes and hence was selected in the present work. The mobile composition of 15 mM ammonium formate (pH 5.5, adjusted with formic acid)-acetonitrile (25:75, v/v) at a flow rate of 1.0 mL/min gave a retention time of 1.82 and 1.44 min for FESO and 5-HMT respectively. Additionally, reinjection reproducibility (for retention time) expressed as % CV was ≤0.8% for 100 injections on the same column.

The chromatograms in Fig. 4a–e of blank plasma spiked with IS, FESO and 5-HMT at LLOQ and an actual subject sample at $C_{max}$ demonstrates the selectivity of the method to differentiate and quantify the analytes from endogenous components in the plasma matrix or other components in the sample. None of the commonly used medications by human volunteers interfered at the retention of analytes and ISs.

### *3.2. Validation results*

#### *3.2.1. System suitability and auto-sampler carryover*

The precision (% CV) of system suitability test was observed in the range of 0.11–0.24% for the retention time and 0.75–0.98% for

the area response of both the analytes and ISs. The signal to noise ratio for system performance was ≥50 for both the analytes and ISs. Auto-sampler carry-over evaluation was performed to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carry over (≤0.10%) observed during auto-sampler carryover experiment.

#### 3.2.2. Linearity, intra-batch and inter-batch accuracy and precision

Both the analytes showed good linearities ($r^2 \geq 0.9980$) through the studied concentration range of 0.01–10 ng/mL. The mean linear equations for calibration curve concentrations were $y = (1.86e{-}2 \pm 2.97e{-}3)x - (3.29e{-}3 \pm 2.20e{-}4)$ and $y = (1.97e{-}1 \pm 3.16e{-}3)x - (7.00e{-}3 \pm 4.00e{-}4)$ for FESO and 5-HMT respectively. The accuracy and precision (% CV) observed for the calibration curve standards ranged from 97.1 to 104.8% and 1.27 to 4.01% respectively for both the analytes. The lowest concentration (LLOQ, 0.01 ng/mL) in the standard curve was measured at a signal-to-noise ratio (S/N) ≥ 50. The LOD value obtained was 0.003 at S/N ≥ 10 for both the analytes.

The intra-batch and inter-batch precision and accuracy results at six QC levels are shown in Table 2. The intra-batch precision (% CV) ranged from 1.99 to 3.68 and the accuracy was within 96.25–104.16% for both the analytes. Similarly for inter-batch experiments, the precision varied from 1.82 to 3.73 and the accuracy was within 96.68–104.70%.

#### 3.2.3. Recovery and ion suppression

The extraction recovery, absolute matrix effect and process efficiency data is presented in Table 3. The average recovery for both the analytes varied from 94.32 to 98.81 at all levels. The process efficiency/absolute recovery obtained for both the analytes and ISs was ≥89% across QC levels. The coefficient of variation (% CV) of the slopes of calibration lines for relative matrix effect in ten different plasma lots did not exceed 1.5% for both the analytes. Post-column infusion chromatograms in Fig. 5 indicate no ion suppression or enhancement at the retention time of analytes and ISs. The average matrix factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the LLOQ level was between 0.99 and 1.01 for both the analytes.

#### 3.2.4. Stability, dilution reliability and method ruggedness

Samples for short term and long term stock solution stability remained unchanged up to 32 h and 121 days respectively for both the analytes and ISs. Bench top stability of analytes in acidified plasma (wet ice bath) was established up to 6 h and for minimum of six freeze and thaw cycles at −20 °C and −70 °C. Auto sampler stability of the spiked quality control samples maintained at 5 °C was determined up to 26 h without significant loss of analytes. Spiked acidified plasma samples stored at −20 °C and −70 °C, for long term stability experiment were found stable for a minimum period of 122 days. For blood sample stability the percentage change was less 4.3% at both the QC levels. The detailed results for stability study are presented in Table 4.

The precision (% CV) for dilution reliability of 1/5th and 1/10th were between 1.14 and 2.76%, while the accuracy results were within 96.3 and 103.6% respectively for both the analytes, which is within the acceptance limit of 15% for precision (% CV) and 85–115% for accuracy. For method ruggedness the precision (% CV) and accuracy values for two different columns ranged from 2.08 to 2.84% and 97.4 to 102.1% respectively at all six QC levels. For the experiment with different analysts, the results for precision and accuracy were within 1.24–3.55% and 97.4–102.8% respectively at these levels for both the analytes.

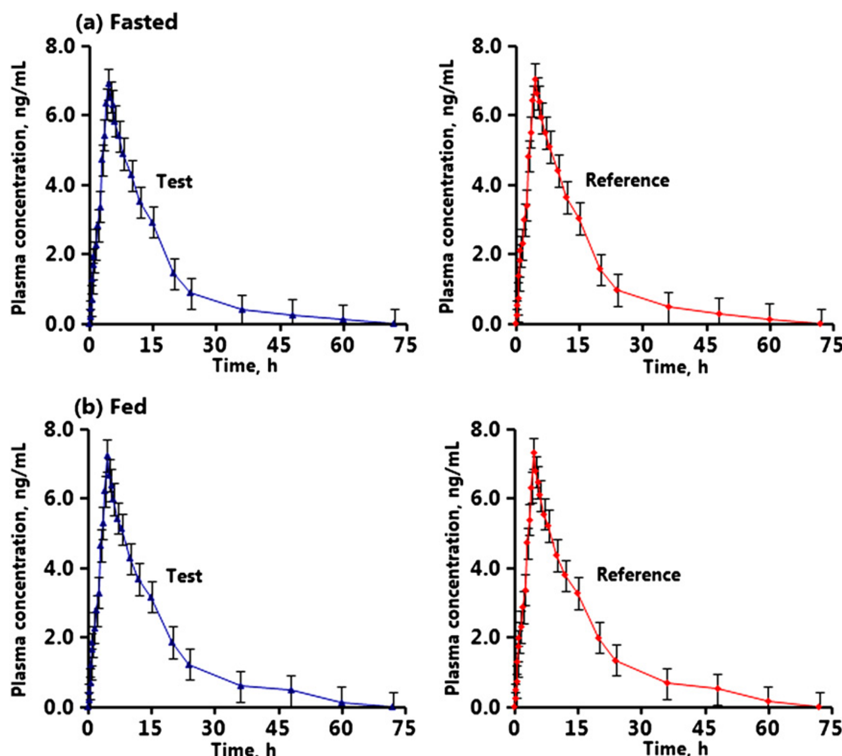


**Fig. 6.** Mean plasma concentration-time profiles of 5-HMT under (a) fasted and (b) fed condition after oral administration of test (8 mg fesoterodine fumarate extended release tablets of an Indian Pharma Company) and a reference (Toviaz® extended release tablets containing 8 mg fesoterodine fumarate, distributed by Pfizer Labs, NY, USA) formulation to 12 healthy Indian male volunteers with standard errors.

### 3.3. Application of the method to subject sample analysis and ISR results

So far there are no reports on the pharmacokinetics of fesoterodine fumarate in healthy Indian volunteers. The proposed validated method was successfully applied to quantify 5-HMT (formed from FESO) in human plasma samples after oral administration of 8 mg fesoterodine fumarate in healthy Indian subjects. The study was conducted with a higher dose based on draft guidance on FESO fumarate [27]. Fig. 6 shows the plasma concentration *vs.* time profile for 5-HMT under fasted and fed conditions. The sampling schedule was extended up to 72 h, to cover 10 half lives (half life ~7.0 h, wash out period of 7 days) for more accurate assessment of total AUC. Additionally, in our pilot study we had total 28 time points, which included 10 time points during the absorption phase. Sampling was done every 10 min up to 1.0 h during the absorption phase to have greater probability of finding the pro-drug if any. Nevertheless, it was not possible to detect FESO concentration in this study.

More than 2500 samples including the calibration, QC and volunteer samples were run and analyzed during a period of 12 days and the precision and accuracy were well within the acceptable limits. Table 5 summarizes the mean pharmacokinetic parameters obtained for 5-HMT after oral administration of test and reference formulation. As evident the mean $T_{max}$ and $t_{1/2}$ values were not influenced by food, while there was a marginal increase in the $C_{max}$ (fed/fast, ~1.04) and AUC values (fed/fast ~1.03) after high-fat and high-calorie diet. This observation is significantly different from a previous report in healthy white males which showed much higher values for $C_{max}$ (1.30) and $AUC_{0-t}$ values (1.18) in the fed compared to fasted condition [9]. This could be due to several factors such as race of subjects, gender, type of food and others. However, there was no significant variability in the pharmacokinetic parameters expressed as minimum and maximum exposure values as reported previously [28]. The ratios of mean log-transformed parameters and their 90% confidence intervals varied from 95.1 to 101.9%, which is within the acceptance range of 80–125%. The precision (% CV) values for intra-subject variation were within 3.0–8.0% for $C_{max}$, $AUC_{0-t}$ and $AUC_{0-inf}$ for 5-HMT under fasted and fed conditions.

ISR can work as a tool to assess whether the validated method, developed with spiked samples, is truly a representative of study sample. The importance of ISR study can be envisaged from its role in clinical as well as in non-clinical studies. Out of 72 reanalyzed incurred samples, 31 samples showed a change for assay reproducibility within ±5% while the remaining 41 samples were within ±5.0–12%. This authenticates the reproducibility of the proposed method.

## 4. Conclusions

To the best of our knowledge this is the first report for the simultaneous determination of FESO and its active metabolite 5-HMT in samples prepared from human plasma by LC–MS/MS. Due to instability of the parent drug and its rapid conversion to 5-HMT, a thorough study has been done with different stabilizing agents and other extraction conditions to optimize the extraction protocol. The method is adequately sensitive, selective and rapid (chromatographic run time of 2.5 min), requiring 100 μL plasma volume for processing. The lower limit of quantitation (LLOQ, 10 pg/mL) was purposely lowered as compared to previous reports (Refs. [15,16]) in anticipation of measuring fesoterodine during the absorption phase if found present by use of stabilizing agents in whole blood of subjects. Although it was not possible to detect fesoterodine in healthy Indian subject samples (under fasted as well as fed conditions), however, this method can be useful in determining fesoterodine in other biological fluids. The limit of quantification is sufficient to monitor at least five half-lives of FESO and 5-HMT concentration with good intra and inter-assay reproducibility. The reproducibility of study data has been successfully demonstrated through incurred sample reanalysis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.11.010.

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