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Original Article

Bioanalysis and Validation of Fesoterodine, an Anti-muscarinic Agent and its Active Metabolite Using Liquid Chromatography with Tandem Mass Spectrometry

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ABSTRACT

Aim: To establish a bioanalytical method with rapid, specific and sensitiveness with tandem mass spectrometric with liquid chromatography for an anti-muscuranic agent, fesoterodine and its metabolite, 5-hydroxy methyl tolterodine in plasma of human, using their isotopic labeled compounds correspondingly, with internal standards, fesoterodine d_{14} and 5-hydroxy methyl tolterodine d_{14}. **Materials and Methods:** The extraction of analytes is by employing tert-butyl methyl ether, divided with Kromasil 100 column C_{18} using the mixture of mobile phase containing methanol and 5 mM ammonium formate buffer (PH 3.5) at a rate of 1 mL/min. The outstanding linearity was shown in the range of concentrations, 0.1022 to 15.0154 and 0.1022 to 15.0211 ng/mL for fesoterodine and its metabolite simultaneously. The lower limit of quantitation values is 0.1022 and 0.1022 ng/mL respectively for fesoterodine and 5-hydroxy methyl tolterodine. **Results:** The accuracy and repeatability results for four different batches at five different concentration levels were discovered to be in the limits of acceptability for ICH guidelines. **Conclusion:** Stability tests, including benchtop, injector, freeze-thaw cycles, and -20ºC storage, confirmed fesoterodine and its metabolite stability in plasma. The chromatographic elution time of 2.5 min enabled the analysis of 300 samples rapidly in a day for the established technique. Validation results qualified the method for regular analysis and pharmacokinetic studies of fesoterodine and 5-hydroxy methyl tolterodine, its metabolite in the human biological fluid, plasma.

Keywords: 5-hmt, Antimuscuranic agent, Fesoterodine, Freeze thaw cycles, Human plasma, LC-MS/MS, Pharmacokinetic.

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INTRODUCTION

Fesoterodine is a competitive muscarinic receptor antagonist provened by US FDA to administer orally to address excessive bladder activity to alleviate the signs of urgent urination, frequent urination, and desire for incontinence, in 2008 (Figure 1).[1] Fesoterodine fumarate is sold commercially as Toviaz.[2] Fesoterodine Fumarate chemically is isobutyric acid 4-(hydroxymethyl) phenyl ester hydrogen 2-((R)-3-diisopropylammonium-phenylpropyl) Fumarate. The molecular weight is 527.66 and the empirical recipe is $C_{30}H_{41}NO_7$.[3]

Fesoterodine, in the form of extended-release tablets, increases bladder capacity, reduces urgency sensation, and decreases incontinence episodes.[4] After administration orally, Fesoterodine experiences quick and thorough hydrolyses by unspecific esterases converting to active metabolite which has accountability for antimuscarinic activity of fesoterodine.[5] Actually, tolterodine and fesoterodine have the same metabolite 5-Hydroxymethyl Tolterodine (5-HMT),[6] but they differ in their metabolism as fesoterodine metabolises by esterases and tolterodine metabolises through the CYP2D6 enzyme system.[7,8] This makes fesoterodine's metabolism more predictable, allowing for better dose tailoring based on individual responses and reducing the risk of drug interactions.[9,10] Thus, fesoterodine designed as a prodrug, and it ensures consistent exposure to 5-HMT after oral intake, with rapid conversion by esterases makes it effective for over active bladder treatment.[11] Fesoterodine's predictable response, less lipophilic profile which allowed for higher dosages,[12-17] regardless of genetic factors, offers advantages in variability, effectiveness, and reduced central nervous system penetration risk compared to tolterodine. Recently, in phase 3 study,[8] Fesoterodine demonstrated a favorable benefit-risk profile and improvement

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of maximum cystometric bladder capacity in child patients with neurogenic detrusor overactivity, made it a viable treatment option for this condition.

From the literature review, UV spectrometric,[18] HPLC[19-21] in formulation and dissolution samples and reported few LC-MS/MS[22-24] techniques for the estimation of fesoterodine in biological plasma.[11,25,26] The pharmacokinetic analysis of the fesoterodine metabolite in plasma and urine was conducted using tandem liquid chromatography by Malhotra (2009) with a deuterated isotope as the internal standard, ensuring 0.02 ng/mL for plasma as lower limits of quantification and for urine is 1.0 ng/mL. From the available literature to date one LC-MS/MS (Jignesh M. Parekh *et al*. 2013)[26] concluded the determination of fesoterodine and its 5-HMT in plasma of human. This technique optimized to *ex vivo* stability and extraction conditions due to the fast conversion of fesoterodine to its metabolite. This approach involved using methyl tert-butyl ether and n-hexane to perform a liquid-liquid extraction to extract analytes and their deuterated analogues from human plasma of 100 µL. The method shown linearity for the range of 0.01-10 ng/mL concentrations for both drug and its metabolite.

Thus, the main study was to develop an alternate and improved bioanalytical methodology for measuring fesoterodine and its metabolite in plasma of human using LC-MS/MS in Multiple Reaction Monitoring Mode (MRM). The extraction liquid-liquid type was applied to the extraction of analytes in plasma. This method showed improved extraction efficiency, sensitivity, and reproducibility. Ultimately, this method aims to be more robust, specific and effective in detecting trace amounts of fesoterodine and its metabolite simultaneously in human plasma (25 µL) human plasma with the respective d_{14} isotopes as internal standards. The main advantage of our method is shorter run time over the reported methods. The validated method can be used for human pharmacokinetic study.

MATERIALS AND METHODS

Materials

The pure form of fesoterodine fumarate (Purity 98.9%), 5-HMT (Purity 95.70%), were provided by Hetero Drugs Limited in Hyderabad, India. Fesoterodine d_{14} fumarate (Purity 94.37%), and 5- hydroxy methyl tolterodine d_{14} (Purity 98.11%), were procured from Clearsynth Ltd, in Mumbai, India. Methanol of LC-MS and analytical grade tert butyl methyl ether were procured from JT Baker (Phillipsburg, USA). Ammonium formate and disodium hydrogen phosphate brought from Merck (Mumbai, India). Alpha-toluene sulphonyl fluoride was purchased from Alfaaesar, Thermo fisher scientific chemicals, Maharashtra. The plasma sample of human was brought from nearby blood bank (Hyderabad, India). The water purification system of Milli-Q, acquired from (Bangalore, India) Millipore, produced ultra-pure water.

HPLC and MS operating conditions

Chromatography was done on a LC-20 AD Shimadzu HPLC systems (Make: Shimadzu Corporation, Japan). A kromasil column C_{18} 4.6IDx100mm, 5.0 µM, from Akzo nobel, was used for separation. The temperature of the column was set at ambient temperature (25ºC±5ºC). The solvent phase used for chromatography consisted of methanol and 5mM ammonium formate buffer (80:20 v/v) in a unilateral mode at 1.0 mL/min (with splitter 50:50) into the mass spectrometers ESI chamber. The run time was 2.5 min for each injection. The maintained temperature was 10ºC for the autosampler and the sample volume of injection was 15 µL.

AB Sciex API-4000 mass spectrometer (Foster City, CA, USA) was used and it is fitted with an interface of Turboion spray TM running at 5000 V, quantitation was accomplished with MSMS detection for analytes and internal standards in a positive ion mode. A temperature of 550ºC was chosen for the source. The source settings were set at 6 psi for curtain gas, 8 psi for collision gas, 8 psi of nebuliser gas, and 45 psi of auxiliary gas. The compound parameters for fesoterodine are declustering potential,72 V; collision energy 45 V; entrance potential,10 V; and collision cell exit potential, 5 V, and 72, 48, 10 and 5 V for 5-hydroxymethyl tolterodine; 72, 37, 10 and 5 V for fesoterodine-d_{14}; and 72, 37, 10 and 5 V for 5-HMT d_{14}. The Multiple Reaction Monitoring mode (MRM) was used to detect the ions.

The transition pairs of precursor ion m/z 423.10 to m/z 223.10 for fesoterodine, m/z 426.50 precursor ion to m/z 223.00 for 5-Hydroxymethyl tolterodine, m/z 342.10 precursor ion to the m/z 223.20 for the fesoterodine-d_{14} and m/z 356.40 precursor ion to the m/z 223.20 product ion for 5- Hydroxymethyl tolterodine -d_{14}. Quadrupoles Q1 and Q3 were separated by 1 unit. The result was analysed by analyst software TM (version 1.6.1).

Quality control sample and Calibration curve standards preparation

Two distinct solutions of stock (1.0 mg/mL) of 5-hydroxymethyl tolterodine and fesoterodine in methanol were made for utilisation to create quality control samples and calibration curve standards. After five days of storage at 2-8ºC, it was discovered that these stocks remained stable. From the stock solution, a mixed solution of analyte for working was made by diluting it with a 50:50 v/v methanol to water mixture. In the same manner, solutions of stock (1.0 mg/mL) were made with methanol for fesoterodine-d_{14} and 5-Hydroxymethyl tolterodine-d_{14} separately. A solution for working was made with same diluents to fesoterodine -d_{14} (500 ng/mL) and 5- Hydroxymethyl tolterodine-d_{14} (1000 ng/mL). The making of standards and quality controls involved the introduction of the suitable working standard solution for each analyte (fesoterodine and 5-hydroxymethyl tolterodine, 50 µL each) into 950 µL of control K2EDTA plasma.

The preparation of Calibration Curve (CC) standards at 0.1022, 0.2043, 0.5108, 1.0216, 2.0431, 3.0046, 6.0092, 9.0092, 12.0123 and 15.0154 ng/mL for fesoterodine and 0.1022, 0.2044, 0.5110, 1.0219, 2.0439, 3.0057, 6.0114, 9.0126, 12.0169 and 15.0211 ng/mL for 5- hydroxymethyl tolterodine were prepared. For every batch of plasma samples, examination of quality control and the CC samples were done. The concentration levels of fesoterodine (0.1095, Quantification of Lower Limit; LLOQ), 0.3024, Quality control of Lower Concentration (LQC), 1.5122, quality control of Middle Concentration (MQC-1), 7.5611, MQC-2, and 13.0815, quality control of High Concentration (HQC), and 5-hydroxymethyl tolterodine Quantification of lower limit is 0.1097, low quality control is 0.3031, first middle quality control is 1.5156, second middle quality control is 7.5781, and the highest quality control is 13.1109 ng/mL) were utilised to prepare the QC samples. Before analysis, the linear and control bulk samples were separated into small quantities and kept in the freezer at a temperature of -70±10°C in Tarson micro centrifuge tubes (2 mL).

Sample extraction protocol

Prior to analysis, all frozen samples of the subjects, calibration standards, and quality control samples were thawed and brought to room temperature. Before being spiked, for 10 sec, the samples were mixed by vertexer. A 25 µL of plasma sample was spiked with 20 µL of the internal standard solution of working. To this, added 250 µL of 0.3M disodium hydrogen phosphate buffer solution. For sample extraction, 5 mL of tert-butyl methyl ether was added using a Dispensette Organic (GmbH, Germany), followed by vortexing for 15 sec. The sample was then agitated for 20 min on a reciprocating shaker (Scigenics Biotech, India). After agitation, the sample was centrifuged at 4000 rpm for 10 min using a Heraeus Megafuse 3SR centrifuge (Japan). A moderate stream of nitrogen was used to evaporate the transparent organic layer (4 mL) at 45°C in a 5-mL glass dry test tube. Following a reconstitution process using the eluate of 500 µL, the dried extract was put in the auto-injector vials. A volume of 15 µL was introduced into the tandem mass spectrometric LC apparatus for analytical purposes.

Bioanalytical method validation

In accordance with the recommendations set forth by the US FDA,[27] EMEA,[28] and ICH,[29] a thorough validation was conducted. The system suitability experiment involved injecting a clean sample of analytes with internal standards at intermediate concentration levels (3.7498 ng/mL for fesoterodine; 3.7662 ng/mL of 5-HMT). Prior to the commencement of the analysis, a system suitability test was conducted daily or as needed. The carryover effects in subsequent runs were investigated in an order of injecting a blank plasma sample, LLOQ samples for 6 times, blank plasma sample, a sample of ULOQ, and a sample of blank.

Eight distinct K2 EDTA plasma lots, one for each type of lipemic and hemolyzed plasma, were analysed in order to assess the method's selectivity. In addition to this, OTC drugs such as

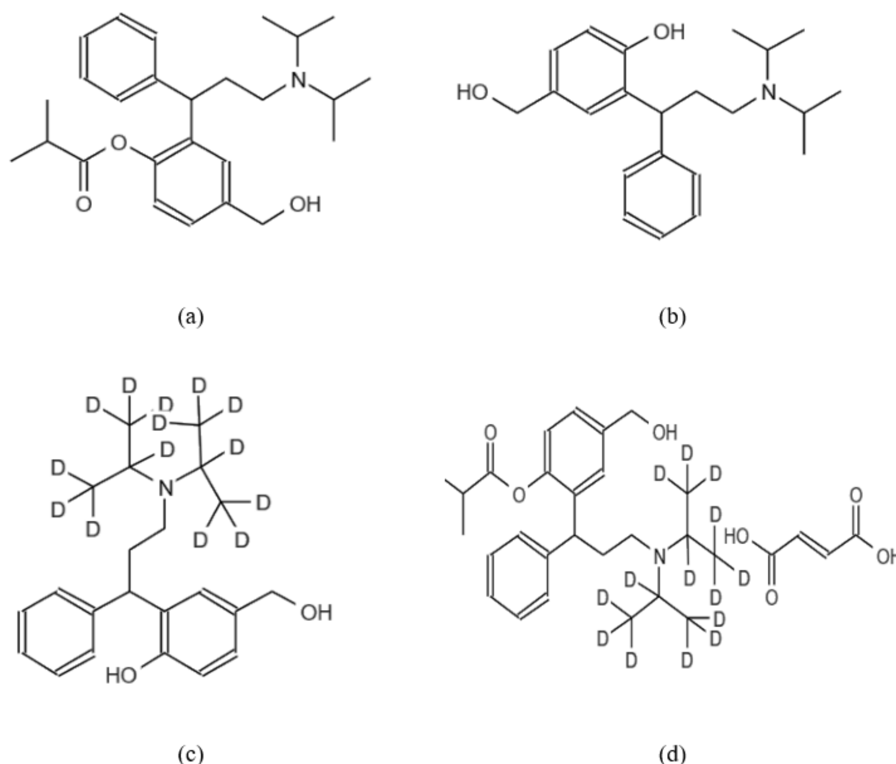

Figure 1: Stuctural representation of (a). Fesoterodine (b). 5-HMT (c). Fesoterodine d_{14} (d). 5- hydroxy methyl tolterodine d_{14}.

paracetamol, ibuprofen, diphenhydramine, nicotine, caffeine and dicyclomine selectivity was evaluated for the method. To determine the sensitivity, spiked LLOQ samples of six sets were analysed. The matrix effect, in the form of IS-normalized Matrix Factor (MF), evaluated by contrasting the response of post-extraction spiked samples mean area with the aqueous samples mean area (neat samples) diluted in mobile phase solutions at the Lower QC and Higher QC levels. The following formula was used to calculate IS-normalized MF:

$$\text{IS normalized MF} = \frac{\text{Ratio of Peak response area in the matrix ions}}{\text{Ratio of Mean peak response without in the matrix ions}}$$

Additionally, the assessment for the effect of matrix using six distinct lots of K2 EDTA plasma was done. From various plasma lots, 3 duplicate samples of every LQC and HQC were created (for a total of 36 QC samples). Calibration curves (Figure 4) with 9 nonzero concentrations were produced in order to establish linearity. Furthermore, one blank plasma sample individually and the other with an internal standard (zero sample) are included in each curve.

By using least square method in the form of linear regression, a CC was examined separately. For intraday accuracy and repeatability results, 6 same injections of the five QC's at Lower limit of quantification, Lower QC, Middle QC-1, Middle QC-2, and High QC sample were analyzed with the curve; for inter-day accuracy and repeatability, 4 batches of samples were analyzed over the course of 3 consecutive days. All concentration levels should have a precision and accuracy should be within 15% from the nominal concentration; the exception is the LLOQ QC, where the CV should be 20%. By carrying out the integrity of dilution experiment, the upper limit of fesoterodine and 5-HMT concentration can be increased. Screened blank plasma was used to dilute six replicates, for each concentration of roughly 1.6 times the highest calibration concentration, four and two times.

The results expressed that the recoveries of fesoterodine and its metabolite were 500 and 1000 ng/mL, respectively, and that the concentrations of fesoterodine-d_{14} and 5-HMTd_{14} were 0.3024 (LQC), 7.5611 (MQC-2) and 13.0815 (HQC) and 0.3031(LQC), 7.5781 (Middle-quality control-2) and 13.1109 (High-quality control) ng/mL, respectively. The analytes and internal standard recovery was estimated by contrasting the response of average peak area of samples spiked before extraction (n=6) with unextracted samples (neat samples; n=6) at every quality control level.

To evaluate the long-term integrity for the analysed analytes during analysis of study sample, a single run included the analysis of a batch of 24,160 bulks drugged QCs, as well as freshly spiked QCs, totaling 195 samples, together with the calibration curve. An alternative instrument of the same manufacturer, processed by an alternate analyst, and utilising an alternate column (an alternate batch number) allowed for determination of one batch precision and accuracy to confirm the ruggedness of procedure.

Stability of stock solution and plasma samples was examined at room temperature by comparing responses at 0 and 9 hr, and in a chilled environment (between 2 and 8°C). At the lower and higher quality control levels, six replicates were used to test the following: bench-top security (13 hr), stability of processed sample (stability of auto sampler for 68 hr, stability of wet extract for 63 hr, and stability of reinjection for 33 hr), freezing-thawing stability (5 cycles), and stability for short-term at -20°C for 4 hr. These stability samples, together with processed newly spiked QC samples measured against fresh spiked standards of calibration curve. Samples were deemed stable if the percentage purity results were between the allowable ranges for precision (≤15% RSD) and accuracy (±15% SD).

RESULTS

Mass spectrometry

The primary objective for this work is to create precise and analytical technique selectively appropriate for the evaluation of pharmacokinetic and bioavailability/bioequivalence studies of fesoterodine. Now-a- days, LC-MS/MS instrumentation is vastly applied for analyzing biological fluids due to its high sensitivity, specificity, and speed. The mass spectrometry conditions were tuned by giving the solution of 100 ng/mL analytes and internals into the ESI source of mass spectrometer in both the ionisation modes. Despite this, the more intense signal was detected in positive mode of ionisation. The source parameters were tuned to obtain maximum area for the analytes. Similarly, the compound parameters were also finalized to enhance the ionization of the analytes thereby obtained maximum response. The highest selectivity and specificity between samples and the internal standards were obtained by using Multiple Reaction Monitoring mode (MRM). The ion transitions monitored for fesoterodine were (parent/product) m/z 412.40 and 223.10, for 5- hydroxymethyl tolterodine were m/z 342.30 and m/z 223.10, for fesoterodine-d_{14} were m/z 426.40 and 223.10 and for 5-hydroxymethyl tolterodine d_{14} m/z 356.40 and m/z 223.10.

Method development

The optimized conditions are critically evaluated during the method development stage. Acetonitrile and methanol in combination with acid additions like acetic and formic acid combined with volatile buffers like ammonium acetate and ammonium formate was employed. Among the other C18 columns tested (including Zorbax, Ace, Inertsil, Grace, Kromasil, Hypersil and Hypurity) the Kromasil 100-5C_{18} column (4.6mm ID x100mm, 5 μM) delivered good peak shapes and responses for the analytes. Kromasil 100-5C_{18} column combined with the eluate of methanol and ammonium formate of 5 mM in an 80:20 (v/v) provided the best chromatographic response. The analyte

ionization was significantly improved with ammonium formate of 5 mM as a mobile phase buffer. We found 1 mL/min flow rate was optimal for proper elution of analytes in 2.5 min run time. The elution time for fesoterodine and 5-HMT, along with internal standards, were 1.30, 1.00, 1.30, 1.00 min respectively. Previous studies (Parekh *et al.*, 2013) used extraction by liquid liquid technique to separate fesoterodine and 5-HMT from human blood samples. Following this approach, various pre-treatment methods for samples were evaluated. Firstly, Protein Precipitation (PP) is attempted using usual HPLC solvents as precipitating agents, but this reported in non-reproducible recoveries, ion suppression, and poor chromatographic peaks for the drug samples.

So, we adopted LLE method using TBME to separate the analytes from plasma samples. This simple approach enabled the simultaneous determination of fesoterodine and 5- hydroxyl methyl tolterodine at low levels of 0.10 and 0.10 ng/mL. While developing the method, Liquid-Liquid Extraction (LLE) examined with various organic solvents, including TBME, dichloromethane, diethyl ether, hexane, and ethyl acetate, as well as combinations. Highest and reproducible recoveries obtained with TBME extraction solvent. Plasma samples were treated with 0.3 M disodium hydrogen phosphate buffer to guarantee reliable and consistent analyte recovery. As internal standards, the stable labeled isotopes fesoterodine d_{14} and 5- hydroxyl methyl tolterodine d_{14} were used for fesoterodine and 5- hydroxyl methyl tolterodine, respectively.

DISCUSSION

Carryover test withSystem suitability

The Coefficient of Variation (CV) for retention time was less than 1% and for the area ratio of fesoterodine and 5-hydroxyl methyl tolterodine was less than 2%. Furthermore, after injecting the greatest quantities of both analytes (upper limit of quantification), no discernible carryover was seen in the ensuing blank sample

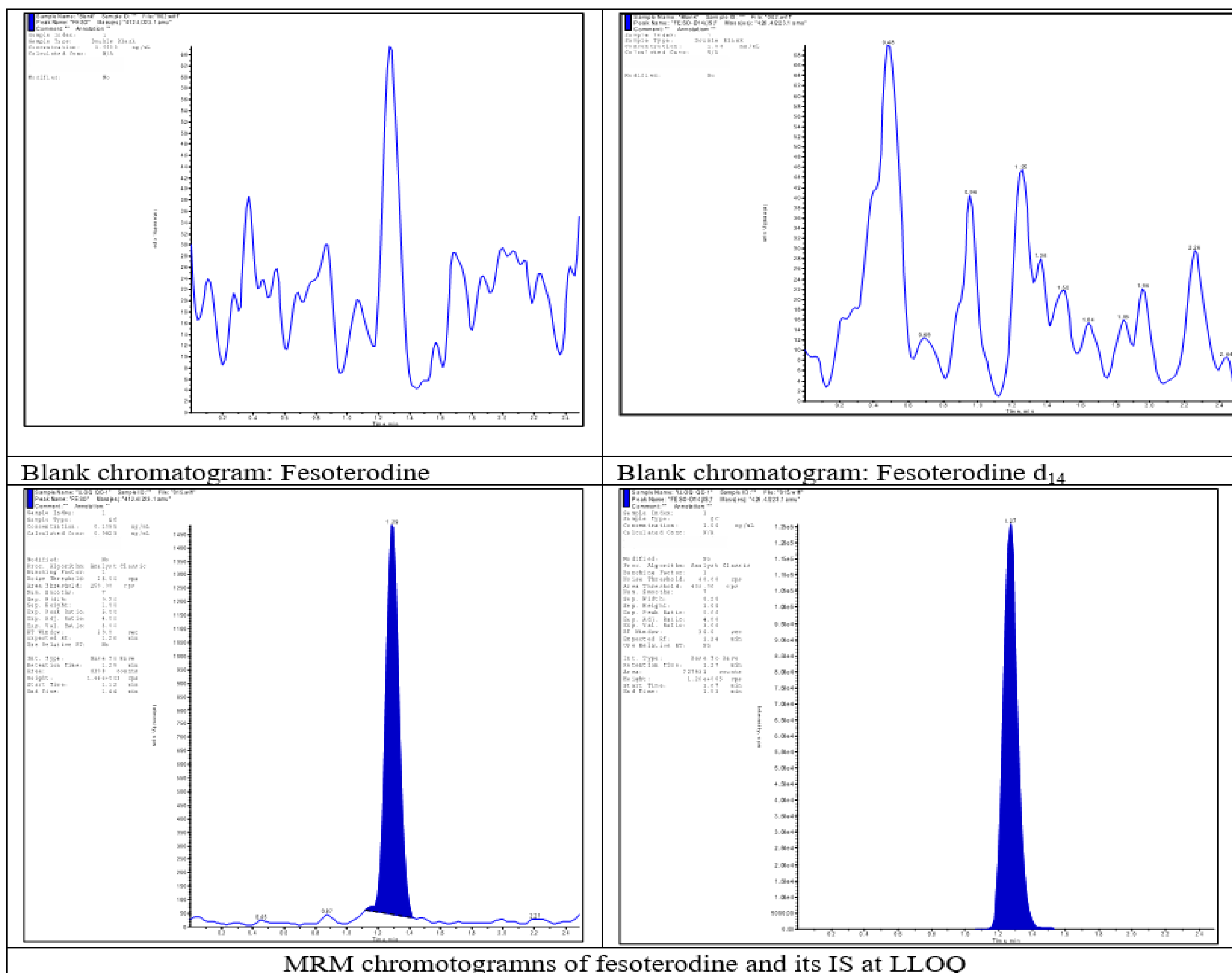

Figure 2: Blank and MRM Chromatograms of Fesoterodine and its IS at LLOQ.

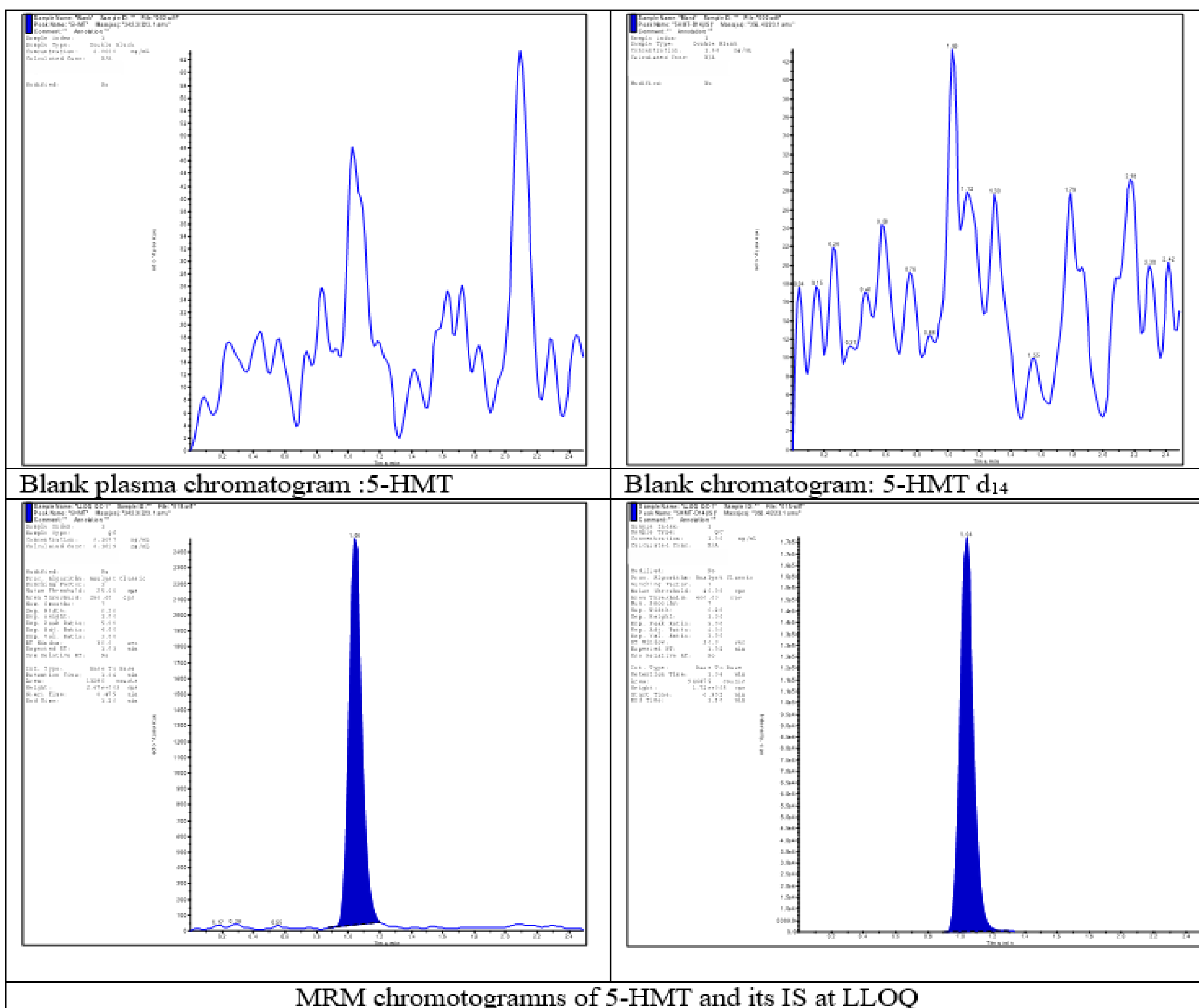

Figure 3: Blank and MRM Chromatograms of 5-HMT and its IS at LLOQ.

runs. This suggests that each analyte was not carried over to the next run.

Selectivity and sensitivity

Selectivity and sensitivity were examined for any potential interference on eight distinct human plasma sources, in which six normal, one lipemic, and one hemolyzed blank and LLOQ samples were considered. The S/N ratios of the added to blank samples from entire samples were greater than 5:1 and no interferences were found in the blank samples from the various sources. This indicates that there were no significant interferences occur at the retention periods from endogenous chemicals in drug-free human plasma. Figures 2 and 3 show the typical fesoterodine and 5-hydroxyl methyl tolterodine chromatograms of the no plasma sample and the QC sample at LLOQ, respectively. Similarly, this demonstrates that no direct intervention was present from the IS in the Multiple Reaction Monitoring (MRM) channel of the analyte. For the Quantification of Lowest Limit (LLOQ) sample, the representative ion chromatogram is 0.1022 ng/mL. Additionally, no intrusion was detected from common medicines such as paracetamol, ibuprofen, diphenhydramine, caffeine, nicotine, and dicyclomine.

Matrix effect

The impact of various plasma lots on value of back calculation for the QC's usual concentration was assessed using the matrix effect method. The precision for fesoterodine and 5-HMT at the LQC level was 1.11% and 1.35%, respectively, while at the HQC level, it was 1.64% and 1.78%, respectively. According to these findings, no observable matrix effect for the analyte at either lower or higher QC concentration in any of the six batches of plasma.

Table 1: Accuracy and Precision for fesoterodine and 5-hydroxy methyl tolterodine.

QC		Same day precsion and accuracy (n-12; 6 from each batch)			Different day precision and accuracy (*n*=30, 6 from each batch)		
Analytes	**Concentration spiked (ng/mL)**	**Concentration found(mean;ng/mL)**	**Precision (%)**	**Accuracy (%)**	**Concentration found (mean; ng/mL)**	**Precision (%)**	**Accuracy (%)**
Fesoterodine	0.1095	0.102±0.008	8.04	93.87	0.104±0.007	7.27	95.33
	0.3024	0.312±0.005	1.73	103.18	0.313±0.007	2.25	103.60
	1.5122	1.486±0.028	1.89	98.31	1.489±0.025	1.73	98.48
	7.5611	7.142±0.085	1.19	94.46	7.161±0.086	1.21	94.71
	13.0815	12.567±0.146	1.16	96.07	12.558±0.124	0.99	96.00
5- hydroxy methyl tolterodine	0.1097	0.097±0.005	5.82	88.63	0.102±0.008	8.70	93.17
	0.3031	0.313±0.015	4.86	103.53	0.311±0.036	4.39	102.81
	1.5156	1.473±0.022	1.51	97.21	1.471±0.020	1.37	97.08
	7.5781	7.237±0.110	1.52	95.51	7.257±0.102	1.14	95.78
	13.1109	12.139±0.158	1.30	92.59	12.119±0.164	1.36	92.44

*Note: As per the latest recommendations from the US FDA and EMEA, the obtained results fell within the predetermined acceptance threshold.

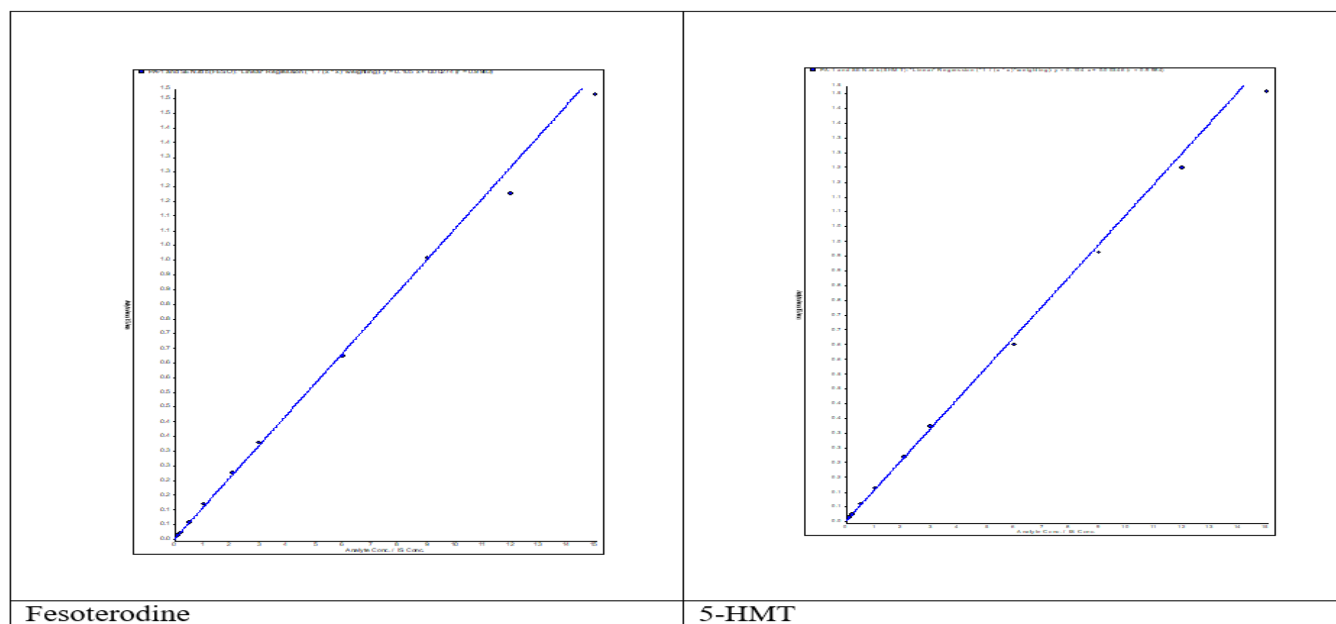

Figure 4: A Representative Calibration Curve for Regression Analysis of fesoterodine and 5-HMT.

Linearity, precision, accuracy and sensitivity

Linearity

For fesoterodine, calibration curve for ten points shows linearity in the range of 0.1022 to 15.015 ng/mL and for 5-hydroxymethyl tolterodine, 0.1022 to 15.0211 ng/mL concentrations (Figure 4). For fesoterodine and 5-hydroxymethyl tolterodine, the calibration curves' mean correlation coefficients were 0.9983 and 0.9989, respectively, and both fell within the acceptable range.

Sensitivity

The quantities 0.1022 and 0.1022 ng/mL, respectively, were established as the LLOQ for fesoterodine and 5-HMT. For fesoterodine and the metabolite, the findings for repeatability and accuracy at this concentration were 3.86% and 108.15%, 3.16% and 108.32%, respectively. At LLOQ concentration, the S/N ratio was evaluated for each analyte, and it was determined to be ≥5.

Precision andAccuracy

Investigations were conducted using QC concentration levels over the entire range and minimum of four runs on three distinct days. Table 1 summarises the findings for repeatability and accuracy for both same-day and different-day measurements in quality control plasma samples. These results demonstrate good precision and accuracy.

Table 2: Results of samples stability of fesoterodine and 5-HMT (*n*=6).

Analyte	Stability test	QC spiked on centration (ng/mL)	mean±SD (ng/mL	Precision (%)	Accuracy/ stability (%)
Fesoterodine	Freeze thaw stability (5 cycles)	0.3024	0.3210±0.01	3.84	106.16
		13.0815	12.630±0.133	1.06	96.56
	Bench top 13 hr	0.3024	0.3154±0.007	2.47	104.30
		13.0815	12.561±0.086	0.68	96.03
	Short term (spiked fresh) QC's	0.3027	0.329±0.009	2.98	108.77
		13.0912	12.760±0.256	2.01	97.47
	Short term (-20c) 4 days	0.3024	0.318±0.008	2.65	96.69
		13.0815	12.655±0.111	0.88	99.25
	Wet extract (63 hr)	0.3024	0.319±0.012	3.87	105.54
		13.0815	12.852±0.196	1.53	98.25
	Auto sampler (68 hr)	0.3024	0.331±0.010	3.21	109.69
		13.0815	12.916±0.128	1.00	98.74
	Reinjection (33 hr)	0.3024	0.315±0.006	1.98	100.99
		13.0815	12.638±0. 006	0.49	100.80
5-HMT	Freeze thaw stability (5 cycles)	0.3031	0.304±0.005	1.94	100.30
		13.1109	11.927±0.225	1.89	90.93
	Bench top 13 hr	0.3031	0.303±0.007	2.32	100.16
		13.1109	11.965±0.083	0.70	91.27
	Short term (spiked fresh) QC's	0.3048	0.3054±0.020	6.63	100.20
		13.1826	11.914±0.184	1.55	90.38
	Short term (-20c) 4 days	0.3031	0.302±0.005	1.71	99.62
		13.1109	11.889±0.109	0.92	100.34
	Wet extract (63 hr)	0.3031	0.303±0.009	3.01	99.82
		13.1109	11.696±0.132	1.13	89.21
	Auto sampler (68 hr)	0.3031	0.297±0.009	3.09	98.06
		13.1109	11.774±0.067	0.57	89.81
	Reinjection (33 hr)	0.3031	0.297±0.005	1.75	92.78
		13.1109	11.816±0.072	0.62	97.27

Extraction efficiency and dilution integrity

To ascertain recovery, six replicates of 5-hydroxymethyl tolterodine and fesoterodine at low, medium, and quality control of high concentrations were made. The liquid-liquid extraction technique proved robust, yielding the best samples. The overall recovery for Fesoterodine was 83.75% with precision ranging from 1.23% to 7.28%, and 5-hydroxy methyl tolterodine, the recovery is 84.04% with precision ranging from 0.37% to 7.20%. The analyte and IS recoveries were reliable and constant, demonstrating the assay's robustness in high-throughput bio-analysis. The integrity of dilution experiment was carried to raise the quantification of upper limit, appropriate for greater dosages of fesoterodine. Fesoterodine and 5-hydroxymethyl tolterodine were used at doses of 24.1505 and 24.2048 ng/mL, respectively, for the dilution integrity test. Fesoterodine's precision and accuracy were determined to be 1.81% and 96.45% at a 1:2 dilution and 2.79% and 98.41% at a 1:4 dilution, respectively. Comparably, for 5-HMT, the repeatability and accuracy were 1.27 and 94.32% at 1:2 dilution and 1.55 and 95.98% at 1:4 dilutions, simultaneously.

The steadiness of the analytes in samples of processed and plasma were evaluated for various circumstances. Benchtop stability (13 hr), stability autosampler for 68 hr, 5 cycles of freeze-thaw cycles, stability of reinjection (33 hr), stability of short term at -20ºC (4 days), and stability of wet extract (63 hr at 20±5ºC) were among the conditions covered by stability experiments. Table 2 shows that the analyte's mean percentage of nominal values at Low QC and High QC levels. Throughout the validation process,

Table 3: Summary of Validation parameters.

Validation Parameters	Fesoterodine		5-HMT	
	% Nominal/%Stability	Precision	% Nominal/%Stability	Precision
Matrix Effect at LQC	0.988	1.34%	0.944	2.31%
Matrix Effect at HQC	1.015	5.21%	0.993	3.98%
Sensitivity	108.15%	3.86%	108.32%	3.16%
Coefficient of correlation (r^2)	0.9966 - 0.9972		0.9958-0.9978%	
Within Batch Precision and Accuracy	92.51%-98.26%	4.36-11.00%	84.84%-102.25%	3.24-4.59%
Intra-Day Precision and Accuracy	93.87%-103.18%	3.21-8.04%	92.44%-102.81%	1.37-8.70%
Between Batch/Inter Day Precision and Accuracy.	94.71%-103.60%	0.99-7.27	88.63%-103.53%	1.30-5.82%
Recovery	83.75%	1.23-7.28%	84.04%	0.37-7.20%
Dilution Integrity: Two times	96.45%	1.81%	94.32%	1.27%
Dilution Integrity: Four times	98.41%	2.79%	95.98%	1.55%
Ruggedness	87.62%-100.41%	1.56-7.87%	85.31%-98.30%	2.01%-7.04%
Drug interactions	96.07%-104.03%	2.06-5.63%	91.96%-100.52%	1.67-3.62%
Steadiness in Whole Blood (3 hr) at LQC	101.43%	1.28-2.43%	97.65%	1.98-8.32%
Steadiness in Whole Blood (3 hr) at HQC.	101.39%	0.89-1.06%	97.59%	1.01-8.64%

**Note: As per the latest recommendations from the US FDA, ICH and EMEA, the obtained results fell within the acceptance criteria.

the reports remained within reasonable bounds. Fesoterodine, 5-hydroxymethyl tolterodine, and ISs solutions of stock were shown to be stable for 5 days at 2-8ºC. Fesoterodine and 5-HMT, Fesoterodine d14, and 5-HMT d_{14} had percentage stabilities (the precision range) of 100.66 (0.52-0.63%), 98.3 (0.99-1.59%), 103.04 (0.67-0.94%), and 98.30% (0.84-1.19%), in that order.

Ruggedness

A separate instrument of the equal make, with a various batch number and reagent combination, was used to test the method's resilience with one precision and accuracy. For fesoterodine, the repeatability (CV) and accuracy values were 1.56-7.87 and 87.62-100.41%, correspondingly. For 5-HMT, the repeatability (CV) and recovery values were 2.01-7.04% and 85.31-98.30%, respectively.

Reinjection reproducibility and run size evaluation

Following the resolution of any problems encountered during the analysis of real subject samples, a reproducibility experiment including re-injection was conducted to verify the instrument's operation. The reports demonstrated that the samples reinjected are remained stable for 33 hr. The steadiness of fesoterodine ranges from 100.80% to 100.99%, with precision between 0.49% and 1.98%. Similarly, the percent stability of 5- hydroxy methyl tolterodine ranged from 92.78% to 97.27%, with precision between 0.62% and 1.75%. A batch of 160 samples, comprising forty sets of lower, middle 1and 2, high quality control samples in addition to 24 newly spiked samples of QC (6 sets at every level), was analysed for long-run evaluation (Table 3).

CONCLUSION

In conclusion, a high-throughput, straightforward, quick, and particular LC-MS/MS technique has built and verified for the determination of fesoterodine and 5-HMTinplasma by means of internal isotope standards. The technique selectivity was also established in hemolysed and lipemic plasma along with the normal EDTA plasma and found the present method was highly selective. The extraction method showed excellent recovery with minimal matrix effect for fesoterodine and 5-HMT in plasma of human. With a quick runtime of 2.5 min, can allow estimating a greater number of samples in each run. According to the most recent regulatory criteria, this approach has been fully validated.

The technique can therefore be used in preclinical, clinical, and Bioavailability and Bioequivalency (BA/BE) investigations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

5-HMT: 5-hydroxy methyl tolterodine; **ICH:** International conference on harmonization; **ULOQ:** Upper Limit of Quantification; **LLOQ:** Lower limit of quantification, **MQC:** Middle quality control; **LQC:** Low quality control; **HQC:** High quality control; **CV:** Coefficient of variation; **IS:** Internal standard.

SUMMARY

The study aimed to develop a precise analytical technique for evaluating the pharmacokinetics and bioavailability of fesoterodine and its active metabolite 5-HMT with corresponding deuterated isotopes d_{14} simultaneously using LC-MS/MS. The sample is extracted by Liquid-Liquid extraction and the optimized conditions employed various combinations of solvents and volatile buffers to achieve good peak shapes and responses. The method demonstrated the linearity in the range 0.1022 to 15.0154 ng/mL for 10 points, high sensitivity, specificity, and reproducibility, with acceptable precision and accuracy within the guidelines of regulatory standards. The method's robustness was confirmed through the recovery of analytes in different matrices, including hemolyzed and lipemic plasma. Stability tests confirmed the integrity of the analytes under various conditions, ensuring reliable quantification in pharmacokinetic studies.

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