LC–MS/MS assay method development and validation for the simultaneous quantification of Solifenacin and Mirabegron in human plasma

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Abstract

The present study intended to develop a simple and novel Liquid Chromatography–Mass Spectrometry/ Mass spectrometry (LC–MS/MS) method for the simultaneous quantification of solifenacin and mirabegron, a combined medication used for the treatment of overactive bladder symptoms in human plasma. Tolterodine drug is used as an internal standard in the study. Both the analytes and internal standard were isolated from 100 µL plasma samples by liquid-liquid extraction and then chromatographed on Zorbax C18 (4.6 mm×50 mm, 5 µm) column with a mobile phase consisting of methanol and 5mM ammonium formate in the ratio of 25:75 (v/v) pumped at 0.3 mL/min. The method had a chromatographic total run time of 5 min. The developed method gave symmetric peak at a retention time of 2.50 min, 2.99 min and 1.18 min respectively for solifenacin, mirabegron and nifedipine satisfying all the peak properties as per USP guidelines. The mass spectral characterization of separated analytes in the LC method was performed using mass detector operated at Multiple Reaction Monitoring mode with precursor-to-product ion transitions at m/z of 363 to m/z of 110 as MH^+ ion for solifenacin, m/z of 397 to m/z of 239 as MH^+ ion for mirabegron. A very sensitive limit of detection of 7.5 ng/mL was observed and showed calibration curve linear over the concentration range of LLOQ to 1000 ng/mL.

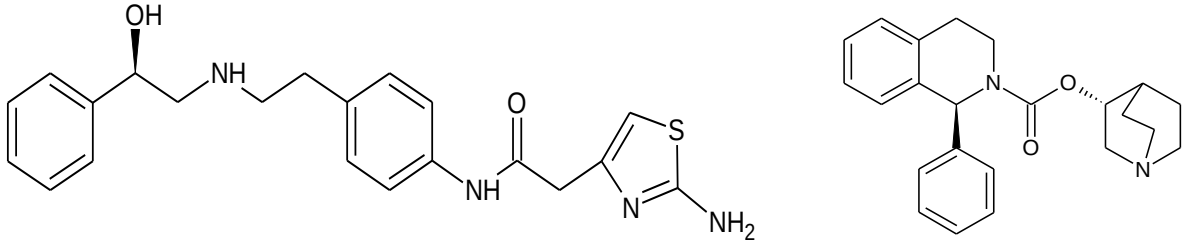
The other validation parameters were found to have acceptable accuracy, precision, linearity and selectivity. The mean extraction concentration was acceptable and very high for both the analytes in HQC, MQC and LOQ levels. Various stability studies of solifenacin and mirabegron such as freeze-thaw, short term, long term, auto-sampler and dry extract stability proved that the method was stable. Based on the results, it can be proved that the method was accurate, precise and specific for the simultaneous analysis of solifenacin and mirabegron in human plasma.

Keywords: Solifenacin, Mirabegron, LCMS analysis, Human plasma, Bio-analytical method.

Introduction

Mirabegron is a β3-adrenoreceptor agonist drug prescribed for the treatment of overactive bladder. It acts as alternative medication to antimuscarinics such as tolterodine or solifenacin for the treatment of overactive bladder[3]. It is also prescribed to treat neurogenic detrusor overactivity, a bladder dysfunction related to neurological impairment in children aged three years and older[2]. The bladder relaxation mechanism includes the activation of β3 adrenergic receptor and results in relaxation of bladder. The common side effects while using the mirabegron include urinary tract infections, urinary retention, headaches, high blood pressure, angioedema and irregular heart rate[6]. It has a molecular mass of 396.51 g/mol with molecular formula $C_{21}H_{24}N_4O_2S$ and its structure was shown in figure 1A.

Solifenacin is an antimuscarinics class medication prescribed to treat neurogenic detrusor overactivity and overactive bladder. It was also used to control involuntary urination, frequent urination and urinary urgency[4]. It is also prescribed to treat neurogenic detrusor overactivity in children aged three years and older. Urinary tract infection, urinary retention, hallucinations, anaphylaxis, QT prolongation, constipation and glaucoma are the possible side effects while using solifenacin[5]. It has a molecular mass of 362.473 g/mol with molecular formula $C_{23}H_{26}N_2O_2$ and its structure is shown in figure 1B.



A) **B)**

Figure 1: Molecular structure of (A) Mirabegron and (B) Solifenacin

The fixed dose combination of mirabegron and solifenacin is available in market with various brands for the treatment of overactive bladder symptoms. Extensive literature survey was conducted for the available analytical methods for the estimation of mirabegron and solifenacin using various analytical techniques. The literature survey confirmed that only one HPTL method reported[1] for the estimation of mirabegron and solifenacin. The other reported methods will be analysis of mirabegron and solifenacin in single or in combination with other drugs using various analytical techniques such as HPLC, LCMS etc. No analytical method is available for the estimation of mirabegron and solifenacin in biological samples such as human plasma. Hence the present work intended to develop and validate a simple LCMS method for the separation and simultaneous estimation of mirabegron and solifenacin in spiked human plasma. Similar class drug tolterodine was selected as internal standard in the study. The molecular structure of tolterodine internal standard is given in figure 2.

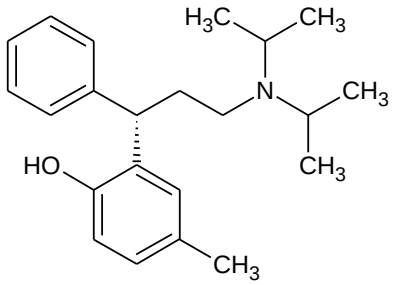


Figure 2: Molecular structure of Tolterodine (Internal Standard)

Material and Methods

Materials: The standard analytical pure drugs in the study viz. solifenacin, mirabegron and nifedipine were procured from Intas Pharmaceuticals Ltd., Hyderabad. The HPLC grade chemicals in the study (methanol, acetonitrile) and milli Q water were purchased from Merck Chemicals, Mumbai. Healthy human blood was procured from nearby diagnostic laboratory. The plasma from the whole blood was separated using Pasteur pipette after centrifugation.

Equipment: The experiment was performed on Waters (Japan) alliance 2695 LCMS system coupled with triple quadrupole mass detector (Waters ZQ, LAA 1369). The system was equipped with auto-injector (0. 1 –1500 µL) and integration was carried on masslynx 4.2 (waters) software. The analytes was separated on Zorbax C18 (4.6 mm×50 mm, 5 µm) column.

Preparation of working standard solutions: The standard stock solution of solifenacin and mirabegron was prepared separately in a 25 mL volumetric flask by accurately weigh and dissolving 25 mg of analyte in 15 mL of methanol. Then the flask was sonicated for 2 min to dissolve the analytes in solvent and the final volume was made up to the mark using the same diluent. The standard solifenacin and mirabegron at a concentration of 1000 µg/mL were obtained separately. Then the stock solutions were diluted successively to obtain a standard solution in the concentration range of 25 to 1000 ng/mL separately for solifenacin and mirabegron. The same procedure was followed for the preparation of nifedipine internal standard solution at a concentration of 200 ng/mL separately.

Preparation of spiked calibration curve standard solutions: The calibration curve dilutions were prepared by spiking 50 µL of prepared standard concentrations of solifenacin and mirabegron in increasing order and 50 µL of internal standard to the blank human plasma. Similarly, a blank without analytes and a zero sample that spiked with internal standard only was prepared. All the spiked plasma samples were treated as per the extraction protocol.

Extraction protocol: Protein precipitation followed by liquid-liquid extraction was performed for the extraction of analytes along with internal standard from the spiked plasma. The protein precipitation of spiked plasma was achieved by adding 1 mL of acetonitrile and the extraction of analytes was performed using 3 mL diethyl ether. The content was vortexed for 2 min and then centrifuged at 10 °C for 5 min at 400 rpm. The supernatant obtained was carefully relocated and concentrated at 60 °C and reconstituted with methanol. The reconstituted solution was used for the LCMS analysis.

Method development: The method development for the separation and analysis of solifenacin and mirabegron in presence of nifedipine internal standard was carried by performing the analysis in different analytical conditions. One parameter was changed at a time and other parameters constant were kept the spiked standard solution was analysed in each changed conditions. The results achieved in such studied conditions were observed and the conditions that give the best results in terms of system suitability, specificity and symmetry were selected for further validation.

Method validation: The developed method for the analysis of solifenacin and mirabegron using LCMS was validated as per the guidelines of ICH[7,8].

Selectivity: The method selectivity was evaluated by analysing and comparing the results achieved for the un-spiked (blank) plasma matrix and the analytes at LLOQ level spiked plasma matrix. The results achieved in theses analysis were compared and the selectivity of the method was assessed.

Linearity and range: The range of solifenacin and mirabegron was assessed by analysing the plasma spiked calibration curve dilutions prepared in the developed method. Peak area response ratio of each analyte to the internal standard was plotted against the concentration of the analyte prepared. The linear regression analysis was performed to assess the linearity of the analytes in the developed method.

Accuracy and precision: The accuracy and precision were performed as intraday and interday studies and were carried in three dissimilar concentrations such as low (LQC), middle (MQC) and high (HQC) concentrations in the linearity range. The selected concentrations in the linearity range were analysed in six replicates for both intraday and interday precision studies. The results in the study were expressed as the % relative standard deviation and the % recovery for all the studied levels. The results concluded are acceptable when the variation results were within ± 15% in the three studied levels.

Recovery: The recovery of the method developed was evaluated by comparing the results observed during the analysis of analytes that were spiked with blank plasma which was exposed to the whole extraction procedure to the results observed for post-extracted plasma samples. The study was conducted at LQC, MQC and HQC levels in the calibration range. It can be considered that the recovery of the analytes must not be 100 % but it is essential that the variability of the results in recovery must be reproducible, precise and consistent in different concentration ranges studied.

Matrix effects: The matrix effect of the method developed for the analysis of solifenacin and mirabegron was evaluated by investigating the effect of blank plasma on the results. In this, the blank plasma of six different batches was spiked with the investigated analytes at LQC and HQC levels and were analysed in the developed method. The %RSD of the peak area response of both the analytes in the study was calculated and a % RSD of < 15 % confirmed that the method is having acceptable matrix effect.

Dilution integrity: The effect of dilution on the accuracy and precision of the analytes in the developed method was assessed in this study. The study was conducted by spiking the analytes at higher than the HQC concentration and then the sample was diluted to HQC and LQC level with the blank plasma matrix. The method was acceptable if the precision and accuracy was within ± 15%.

Stability experiments: The stability analysis was conducted to evaluate the stability of the analytes in plasma matrix under several conditions which simulate the conditions that could occur during sample analysis. Different stability studies such as short term, long term, auto sampler, freeze and thaw and dry extract stability were performed at LQC, MQC and HQC levels utilizing six replicates from each level.

In short term stability, the defrosted samples was stored in room temperature for 6 h and then analysed in the developed method. In long term stability, the samples were stored in freezing temperature and then analysed after 30 days of the incubation. In freeze thaw stability, the QC samples stability was investigated through four freeze–thaw cycles after being kept in freeze for 24 h. Samples were then thawed unassisted at room temperature for 2 h or even more and then kept to freeze again at − 8 °C overnight for every freeze–thaw cycle.

The consequences of infrequent delay of the sample injection in auto-sampler were evaluated in auto-sampler stability. In this the sample was analysed after 24 h of incubation in an auto-sampler. The dry extract stability was assessed by incubating the dry residue at room temperature without reconstitution and was reconstituted after 24 h of incubation. The % recoveries and the % stability in all the studies were calculated in the studied concentration levels for both the analytes.

Results and Discussion

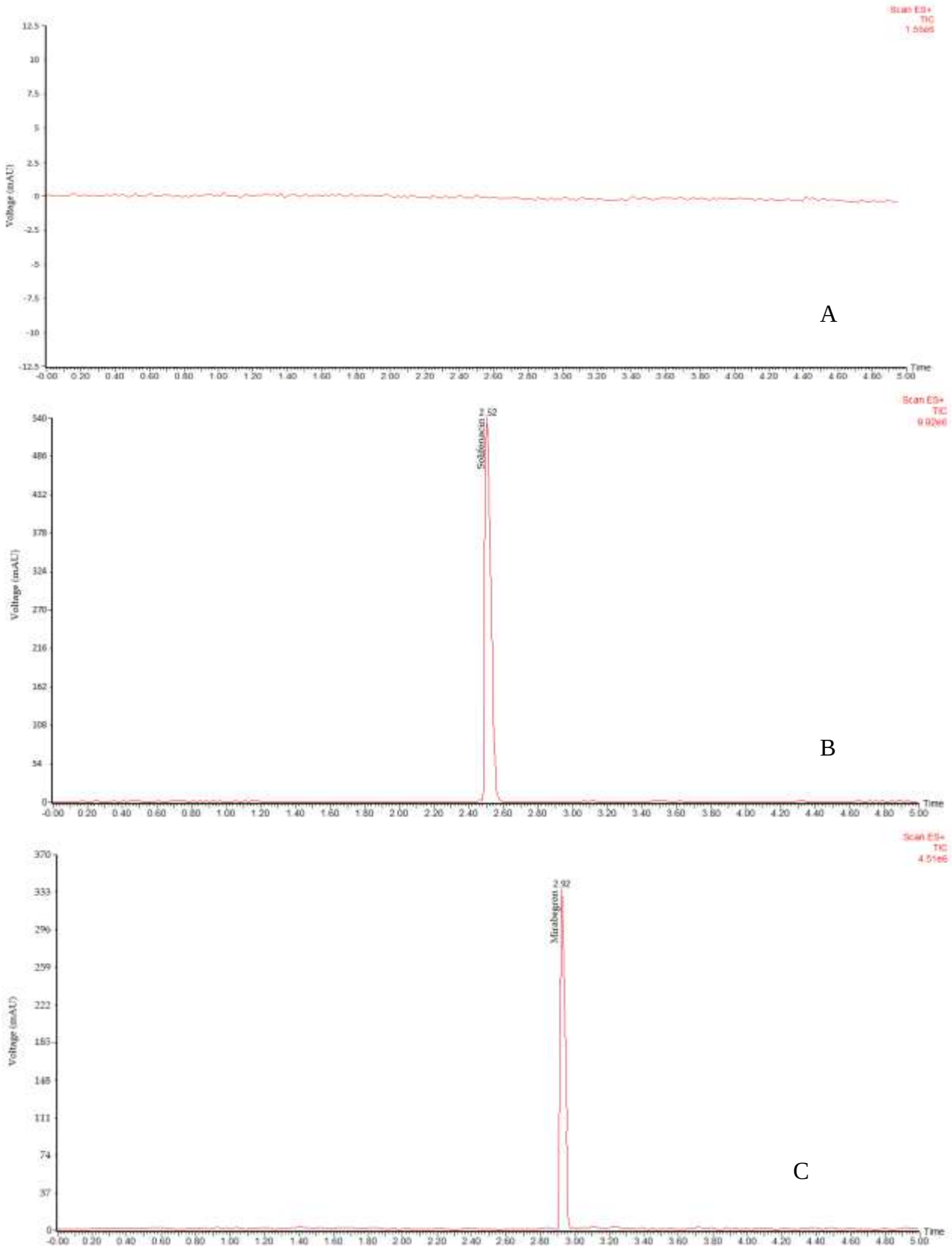
To develop a high sensitive and robust analytical method, the solid-phase extraction and the liquid–liquid extraction was widely used for the preparation of samples from the biological samples. The solid-phase extraction technique was treated as an expensive and it was very difficult to extract the highly polar compounds through liquid – liquid extraction. In view of this, the present study utilised a simple protein precipitation technique for the preparation of samples and it was done using acetonitrile solvent. It facilitates the higher efficiency for precipitations of proteins with very less analyte loss when compared with other solvents such as acetone and methanol. Further the protein precipitation was followed by liquid – liquid extraction using diethyl ether solvent. The extracted samples was analysed in the method development and followed by method validation study using LCMS.

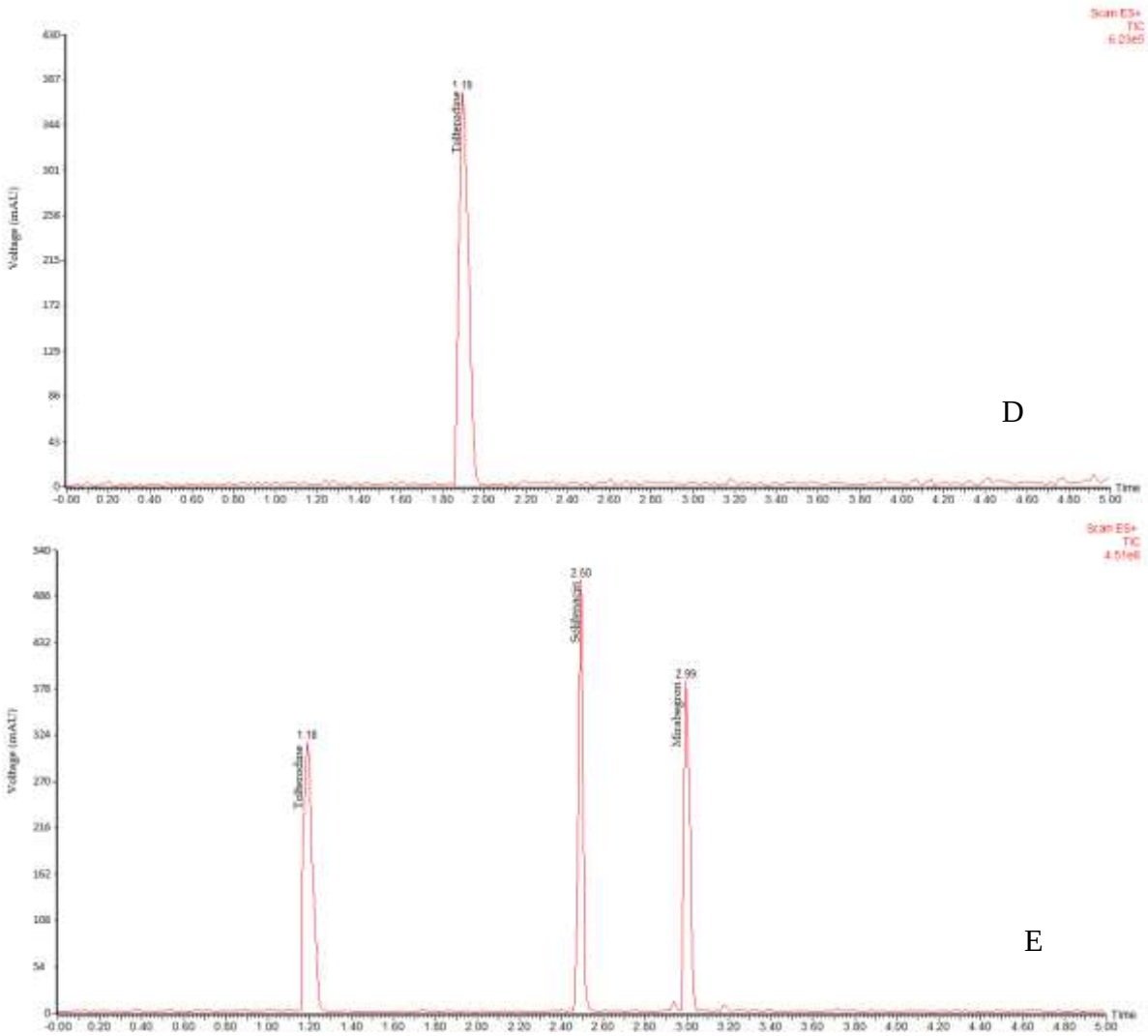
The analytes in the study viz. solifenacin and mirabegron have dissimilar physicochemical properties. Consequently, considerable effort was accomplished to adjust the chromatographic conditions in order to achieve sharp peaks shape and satisfactory response. Various method conditions were adjusted to attain suitable analytical conditions for the separation and simultaneous analysis of solifenacin and mirabegron along with internal standard nifedipine. Very nominal quantity of sample (2 µL) was separated on Zorbax C18 (4.6 mm×50 mm, 5 µm) column at room temperature with 0.3 mL/min flow rate of mobile phase that facilitates less consumption of mobile phase. In the selection of mobile phase, acetonitrile and methanol at various compositions were studied as organic modifier as well as formic acid and ammonium format at various compositions and pH ranges.

The involvement of methanol as mobile phase and ammonium formate as pH modifier produces best response than acetonitrile and formic acid. Hence methanol and 5mM ammonium formate at various compositions were selected for optimization study. It was confirmed that 25:75 (v/v) ratio of methanol and 5mM ammonium formate as the mobile phase was the most suitable for obtaining the best sensitivity, efficiency and peak shape.

The typical chromatograms observed in the developed conditions as shown in figure 3 confirm that the un-spiked

chromatogram does not show any peak throughout the run time whereas the spiked standard chromatogram shows peaks at a retention time of 2.50 min, 2.99 min and 1.18 min respectively for solifenacin, mirabegron and nifedipine. The retention time observed for the analysed in the combined standard solution was comparable with the individual spiked chromatogram. The results confirm that there is no interference of endogenous plasma components during the analysis and hence it was proved that the method was specific for the analysis of analytes in the study.





A) Un-spiked plasma sample; B) spiked with solifenacin; C) spiked with mirabegron; D) spiked with internal standard (nifedipine) and E) spiked with both analytes and internal standard
Figure 3: LCMS chromatograms obtained in the optimized conditions

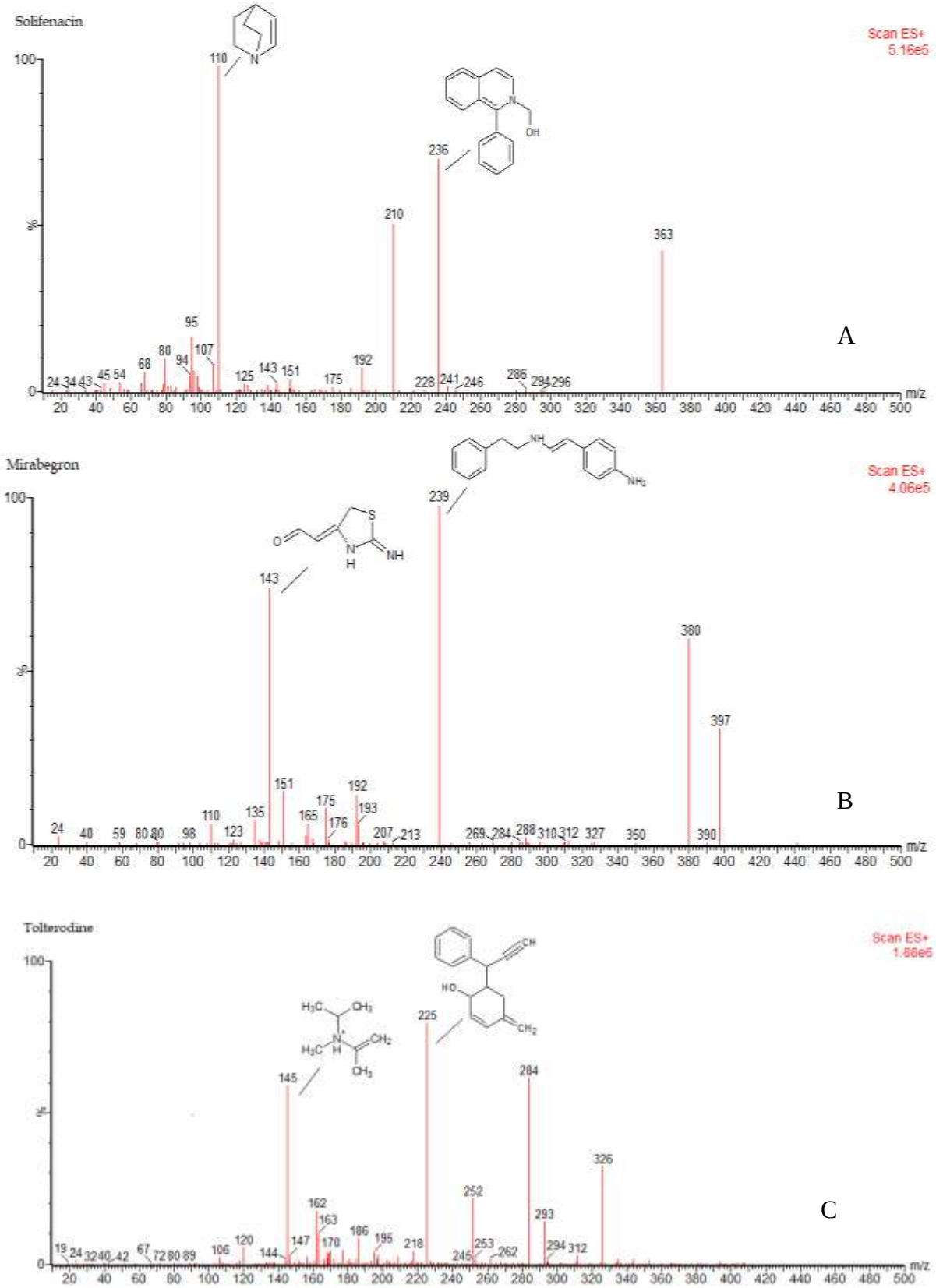
Table 1
LC–MS/MS parameters selected for the quantification of solifenacin, mirabegron using nifedipine as internal standard

S.N.	Parameter	Results observed in the developed method		
		Solifenacin	Mirabegron	Nifedipine
1	Precursor ion (m/z)	363	397	326
2	Product ion (m/z)	110	239	225
3	Declustering potential (v)	41	27	32
4	Entrance potential (v)	10	10	10
5	Collision energy (v)	31	18	37
6	Cell exit potential (v)	21	18	14

Coupling of liquid chromatography to MS/MS detection is an extremely selective technique which results in insignificant interference of endogenous impurities. The mass detector operated in multiple reaction monitoring (MRM) mode was very influential method that produces sensitivity and the selectivity to the analytes. Hence in the study, the mass detector in MRM mode was selected for the detection of analytes. Both +ESI and −ESI were investigated and it was obvious that the signal intensities acquired from the +ESI were higher than those acquired from the −ESI which could be attributed to the capability of the target analytes as well as internal standard to gain protons.

In the full scan, Q1 mass spectra showed predominant protonated $[M+H]^+$ parent ions at m/z of 363, 397 and 326 for solifenacin, mirabegron and nifedipine respectively. The

characteristic most abundant fragment ions found in the product ion mass spectrum were 110, 239 and 225 respectively for solifenacin, mirabegron and nifedipine. The mass spectral parameters were summarized in table 1 and full scan mass spectra observed for the analytes in the study were given in figure 4.



A) Solifenacin; B) Mirabegron and C) Nifedipine
Figure 4: Full scan mass spectra observed in the optimised conditions

The calibration curve was observed to be linear in the concentration range of 25 to 1000 ng/mL for both the analytes with regression equation of y = 0.0062x + 0.2284 (R^2 = 0.9995) and y = 0.0057x + 0.1592 (R^2 = 0.9993) for solifenacin and mirabegron respectively. The results of the linearity (table 2) confirm that the method has broad and sensitive calibration curve.

Accuracy and precision: The precision and accuracy of the method developed for the analysis of solifenacin and mirabegron using LCMS were evaluated in HQC, MQC and LQC levels. The % accuracy in intraday precision was observed to be in the range of 99.83, 99.77 and 98.83 % for solifenacin and 100.09, 99.37 and 98.08 % for mirabegron in HQC, MQC and LQC respectively. The % accuracy in interday precision was observed to be in the range of 98.07, 96.52 and 96.32 % for solifenacin and 97.59, 98.49 and 96.39 % for mirabegron in HQC, MQC and LQC respectively.

The % RSD was observed to be within the acceptable limit for both the precisions in the studied levels for solifenacin and mirabegron confirming that the method was precise and accurate. Table 3 shows the intraday and interday precision study results observed for the method developed in the study for the analysis of solifenacin and mirabegron.

Recovery: The recovery study confirms the efficiency of the extraction of both the analytes along with internal standard and the % recovery was calculated in three QC levels. The % recovery was observed to be in the range of 90.36 to 106.71 % for solifenacin and 92.84 to 103.57 % for mirabegron. The % recovery was observed to be within the acceptable limit for solifenacin and mirabegron in the developed method. Hence it can be confirmed that the extraction protocol introduced by the proposed method was efficient for the separation and analysis of solifenacin and mirabegron.

The matrix effect defines the efficiency of the ionization of the analytes in the ion source. The matrix effect was examined for solifenacin and mirabegron in three QC levels. The results confirm that there was no significant matrix effect on the ionization (suppression or enhancement) of the analytes which proves that the utilized conditions for sample processing have efficaciously removed any probable interference from the matrix. The impact of sample dilution on the accuracy and precision of the developed method was evaluated in dilution integrity study.

Table 2
Linearity results observed in the developed method

S.N.	Peak area of solifenacin	Peak area of mirabegron	Peak area of IS	Area ratio of solifenacin and IS	Area ratio of mirabegron and IS
1	25	58747.0	35157.9	134157.8	0.438
2	50	65253.4	65855.1	131252.7	0.497
3	100	112565.8	104151.8	133262.8	0.845
4	250	232683.4	198696.3	134154.8	1.734
5	500	444708.5	401235.8	132907.1	3.346
6	750	661241.8	596847.1	133251.8	4.962

Table 3
Intra and interday precision and accuracy results for solifenacin and mirabegron in the developed method

Analyte	QC level	Intraday precision (n=6)			Interday precision (n=6)		
		Conc. found (ng/mL)	Accuracy %	RSD %	Conc. found (ng/mL)	Accuracy %	RSD %
Solifenacin	HQC (1000 ng/mL)	998.33	99.83	1.72	980.65	98.07	1.24
	MQC (250 ng/mL)	249.42	99.77	1.42	241.31	96.52	1.45
	LQC (25 ng/mL)	24.71	98.83	1.83	24.08	96.32	1.63
Mirabegron	HQC (1000 ng/mL)	1000.85	100.09	0.78	975.87	97.59	1.21
	MQC (250 ng/mL)	248.43	99.37	1.22	246.22	98.49	0.80
	LQC (25 ng/mL)	24.52	98.08	1.71	24.10	96.39	1.98

Table 4
Different stability study results of solifenacin and mirabegron in the developed method

S.N.	Test	QC level	Solifenacin			Mirabegron		
			Conc. found (ng/mL)	% stability	RSD %	Conc. found (ng/mL)	% stability	RSD %
1	Short term stability	HQC (1000 ng/mL)	975.75	97.58	1.40	990.85	99.08	1.72
2		MQC (250 ng/mL)	243.74	97.50	1.72	244.37	97.75	0.45
3		LQC (25 ng/mL)	24.05	96.21	1.96	24.12	96.46	0.69
4	Long term stability	HQC (1000 ng/mL)	917.42	91.74	1.32	921.08	92.11	1.49
5		MQC (250 ng/mL)	229.11	91.64	2.05	224.17	89.67	1.19
6		LQC (25 ng/mL)	22.74	90.98	2.38	22.14	88.55	1.38
7	Freeze–thaw stability	HQC (1000 ng/mL)	949.18	94.92	2.01	925.52	92.55	2.90
8		MQC (250 ng/mL)	241.42	96.57	2.40	230.85	92.34	2.11
9		LQC (25 ng/mL)	23.17	92.68	2.51	22.42	89.68	1.71
10	Auto-sampler stability	HQC (1000 ng/mL)	882.40	88.24	1.28	930.00	93.00	2.26
11		MQC (250 ng/mL)	222.04	88.82	2.35	236.35	94.54	3.45
12		LQC (25 ng/mL)	21.56	86.24	1.10	24.01	96.05	3.10
13	Dry extract stability	HQC (1000 ng/mL)	976.94	97.69	2.75	994.61	99.46	3.13
14		MQC (250 ng/mL)	245.28	98.11	2.43	245.89	98.36	2.28
15		LQC (25 ng/mL)	23.99	95.96	2.95	24.48	97.91	2.90

The 2 factor higher concentration than HQC level was prepared and diluted to the HQC level prior to the analysis. The accuracy in the dilution integrity study was observed to be 95.13 % and 94.29 % respectively for solifenacin and mirabegron confirming that the method was accurate and precise. The stability studies such as short term, long term, freeze–thaw, auto-sampler and dry extract stability showed that the mean% nominal values of the analytes were within ± 15% of the predicted concentrations for the analytes at their LQC, HQC and LQC levels. The results of the stability studies were shown in table 4 confirming that the stabilities were acceptable limits and proved the good stability of solifenacin and mirabegron.

Conclusion

A novel HPLC–MS/MS method was developed and subsequently validated for the simultaneous estimation of solifenacin and mirabegron as a combined medication prescribed for the treatment of overactive bladder symptoms in spiked human plasma. The method reports a wide calibration range of 25-1000 ng/mL with sensitive detection limit of 7.5 ng/mL for both solifenacin and mirabegron. The method validation proved that the method shows satisfactory results in terms of its selectivity, recovery, accuracy and precision.

Various stability studies also were performed and results were in the acceptable limit. Hence it can be concluded that the method was suitable for the separation and simultaneous analysis of solifenacin and mirabegron and may be applicable for the pharmacokinetic profiling of the studied drugs.

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