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Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

VALIDATED LC-MS/MS METHOD FOR THE DETERMINATION OF THE MUSCARINIC RECEPTOR ANTAGONIST (MRA) SOLIFENACIN FROM HUMAN PLASMA

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ABSTRACT. The purpose of this study was the development and validation of an LC-MS/MS method, for the determination of solifenacin from human plasma. The sample workup involved a simple protein precipitation procedure. A core/shell type analytical column (50×2,1 mm, 2.6 Å) was used with C18 stationary phase. The mobile phase consisting of 65% acetonitril and 35% water provided good peak shape, accuracy and precision. The mass spectrometer was operated in positive electrospray ionization mode for analyte and internal standard. The following parameters were evaluated for validation purpose: Selectivity, sensitivity, matrix effect, anticoagulant effect, linearity, precision and accuracy, recovery, short and long term analyte/IS stability in solvent/matrix and carryover. The validated calibration range was 0.71-71.28 ng/ml. The correlation coefficient R^2 was at least 0.99 in all validation batches. The validated method has been successfully used for the evaluation of bioequivalence of generic solifenacin 10 mg formulations.

Keywords: *solifenacin, muscarinic antagonist, method validation, bioequivalence trial, LC-MS/MS*

INTRODUCTION

Solifenacin (1*S*,3′*R*)-1-Azabicyclo[2.2.2]oct-8-yl-1-phenyl-3,4-dihydro-1*H*-isochinolin-2-carboxylate with the empirical formula $C_{23}H_{26}N_2O_2$ is a competitive muscarinic receptor antagonist indicated for the treatment of the overactive bladder with associated symptoms such as urge urinary incontinence

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and increased urination frequency. Although acetylcholine and muscarinic receptors mediate different actions in various organs, the major therapeutic target organ for solifenacin is the urological tract [1,2]. Solifenacin is generally used in form of succinate salt (Figure 1).

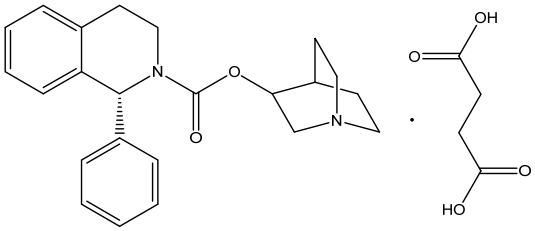

Figure 1. Structure of solifenacin succinate

RESULTS AND DISCUSSION

Determination of acquisition parameters

There are only a few methods known in the literature for the determination of solifenacin in human plasma or pharmaceutical formulations, using LC, UV [3,7] or LC-MS/MS methods [4-6].

The LC/UV methods presented in papers [3,7] uses UV only detection, with LLOQ values of 2ng/ml and 10 µg/ml, unsuitable for bioanalytical assays. Besides there are MS incompatible buffers used for setting the pH of the mobile phase (phosphate). Retention times of up to 25 min are unsuitable for a high throughput analysis.

The LC-MS/MS methods reviewed [4-6] are of a suitable sensitivity (LLOQ between 0.47-0.60 ng/ml), they used a more laborious and time consuming liquid-liquid extraction method. However, analysis time of 3 min. assure a rapid analysis more than 350 samples/day.

The m/z transitions used for multiple reaction monitoring (MRM) were chosen based on the spectra from Figures 2 and 3. The monitored transitions should not interfere in their m/z value, specific for a given analyte. Their intensity should be convenient for the qualifiers, and the qualifier/quantifier ratio should remain stable over the time. Taking into account the considerations above the following transitions were chosen for the quantitative assay method:

Solifenacin: m/z 363.3→110.2, (363.3→193.2 qualifier ion) CE 30V,

Losartan (IS): m/z 423.2→207.2 (423.2→377.3 qualifier ion) CE 15V.

(CE – Collision Energy)

For analyte and IS (Internal Standard) the single charged molecular ions were used as precursors.

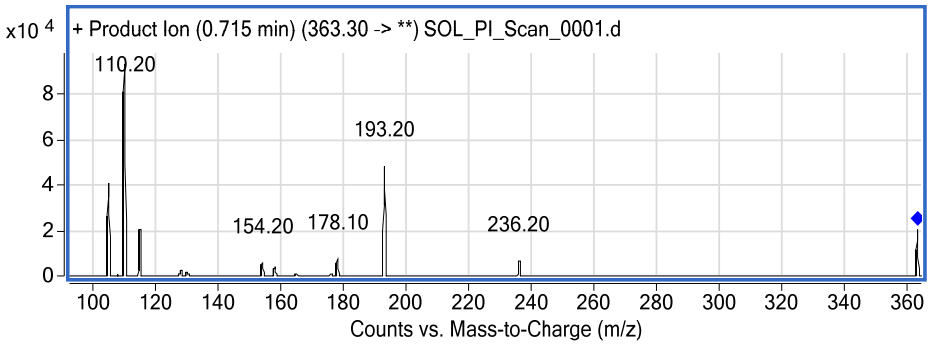

Figure 2. ESI (+) Spectrum of Solifenacin

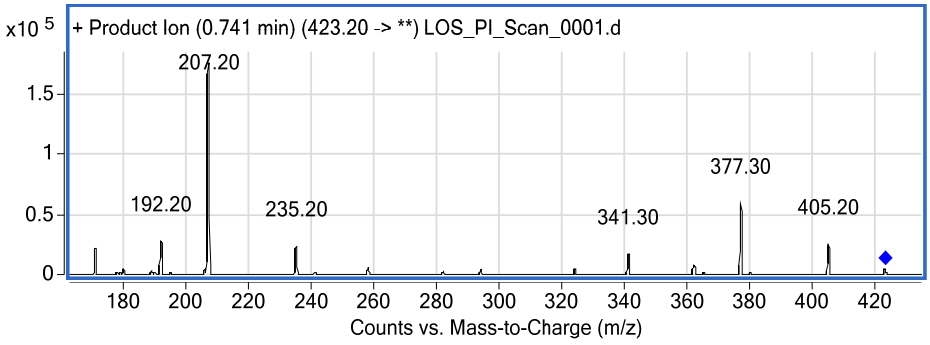

Figure 3. ESI (+) Spectrum Losartan (IS)

Figure 4 shows a typical MRM total ion chromatogram for an ULOQ (upper limit of calibration) sample. The analyte and IS are practically co-eluting at 0.73 min. Values are back calculated concentrations for each analyte.

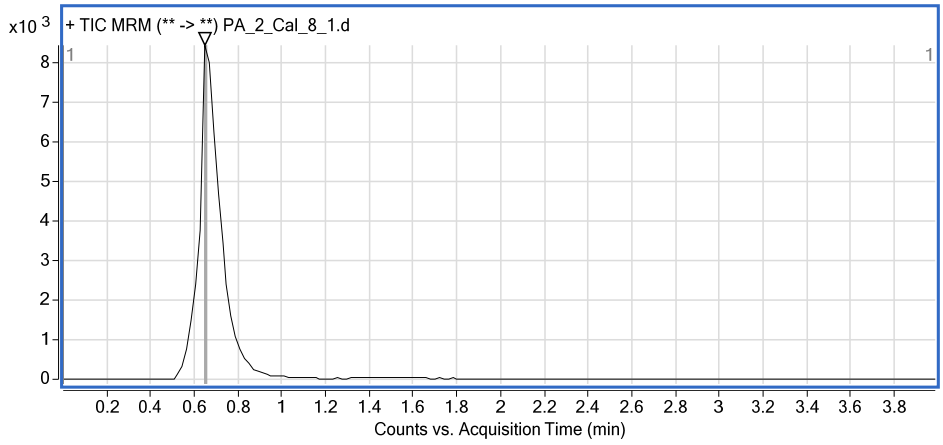

Figure 4. MRM chromatogram of Cal8 (solifenacin 71.28 ng/ml, Losartan 100.75 ng/ml)

The use of the co-eluting internal standard will compensate the matrix effect, and it's a convenient alternative to the stable isotope labeled solifenacin. Moreover, it is easily soluble in the sample solvent resulting after plasma protein precipitation (methanol:water 3:1).

It's noticeable, that no significant spectral response has been observed at the retention time of the analyte/IS in matrix blank samples (Figure 5.).

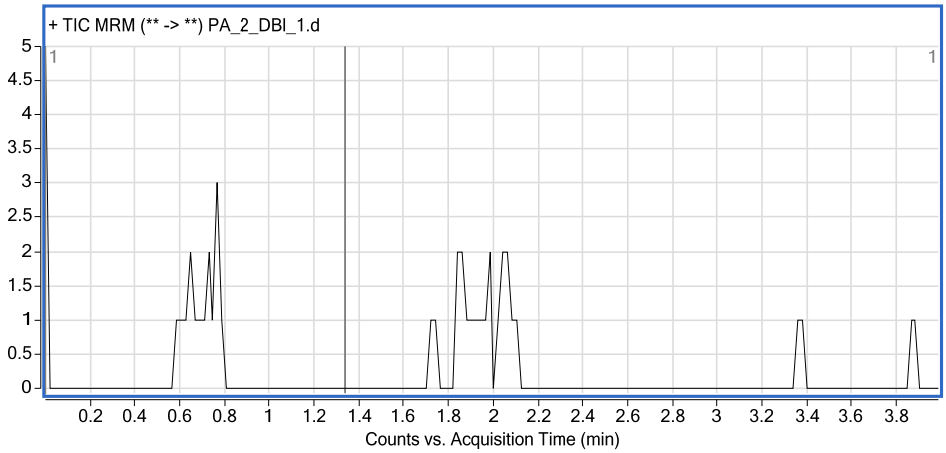

Figure 5. MRM chromatogram of DBI1 (matrix blank 0 ng/ml analyte/IS)

Bioanalytical method validation

The analytical method was validated according to the EMEA/ CHMP/EWP/192217/2009 Guideline on validation of bioanalytical methods [8].

The tested parameters were: selectivity, sensitivity, matrix effect, anticoagulant effect, intra/interbatch precision and accuracy, recovery, short/long term stability of stock solutions of analyte, short term stability of working solutions of analyte, bench top stability in biological matrix, freeze thaw stability in biological matrix, injector/autosampler stability of the processed samples, stability during delayed processing, dilution integrity, carryover. All tests were performed using 6 replicates at the mentioned QC (Quality Control) levels.

The calibration curve range is established according to literature data about plasma concentrations of the analyte. $C_{max\ average\ from\ literature}$ for Solifenacin was found of ca. 15 ng/ml, after administration of a 10 mg dose. [2,6]

A summary of main results of validation batches is presented in Table 1.

The validated calibration range was 0.71-71.28 ng/ml. The calibration curves were obtained using a linear weighted (1/x) regression analysis of the

peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. The lower limit of quantitation was set smaller than 5% of expected average C_{max} values.

Linearity summary results for solifenacin are presented in Table 2. The limit of quantitation was 0.71 ng/ml and the linear dynamic range of the curve was from 0.71 to 71.28 ng/ml.

Summary of method validation

Table 1. Bioanalytical method validation summary for solifenacin

Calibration concentrations (ng/ml)	0.71, 2.14, 5.35, 10.69, 21.39, 32.08, 42.77, 71.28
Lower limit of quantitation (ng/ml)	LLOQ, 0.71 Accuracy 98.84 %, RSD 2.91
QC Concentrations (ng/ml)	LLOQ-QC, LQC, MQC, HQC 0.71, 2.14, 21.39, 42.77
Between-run accuracy (%)	LLOQ-QC, LQC, MQC, HQC 104.99, 103.65, 102.23, 100.81
Between-run precision (RSD)	LLOQ-QC, LQC, MQC, HQC 9.08, 3.67, 2.39, 4.07
IS normalized Matrix factor (MF) RSD	LQC 1.21 4.44
Recovery (%)	LQC MQC HQC 100.32 97.97 101.17
Long term stability of stock solution and working solutions (Observed change %)	Confirmed up to 53 days at +4 °C LQC Stab. 101.09, change +1.09 % HQC Stab. 94.35 change –5.65 % IS Stab. 101.38 change + 1.62%
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	Confirmed up to 23.80 h LQC Stab. 102.68, change +2.68 % HQC Stab. 101.70 change +1.70 %
Long term stability in biological matrix (Observed change %)	Confirmed up to 189 days at –50 °C LQC Stab. 94.97, change –5.03 % HQC Stab. 93.79 change –6.21 %
Autosampler storage stability (Observed change %)	Confirmed up to 76.1(6) h LQC Stab. 95.34, change –4.66 % HQC Stab. 92.21 change –7.79 %
Freeze and thaw stability (Observed change %)	-50 °C , 3 cycles LQC Stab. 101.58, change +1.58% HQC Stab. 102.09, change +2.09%
Dilution integrity	Concentration diluted (2-fold) 103.97 %; RSD 3.78 % Concentration diluted (4-fold) 105.87%; RSD 3.04 %

PA – Precision and Accuracy batch
LLOQ-QC/LQC/MQC/HQC – Lower Limit of Quantitation/Low/Medium/High Quality Control sample

Table 2. Linearity summary results for solifenacin

Calibration level	Nominal conc. (ng/ml)	Mean conc.±S.D. (ng/ml)	RSD %	Accuracy %
Cal_1_1	0.71	0.71±0.05	7.21	100.29
Cal_1_2	0.71	0.70±0.06	8.19	98.62
Cal_2	2.14	2.11±0.03	1.49	98.60
Cal_3	5.35	5.39±0.08	1.49	100.78
Cal_4	10.69	10.86±0.05	0.45	101.55
Cal_5	21.39	21.59±0.41	1.92	100.93
Cal_6	32.08	32.28±0.72	2.22	100.64
Cal_7	42.77	42.12±1.18	2.80	98.49
Cal_8_1	71.28	71.62±1.46	2.03	100.47
Cal_8_2	71.28	71.02±1.05	1.48	99.63

CONCLUSIONS

A rapid and robust method has been developed and validated for the determination of solifenacin in human plasma. The quantitation was performed on an Agilent 1200 series HPLC system, coupled to an Agilent 6410 triple quadrupole mass spectrometer, using electrospray ionization technique. The components were detected in positive ionization mode.

The developed method involves a rapid sample workup using protein precipitation versus liquid-liquid extraction. Even so, the sample matrix presents no interferences or significant matrix effects at the retention time on analyte/IS. An analysis time of 4 min. is adequate for the elution of all analytes/matrix components from the column, and ensures a capacity of more than 350 samples/day on the used LC-MS/MS system. The usage of a non-labeled internal standard beside the protein precipitation, makes the method more cost-effective.

The method was successfully used for the evaluation of bioequivalence of a generic formulation of solifenacin 10 mg film-coated tablets in human subjects.

EXPERIMENTAL SECTION

Solvents and reference materials used

All used solvents are of HPLC grade. Acetonitril was purchased from VWR, formic acid from Merck KGaA, HPLC water was obtained using a Millipore Simplicity UV water purification system. Certified reference materials of Solifenacin succinate and Losartan potassium (internal standard-IS) were obtained from Ak Scientific Inc., respectively Sigma-Aldrich and are of analytical standard grade. Blank human plasma was obtained from the regional blood transfusion center (CRTS) Cluj.

Instrumentation and working parameters

An Agilent 1200 series HPLC system with a Phenomenex Kynetex C18 column (50 × 2.10 mm) equipped with Phenomenex Security Guard (4 ×2.0 mm) was used for separation. The used mobile phase was an isocratic mixture of 65:35 acetonitrile:water (containing 0.5% formic acid). The used flow rate was 0.3 ml/min, the column temperature was set to 35 ºC. An Agilent 6410 triple Quadrupole Mass Spectrometer (Agilent Technologies, USA), equipped with electrospray ion source was used for the LC-MS/MS analyses. The runtime was 4 min/sample. The data acquisition and processing were carried out using MassHunter software. The whole system (software and hardware) was validated. The mass spectrometer was operated in positive ionization mode for analyte and IS. Nitrogen was used as nebulizing gas and collision cell gas. The temperature of the ESI source was set to 350 ºC, and the needle voltage to 4000V.

The quantitation was performed using MRM (multiple reaction monitoring) of the transitions: m/z 363.3→110.2, (363.3→193.2 qualifier ion) collision energy 30V, for solifenacin and 423.2→207.2 (423.2→377.3 qualifier ion) collision energy 15V for losartan (IS).

The mass spectrometer was operated at unit resolution with a dwell time of 300 ms per transition.

Stock and working solutions preparation

Stock solutions of solifenacin (1.0 mg/ml) were prepared in ultrapure water dissolving accurately weighed amounts of reference material. Stock solutions of losartan (1.0 mg/ml) were prepared in methanol/water 50/50 (w/w) dissolving accurately weighed amounts of losartan. They were stored between 2-8 °C. Correction factors were applied to the weighed amounts of reference materials to calculate the content of the pure substance (Table 3). Correction factors are derived from the purity and the chemical form (salt, etc.). Water content will be substracted from the purity.

Table 3. Correction factors for reference materials

Reference material	**Solifenacin succinate**	**Losartan potassium**
Purity (%)	98.3	99.6
Water (%)	0.23	0.27
Chemical form correction factor	0.7543	0.9174
Correction factor	**0.7397**	**0.9113**

Working solutions of analyte and internal standard were prepared freshly before use by successive dilutions from stock solutions to appropriate levels, using water as solvent. They were used for spiking human plasma used for calibrators and QC samples preparation.

Calibrators and QC samples preparation

To 400 µl of blank human plasma, 50 µl of spiking solution of analyte and 50 µl of spiking solution of internal standard were added in polypropylene tubes, to yield final concentrations of 0.71, 2.14, 5.35, 10.69, 21.39, 32.08, 42.77, 71.28 ng/ml for solifenacin.

Sample preparation (workup)

To precipitate plasma proteins, 1500 µl of acetonitrile was added to the spiked samples, then vortexed for 20 minutes at 1500 rpm. Further the samples were centrifuged at 4 °C for 10 minutes at 4000 rpm. 800 µl of supernatant was transferred to HPLC autosampler vials and injected into the analytical system (20 µl/sample).

Calibration curve parameters

The linearity of the method was evaluated using spiked plasma samples in the concentration range mentioned above using the method of least squares. Three linearity curves were analyzed.
Each calibration batch (curve) consisted of: blank samples in duplicate, zero samples (blank with IS) in duplicate and eight non-zero concentration levels, of which the lower and upper limit of quantitation samples were in duplicate. The calibration curves were obtained by using a linear weighted (1/x) regression analysis of the peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. Study samples concentrations were obtained by interpolation from the calibration curve.

The linearity results are summarized in Table 2 in the 'Results and Discussion' section.

ACKNOWLEDGMENTS

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