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Dedicated to Professor Ioan Bâldea on the Occasion of His 80th Anniversary

VALIDATED LC-MS/MS METHOD FOR THE DETERMINATION OF TADALAFIL – A COMPETITIVE PHOSPHODIESTERASE 5 INHIBITOR (PDE5) – 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 tadalafil 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 30% acetonitril and 70% water provided good peak shape, accuracy and precision (stable ionization). 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 22.2-1111.3 ng/ml. The correlation coefficient R^2 was at least 0.9995 in all validation batches. The validated method has been successfully used for the evaluation of bioequivalence of generic tadalafil 20 mg formulations.

Keywords: *tadalafil, phosphodiesterase 5 inhibitor, method validation, bioequivalence trial, LC-MS/MS*

INTRODUCTION

Tadalafil, (6R,12aR)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexa-hydro-2-methylpyrazino[1′,2′:1,6]pyrido[3,4-b]indole-1,4-dione with the empirical formula $C_{22}H_{19}N_3O_4$ (Figure 1) is a reversible cyclic guanosine

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monophosphate(cGMP)-specific phosphodiesterase Type 5 inhibitor indicated for the treatment pulmonary arterial hypertension, benign prostatic hyperplasia but mainly used for the treatment of erectile dysfunction (impotence) [1, 2, 13]. Tadalafil acts as a muscle relaxant of blood vessels, increasing blood flow to particular part of the human body.

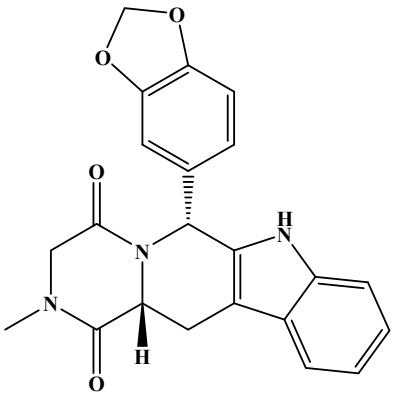

Figure 1. Structure of tadalafil

RESULTS AND DISCUSSION

Determination of acquisition parameters

There are various methods known in the literature for the determination of tadalafil in human blood, plasma or urine [3, 4, 7-9], animal plasma [5] or pharmaceutical formulations/dietary supplements [11], using LC-MS/MS methods.

The m/z transitions used for multiple reaction monitoring (MRM) [6, 10, 12] 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 whole calibration range and in time. Taking into account the considerations above the following transitions were chosen for the quantitative assay method:

Tadalafil: m/z 390.2→268.1, (390.2→135.0 qualifier ion) CE 15V,

Losartan (IS): m/z 423.2→207.1 (423.2→377.2 qualifier ion) CE 15V.
(CE – Collision Energy)

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

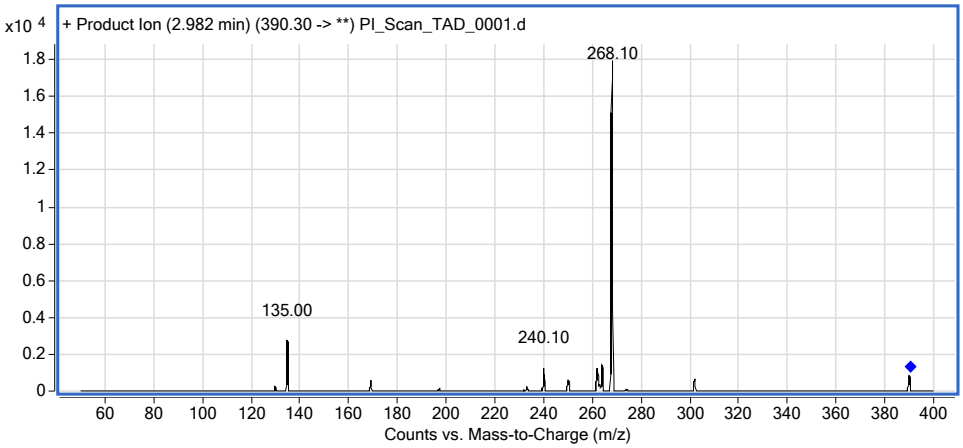

Figure 2. ESI (+) Spectrum of Tadalafil

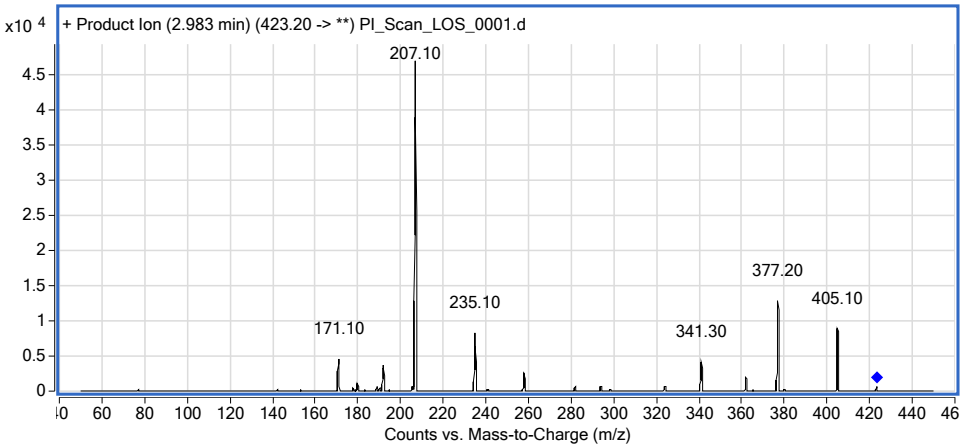

Figure 3. ESI (+) Spectrum Losartan (IS)

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 3.0 min. Values are back calculated concentrations for each analyte.

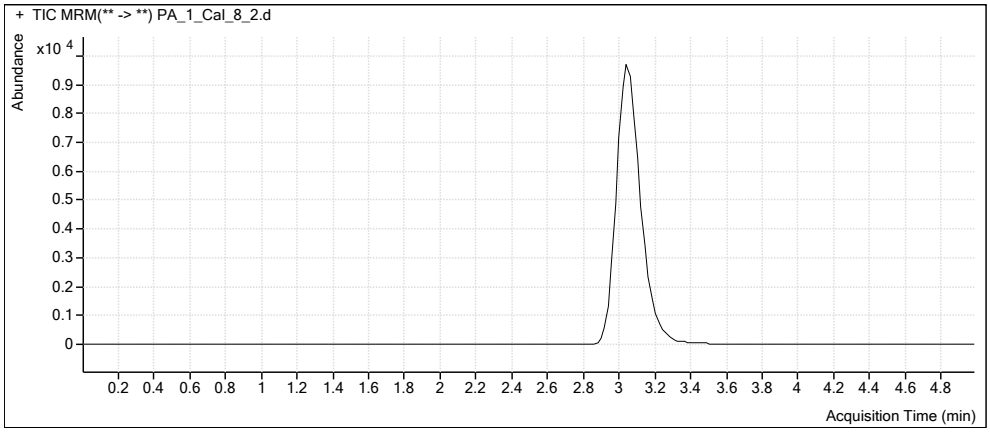

Figure 4. MRM chromatogram of Cal8 (tadalafil 1111.3 ng/ml, Losartan 39.9 ng/ml)

The use of the co-eluting internal standard leads to a minimization of the matrix effect, and it's a convenient alternative to the stable isotope labeled tadalafil. Moreover, it is easily soluble in the sample solvent resulting after plasma protein precipitation (methanol:water 4:1).

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).

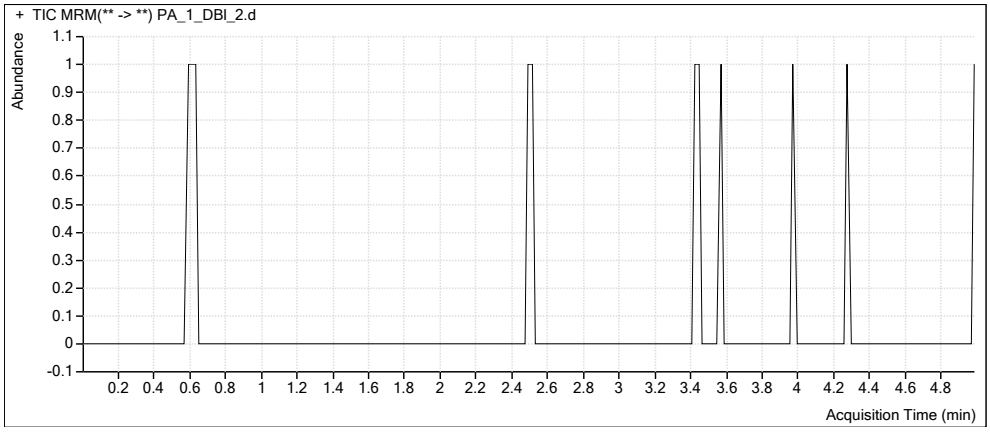

Figure 5. MRM chromatogram of DBl2 (matrix blank 0 ng/ml analyte/IS)

Bioanalytical method validation

The analytical method was validated according to the EMEA/CHMP/EWP/192217/2009 Guideline on validation of bioanalytical methods [14].

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, tabletop stability in biological matrix, freeze thaw stability in biological matrix, injector/autosampler stability of the processed samples, stability during delayed processing (worst case scenario), dilution qualification, analyte and internal standard carryover. All tests were performed using 6 replicates at the mentioned QC (Quality Control) concentration levels.

The calibration curve range is established according to literature data about plasma concentrations of the analyte. $C_{max\ average\ from\ literature}$ for Tadalafil was found to be ~ 450-500 ng/ml, after administration of a 20 mg dose [2-4, 8].

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

The validated calibration range was 22.2-1111.3 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 tadalafil are presented in Table 2. The limit of quantitation was 22.2 ng/ml and the linear dynamic range of the curve was from 22.2 to 1111.3 ng/ml.

Summary of method validation

Table 1. Bioanalytical method validation summary for tadalafil

Calibration concentrations (ng/ml)	22.23, 22.23, 44.45, 83.35, 166.70, 333.40, 611.23, 833.50, 1111.33, 1111.33 (ng/ml)
Lower limit of quantitation (ng/ml)	LLOQ, 22.23ng/ml, Accuracy 97.91%, RSD 2.98
QC Concentrations (ng/ml)	LLOQ-QC, LQC, MQC, HQC 22.23, 44.45, 333.40, 833.50 (ng/ml)
Between-run accuracy (%)	LLOQ-QC, LQC, MQC, HQC 99.74, 99.50, 99.11, 99.30
Between-run precision (RSD)	LLOQ-QC, LQC, MQC, HQC 2.18, 2.82, 3.76, 3.97
IS normalized Matrix factor (MF) RSD	0.96 (1.32)

Recovery (%)	LQC, MQC, HQC 100.38, 98.29, 97.14
Long term stability of stock solution and working solutions (Observed change %)	Confirmed up to 20 days at + 4 °C LQC Stab. 111.86, change +11.86% HQC Stab. 105.35 change +5.35%
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	Confirmed up to 23.58(3) h LQC Stab. 106.13, change +6.13% HQC Stab. 106.66, change +6.66%
Long term stability in biological matrix (Observed change %)	Confirmed up to 134 days at -50 °C LQC Stab. 106.38, change +6.38% HQC Stab. 103.23 change +3.23%
Autosampler storage stability (Observed change %)	Confirmed up to 73.38(3) h LQC Stab. 103.53, change +3.53% HQC Stab. 100.59, change +0.59%
Freeze and thaw stability (Observed change %)	-50 °C , 3 cycles LQC Stab. 100.77, change +0.77% HQC Stab. 99.01, change –0.99%
Dilution integrity	Concentration diluted (2-fold) 99.97%; RSD 1.91% Concentration diluted (4-fold) 97.73%; RSD 4.35%

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 tadalafil

Calibration level	Nominal conc. (ng/ml)	Mean conc.±S.D. (ng/ml) n=6	RSD %	Accuracy %
Cal_1_1	22.23	22.21±0.60	2.70	99.92
Cal_1_2	22.23	22.15±0.54	2.43	99.64
Cal_2	44.45	45.24±0.55	1.22	101.78
Cal_3	83.35	83.94±0.77	0.92	100.70
Cal_4	166.70	162.58±3.25	2.00	97.53
Cal_5	333.40	335.99±4.30	1.28	100.78
Cal_6	611.23	603.09±8.76	1.45	98.67
Cal_7	833.50	841.03±2.53	0.30	100.90
Cal_8_1	1,111.33	1103.99±6.81	0.62	99.34
Cal_8_2	1,111.33	1119.52±8.15	0.73	100.74

CONCLUSIONS

A rapid and robust method has been developed and validated for the determination of tadalafil 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 method was successfully used for the evaluation of bioequivalence of a generic formulation of tadalafil 20 mg film-coated tablets versus *Cialis*® 20 mg film-coated tablets, Eli Lilly, in 26 healthy Caucasian male subjects.

EXPERIMENTAL SECTION

Solvents and reference materials used

All used solvents are of HPLC grade. Acetonitril and Methanol were purchased from VWR, formic acid from Merck KGaA, HPLC water was obtained using a Millipore Simplicity UV water purification system. Certified reference materials of Tadalafil and Losartan potassium (internal standard-IS) were obtained from European Pharmacopoeia (Ph.Eur.), respectively Fluka 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 30:70 acetonitrile:water (containing 0.1% formic acid). The used flow rate was 0.3 ml/min, the column temperature was set to 45 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 5 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 390.2→268.1, (390.2→135.0 qualifier ion) collision energy 15V, for tadalafil and 423.2→207.1 (423.2→377.2 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 tadalafil (1.0 mg/ml) were prepared in methanol dissolving accurately weighed amounts of reference material. Stock solutions of losartan-K (1.0 mg/ml) were prepared in methanol/water 50/50 (w/w) dissolving accurately weighed amounts of losartan-K. They were stored at 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.).

Table 3. Correction factors for reference materials

Reference material	**Tadalafil**	**Losartan potassium**
Purity (%)	99.9	99.6
Chemical form correction factor	1.0000	0.9152
Correction factor	**0.9990**	**0.9115**

Working solutions of analyte and internal standard were prepared freshly before use by successive dilutions from stock solutions to appropriate levels, using methanol/water 50/50 (w/w) 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 22.23, 44.45, 83.35, 166.70, 333.40, 611.23, 833.50, 1111.33 ng/ml for tadalafil.

Sample preparation (workup)

To precipitate plasma proteins, 2 ml of methanol was added to the spiked samples, then vortexed for 20 minutes at 1500 rpm. Further the samples were centrifuged at 4 °C for 20 minutes at 4000 rpm. 450 µl of supernatant was transferred to HPLC autosampler vials diluted with 450 µl of water and injected into the analytical system (15 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. Two identical validated analytical systems has been used, three linearity curves were analyzed on each system.

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.

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