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Development and validation of a GC/MS method for the determination of tadalafil in whole blood

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ABSTRACT

Tadalafil is a phosphodiesterase type 5 (PDE-5) inhibitor and it is used in the treatment of pulmonary arterial hypertension and erectile dysfunction. A sensitive and specific method is described for the determination of tadalafil in whole blood. Tadalafil and its internal standard (protriptyline) were isolated from the matrix by solid phase extraction, and were analyzed by gas chromatography/mass spectrometry (GC/MS) after derivatization by N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS). Limits of detection and quantification for tadalafil were 0.70 and 2.00 μg/L, respectively. The calibration curve was linear between 2.00 and 500.0 μg/L, with a correlation coefficient higher than 0.991. The values obtained for intra- and inter-day accuracy was found to be between −10.5 to 8.5% and −4.2 to 4.5%, respectively, while intra- and inter-day precision were less than 8.4 and 11.2%, correspondingly. Absolute recovery was determined at three concentration levels and ranged from 92.1 to 98.9%. The proposed method is the first fully validated GC/MS method for the determination of tadalafil in whole blood and it can be routinely applied by toxicological laboratories, for pharmacokinetic studies, for therapeutic drug level monitoring or for the investigation of related forensic cases.

1. Introduction

Tadalafil is one of the three selective phosphodiesterase type 5 (PDE-5) inhibitors and it is the active compound of Cialis®, a prescription drug approved by United States Food and Drug Administration in 2003 for the treatment of penile erectile dysfunction and in 2009 for the treatment of the pulmonary arterial hypertension [1–4]. The therapeutic dose of tadalafil ranges from 2.5 to 20 mg, daily, and its absorption is not affected by food. Tadalafil is effective about 16 min after administration while its peak efficacy occurs after about 2 h and it is maintained for up to 36 h [2,5]. Due to its long plasma half-life (17.5 h) and the clinical efficacy of different doses taken once daily, that last 36–48 h, tadalafil is preferred as a therapeutic alternative for men, who may be looking for a dosing option that avoids scheduling sexual activity and they want more convenience and spontaneity in their sexual life [5]. The drug is predominantly metabolized by the liver CYP3A and eliminated primarily in the feces and urine, while inhibitors of the CYP3A such as clarithromycin may lead to elevated serum concentrations [6].

Tadalafil is also the first once-daily PDE-5 inhibitor used in the treatment of pulmonary arterial hypertension, usually at doses of 40 mg, and it has been shown to improve exercise capacity and tolerance, pulmonary hemodynamics and quality of life [6–8]. Furthermore, tadalafil could be the active substance or an adulterant in some dietary supplements, herbal remedies and food products, which mainly claim to enhance male sexual function [1,9,10]. Adverse effects of tadalafil are the same as with the other two PDE-5 inhibitors (sildenafil and vardenafil), like headache, facial flushing, nausea, dyspepsia, abdominal pain, visual disturbances, muscle aches, hypotension, tachycardia and rarely, heart attack. It is important to be noticed that PDE-5 inhibitors are prescription drugs and should be used under medical supervision. Physicians should advise patients to seek immediately medical attention in the event of an adverse effect [10,11]. Furthermore, patients taking nitrate medications should not use tadalafil or other PDE-5 inhibitor, as this combination might provoke potentially life-threatening hypotension [10].

There are some analytical methods for the determination of tadalafil in plasma [12,13] or urine [14], but mainly in non biological materials such as pharmaceutical tablets [15] and dietary supplements [9,10,16] or herbal products [1,16–18]. Most of these methods are based on liquid chromatography [1,9,10,12,13,15–17] combined with UV [1,9,12,13,15,16], MS [1,16] or MS/MS [9,10,17],

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whereas there are only two GC/MS published methods for the determination of tadalafil, one in dietary supplements, herbal and food products [18] and one in urine [14]. To the best of our knowledge, there is no method published concerning the determination of tadalafil in whole blood. The aim of our study was the development, optimization and validation of a simple, sensitive and specific GC/EI-MS method for the determination of tadalafil in whole blood. The developed method could be used for drug level monitoring during clinical practice, for pharmacokinetic and pharmacodynamic studies, as well as for the investigation of forensic cases. As it is mentioned above, there are no validated methods published concerning the determination of tadalafil in blood. Whole blood is used for therapeutic drug monitoring and it is the biological matrix of choice in forensic cases. The two already published HPLC methods are in plasma [12,13] and lack sensitivity as they show quite high LOQ values. Gas chromatography–mass spectrometry instrumentation is available almost in all toxicological laboratories. It offers a low cost analysis and at the same time provides the necessary identification criteria for drug analysis. A GC/MS method can easily be applied in routine every day analysis by clinical and forensic laboratories.

2. Experimental

2.1. Chemical and reagents

Reference standard of tadalafil (>99.9% pure) was donated by Eli Lilly (Surrey, UK) and was kept according to the instructions of its commercial certificate. Protriptyline (>99.9% pure) was purchased from Sigma–Aldrich (St. Louis, USA). Analytical or HPLC grade solvents were used (methanol, hexane, ethyl acetate, dichloromethane, acetonitrile, isopropanol, ammonium hydroxide) and were purchased from Merck (Darmstadt, Germany). Analytical reagents were purchased as follows: pentafluoropropionic anhydride (PFPA) 99%, heptafluorobutyric anhydride (HFBA) 99%, N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS), N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and trifluroacetic acid (TFAA) 99% from Fluka (Steinheim, Germany), acetic anhydride 97% from Mallinckrodt (St. Louis, USA) and pyridine 99.5% from Ferak (Berlin, Germany). In this study SPE columns (Abselut Nexus, Bond Elut LRC Certify I and II) were obtained from Varian (Houten, Netherlands). Human drug free whole blood was obtained in test tubes containing anti-coagulant (EDTA K_3) from healthy donors, after informed consent, and before its use it was screened by GC/MS for the presence of tadalafil and protriptyline.

2.2. Calibrators and controls

Stock standard solutions of tadalafil and protriptyline at concentration of 1.00 mg/mL were prepared by dissolving the appropriate amount of drug in methanol. Working standard solutions of tadalafil at concentrations: 0.04, 0.10, 0.12, 0.40, 1.00, 2.00, 4.00, 8.00 and 10.0 μg/mL, and the working internal standard solution of protriptyline at a concentration of 2.00 μg/mL were prepared by diluting the appropriate volume of the corresponding stock standard solution with methanol. Fresh working solutions were prepared on a daily basis. Six blood calibrators containing tadalafil at concentrations of 2.00, 5.00, 20.0, 50.0, 200.0 and 500.0 μg/L were prepared by spiking 950 μL of blank human whole blood with 50 μL of the working standard solutions. The tadalafil concentrations of the three blood quality control (QC) samples were 6.00, 100.0 and 400.0 μg/L, and were prepared in a similar way to the calibrators.

2.3. Instrumentation

Chromatographic analysis was performed using a Shimadzu GC-2010 gas chromatograph equipped with a model AOC-20i autosampler system (Shimadzu, Japan) and interfaced with a QP 2010S mass spectrometer (Shimadzu). A cross-linked DB-1MS (Agilent, USA) capillary column (12 m × 0.20 mm i.d., 0.33 μm film thickness) was used. Additional apparatus used in this study were: a 691 digital pH-meter (METROHM, Switzerland) with a glass electrode, a vortex (Chiltern, Model MT 19), a cooling centrifuge (Sigma 4K10, Germany), an SPE manifold (VacMaster®-10, IST) and an evaporator using nitrogen (Reacti-Vap PIERCE, Model 18780, Rockford, IL).

2.4. GC/MS conditions

The chromatographic conditions were as follows: initial oven temperature was 150 °C for 1 min, which was increased by 40 °C/min to 300 °C and held there for 5 min. The temperatures of the injection port, ion source and interface were 280, 250 and 300 °C, respectively. Helium was used as carrier gas at a flow rate of 0.6 mL/min. The mass spectrometer (MS) was operated in electron impact ionization/selective ion monitoring (EI/SIM) mode (70 eV). The identification mass fragments used in this method were: *m/z* **461**, 340 and 334 for derivatized tadalafil and *m/z* **116**, 142 and 335 for the derivatized internal standard protriptyline (quantification ions are bold marked). The mass spectrum of silylated tadalafil is shown in Fig. 1. The retention times of silylated tadalafil and protriptyline were 8.9 and 4.3 min, respectively.

2.5. Sample preparation

A volume of 50 μL of the working internal standard solution was added to the calibrator, QC and patient samples (1000 μL) and all were vortex mixed for 15 s. Therefore, all samples contained 100.0 μg/L of protriptyline. The pH of all samples was adjusted to 7 with the addition of 5.00 mL of a mixture of acetate buffer (pH 7.00) with methanol (95:5, v/v, mixture A) and the samples were centrifuged at 2000 rpm for 10 min. Bond Elut LRC Certify II SPE columns were conditioned with 2 mL of methanol and 2 mL of mixture A prior to sample loading. The samples were applied onto the columns at a flow rate of approximately 1.0 mL/min. Then, the columns were washed successively with 2 mL of mixture A and they were dried under high vacuum (≥10 mm Hg) for 3 min. The analytes were eluted twice with 1.5 mL of a freshly prepared mixture of dichloromethane:isopropanol:ammonium hydroxide (85:15:2, v/v/v). The eluates were collected in clean tubes and evaporated to dryness under a gentle stream of N_2 at 40 °C. The extracted samples were derivatized by adding subsequently 30 μL of acetonitrile and 30 μL of BSTFA with 1% TMCS, vortex mixing and heating at 70 °C for 30 min. After cooling, the samples were injected (1 μL) into the GC/MS system (splitless mode).

3. Results and discussion

3.1. Method development

A GC/MS method was developed for the determination of tadalafil in whole blood that includes isolation of tadalafil from the samples by SPE followed by derivatization (silylation) using BSTFA with 1% TMCS. To the best of our knowledge, it is the first validated GC/MS method for the determination of tadalafil in whole blood.

During the optimization of the derivatization step, different derivatization reagents, such as PFPA, HFBA, TFAA, BSTFA with 1% TMCS, MTBSTFA and a mixture of acetic anhydride in pyridine were tested. Tadalafil was derivatized only after silylation by BSTFA with 1% TMCS, while MTBSTFA led to extremely low yield (≤10%). When

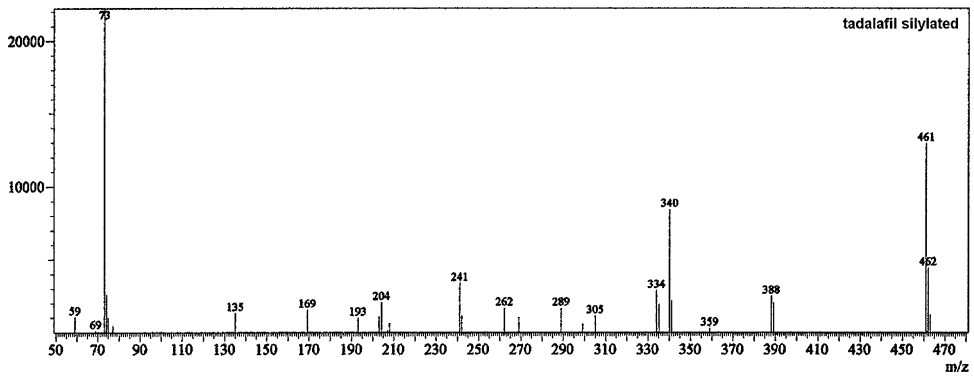

Fig. 1. Mass spectrum of silylated tadalafil.

the other derivatization reagents (PFPA, HFBA, TFAA, mixture of acetic anhydride in pyridine) were used, tadalafil was not derivatized under any condition tested (temperature and duration of the reaction). So, BSTFA with 1% TMCS was selected as the derivatization reagent and the conditions of the reaction (temperature and duration) were optimized. During the optimization of the derivatization procedure, different temperatures (room temperature, 50, 60, 70, 80 and 90 °C) were tested and the peak area of silylated tadalafil was found to be maximum at 70 °C. The optimum derivatization duration was found to be 30 min, while after that time there was not significant effect up to 60 min. Chromatographic conditions of the method were optimized and different values of each parameter were tested like injector (240, 260, **280**, 300 °C), interface (280, 290, **300**, 310 °C), initial (80, 100, 120, **150** °C) and final column (280, 290, **300**, 310 °C) temperatures, column temperature rate (10, 20, 30, **40**, 50 °C/min) and carrier gas flow rate (0.5, **0.6**, 0.7, 0.8, 0.9, 1.0 mL/min). The optimized GC separation was achieved using the bold marked chromatographic conditions.

During the optimization of the sample preparation step of the method, liquid–liquid extraction (LLE) and solid-phase extraction (SPE) of blood samples were checked. The LLE of blood samples with a plethora of organic solvents [*tert*-butyl methyl ether, hexane, toluene] and mixtures of solvents [hexane:ethyl acetate (80:20, v/v), hexane:ethyl acetate:isopropanol (80:15:5, v/v/v), hexane:dichloromethane:ethyl acetate (2:1:1, v/v/v)] at basic pH [8.0, 9.0 and 10.0] led to dirty samples unsuitable for derivatization. The back extraction technique [12] gave clean extracts but very low recovery results (≤32%). When SPE was tested, three different types of SPE columns were chosen: Abselut Nexus (Varian), Bond Elut LRC Certify (Varian) and Bond Elut LRC Certify II (Varian). The lower recovery results for tadalafil (≤48%) were found when Bond Elut LRC Certify SPE columns used, as well as Abselut Nexus SPE columns led also to low recovery results (≤61%). The higher recovery results for both tadalafil and protriptyline without any interference from endogenous whole blood compounds were achieved using Bond Elut LRC Certify II SPE columns. The extraction efficiency for the analyte and its internal standard was reproducible when using these SPE columns and the extracts were suitable for derivatization. Subsequently, the SPE procedure was further optimized by: (a) modifications of the washing of the columns (methanol; **mixture A**), and (b) using different solvent mixtures for the elution of the analytes [ethyl acetate:ammonium hydroxide (100:2, v/v); methanol:ammonium hydroxide (100:2, v/v) **dichloromethane:isopropanol:ammonium hydroxide (85:15:2, v/v/v)**; ethyl acetate:isopropanol:ammonium hydroxide (85:15:2, v/v/v); and ethyl acetate:acetonitrile:ammonium hydroxide (85:15:2, v/v/v)]. The optimum SPE conditions are bold marked.

3.2. Method validation

The following criteria were used to evaluate the GC/MS method according to FDA [19] and ICH [20] guidelines: selectivity, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, absolute recovery, robustness and stability. Selectivity, linearity, precision and accuracy of the method were validated through six analytical runs at six different days.

Selectivity was studied by analyzing six different blank samples and the matrix effect was assessed. All blank blood samples were free of co-eluting peaks at the retention times of the analyte and the internal standard (Fig. 2A). The selectivity of the method was adequate with minimal matrix effect at all blank samples.

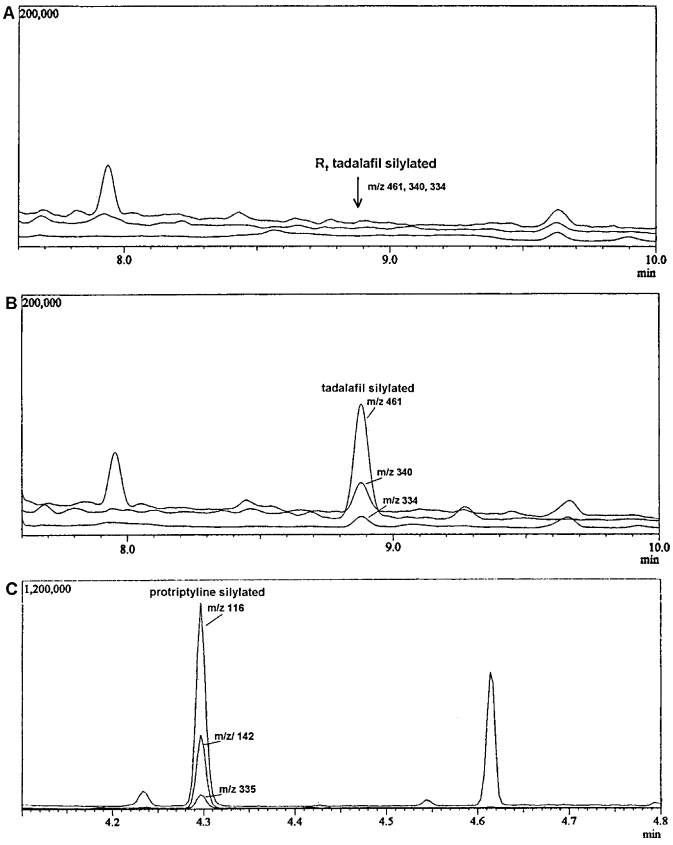

Fig. 2. SIM chromatograms of a: (A) blank, (B) spiked blood sample at the LOQ concentration (tadalafil: 2.00 μg/L), and (C) internal standard (protriptyline: 100.0 μg/L).

Table 1
Linearity results of the developed method for the determination of tadalafil in whole blood.

Concentration range	Linear regression equation $y = a(\pm s_a)x + b(\pm s_b)$	R^2	LOD (μg/L)3.3 (s_b/a)	LOQ (μg/L)10 (s_b/a)
(2.00–500 μg/L)	$y = 0.01033\ (\pm 0.00081)x - 0.0095\ (\pm 0.0042)$	≥0.991	1.34	4.07

y = peak area ratio of analyte/internal standard.

Specificity was determined by analyzing a standard mixture of commonly used illicit and licit drugs as well as their metabolites (sildenafil, vardenafil, morphine, codeine, 6-acetyl-morphine, methadone, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, cocaine, ecgonine methylester, benzoyloecgonine, diazepam, nordiazepam, bromazepam, alprazolam, 7-amino-flunitrazepam, phenobarbital, amitriptyline, clomipramine, venlafaxine, citalopram, mirtazapine, metoprolol, atenolol, diltiazem, amphetamine, methamphetamine, MDMA, ephedrine, ketamine) at a concentration of 10 mg/L. Spiked blood samples with these substances ($n = 6$) at a concentration of 1 mg/L were analyzed. The specificity study showed that blood concentrations of 1 mg/L of all drugs tested do not interfere with the accurate determination of tadalafil and protriptyline in whole blood.

The *LOD* and *LOQ* for tadalafil were determined as the lowest concentration yielding signal-to-noise ratios of at least 3:1 and 10:1, respectively, with correct relative ions intensities and a retention time within ±0.2 min of the average calibrator retention time. LOD and LOQ were 0.70 and 2.00 μg/L, respectively. Representative SIM chromatograms of a spiked blood sample at the LOQ concentration (tadalafil: 2.00 μg/L) and its internal standard (protriptyline: 100 μg/L) are shown in Fig. 2B and C, respectively. The limits of detection and quantification were also calculated from the standard deviation of the intercept (s_b) of the calibration curve and its slope (a) according to the following equations: LOD = 3.3(s_b/a) and LOQ = 10(s_b/a). The results are presented in Table 1.

Linearity was determined by the calculation of the regression line using the method of least-squares with a weighting factor of $1/x^2$ and it was expressed by the correlation coefficient (R^2). Calibration curves (based on the peak area ratio of each analyte to the internal standard) were plotted every day and were used for the determination of the analyte's concentration in the QC and the patient blood samples.

The linear dynamic range was 2.00–500.0 μg/L, with correlation coefficients (R^2) exceeding 0.991 and the % RSD of slopes was found to be 7.8%. Linearity results of the validated method are presented in Table 1.

Absolute recovery of the method was assessed by running six replicates at the three quality control concentrations (6.00, 100.0 and 400.0 μg/L). Absolute recovery of the method was calculated as the percentage of the response of the analyte in the sample compared to that of a methanolic solution containing the analyte at the same concentration. The following equation was used:

$$\%\ \text{absolute recovery} = \frac{\text{peak area of spiked blood sample} \times 100}{\text{mean peak area of 6 runs of methanolic solution}}.$$

Combination of SPE with derivatization using BSTFA with 1% TMCS improved significantly the absolute recovery (calculated at three QC concentration levels) of the method for tadalafil and it was found to be between 92.1 and 98.9%. Absolute recovery values at the three quality control levels are shown in Table 2.

Precision and accuracy of the method (intra-day $n = 6$ and inter-day $n = 36$) were calculated by analyzing the LOQ concentration (2.00 μg/L) and the three QC levels within analyte's linear range (6.00, 100.0 and 400.0 μg/L). The concentration of the LOQ and QC samples was calculated by the calibration curve of each day of analysis. Precision was expressed as the relative standard deviation (% RSD). Accuracy of the method was calculated as the percentage difference from the expected concentration (% E_r). Intra- and inter-day accuracy was found to be between −10.5 to 8.5% and −4.2 to 4.5%, while intra- and inter-day precision were less than 8.4 and 11.2%, respectively (Table 3).

Robustness of the entire method was studied by changing several parameters of the procedure (pH of samples was adjusted to 6.50 and 7.50 instead of 7.00, derivatization temperature 65 and 75 °C instead of 70 °C, and the ratio of the solvents in the mixture of elution 80:10:2 and 80:20:2, v/v/v instead of 85:15:2, v/v/v) as well as chromatographic parameters (flow rate of carrier gas: 0.57 and 0.63 mL/min, injector temperature: 275 and 285 °C and 3% lower detector voltage). The differences of the mean area of the analyte, for each parameter of the method changed, as well as their standard deviation were calculated. Neither a single parameter nor a combination of the ones changed, showed a significant influence on the results of the method, which proved to be sufficiently robust.

The stability of tadalafil was assessed by analyzing spiked blood with tadalafil at low and high QC concentrations (6.00 and 400.0 μg/L) and keeping the samples at 4 °C for 7 days and at −20 °C for 30 days. Furthermore, frozen spiked blood samples were subjected to three freeze–thaw cycles. Tadalafil in whole blood was found to be stable at the conditions studied. The loss, at two QC concentrations (low and high) was calculated less than 13.2, 11.5 and 8.8% at 4 °C for 7 days, at −20 °C for 30 days and at three freeze–thaw cycles, respectively.

HPLC is a well established and robust analytical technique but the two previously published HPLC methods [12,13] for the determination of tadalafil have relatively low sensitivity (LOQs: 5.00 and 10.0 μg/L, respectively) and they show quite low extraction efficiency (recovery values: 66.1 and 76.6%, respectively). The proposed method, when compared with the two previously described HPLC methods for the determination of tadalafil in plasma, shows improved sensitivity with lower LOD and LOQ concentrations, higher extraction recoveries, enhanced accuracy and precision data. The only already published GC/MS method [14] is applied in urine, it includes LLE that proved unsuitable when blood samples were used and lacks sensitivity as its LOQ is significantly higher (25.0 μg/L) than our method. To the best of our knowledge, the proposed method is the only published GC/MS method for the determination of tadalafil in blood samples providing full validation data and it can be used for therapeutic drug level monitoring of patients with pulmonary arterial hypertension or erectile dysfunction under tadalafil treatment. The method could also be used for pharmacokinetic and pharmacodynamic studies, as well as for forensic purposes.

Table 2
% Absolute recoveries of tadalafil at three quality control concentrations.

Tadalafil (μg/L)	% Absolute recovery mean ± SD ($n = 6$)
6.00	96.4 (±4.3)
100.0	98.9 (±2.1)
400.0	92.1 (±3.9)

Table 3
Intra- and inter-day accuracy and precision of the developed method for the determination of tadalafil in whole blood at LOQ and three quality control concentrations.

Concentration added (μg/L)	Intra-day (n = 6)			Inter-day (n = 36)		
	Concentration found mean ± SD (μg/L)	Accuracy (% E_r)	Precision (% RSD)	Concentration found mean ± SD (μg/L)	Accuracy (% E_r)	Precision (% RSD)
2.00	1.79 (±0.13)	−10.5	7.3	2.09 (±0.17)	4.5	8.1
6.00	5.42 (±0.35)	−9.7	6.5	5.88 (±0.66)	−2.0	11.2
100.0	108.5 (±4.9)	8.5	4.5	95.8 (±6.6)	−4.2	6.9
400.0	369 (±31)	−7.8	8.4	410 (±27)	2.5	6.6

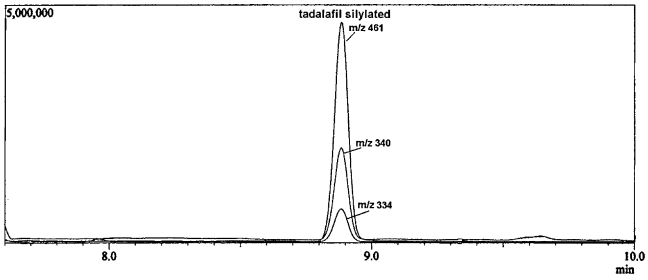

Fig. 3. SIM chromatogram of a real blood sample (patient A, tadalafil: 56.8 μg/L).

3.3. Method application

The validated method was applied to blood samples obtained from two male patients, who were taking occasionally 20 mg of tadalafil (Cialis®, Eli Lilly) for erectile dysfunction, after their informed consent. The study was approved by the ethical committee of the National Drug Organization of Greece. All the patients were not receiving any other medication. The blood samples were collected 1 or 2 h after the tadalafil administration in test tubes with anti-coagulant (EDTA K_3). The concentrations of tadalafil in blood of the patients were found to be: 56.8 μg/L (patient A, 1 h after administration) and 79.2 μg/L (patient B, 2 h after administration). A representative SIM chromatogram of a real blood sample of patient A is shown in Fig. 3. The developed method was proved to be adequately sensitive and suitable for the successful measurement of tadalafil levels in human blood samples.

4. Conclusions

A simple, sensitive and specific GC/MS method for the determination of tadalafil in whole blood, after SPE and derivatization using BSTFA with 1% TMCS, was developed and validated according to the international guidelines. The developed method was successfully applied to real blood samples and it was demonstrated that it is suitable for the investigation of related clinical or forensic cases, or for pharmacokinetic studies and therapeutic drug level monitoring of tadalafil patients.

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