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Simultaneous determination of sildenafil and *N*-desmethyl sildenafil in human plasma by high-performance liquid chromatography method using electrochemical detection with application to a pharmacokinetic study

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Abstract

A method which employed high-performance liquid chromatography coupled with electrochemical detection was developed for the simultaneous determination of sildenafil and its metabolite, *N*-desmethyl sildenafil, in human plasma has. The method was developed and validated for purposes of its application to a pharmacokinetic study in healthy volunteers after an oral dose of 50 mg/tablet under fasting conditions. High precision and accuracy were demonstrated. A one-step liquid–liquid extraction further provides a simple and practical way to process plasma samples containing sildenafil with good quantitative recovery. Sampling lasted for 24 h after dosing; consequently a limit of quantitation (LOQ) of 7.858 ng/mL was achieved for sildenafil whereas a LOQ of 8.675 ng/mL was obtained for *N*-desmethyl sildenafil. The mobile phase consisted of acetonitrile, methanol and phosphate buffer (0.05 M) (18.5:34.5:47.0, v/v/v) pH 7.68. The stationary phase was a C_8 (150 mm × 4.6 mm), 5 μm particle size operated at 27 °C. All analytes were stable at the pH of the supernatant, and during the analytical time window. At the applied potential of +1.20 V versus Ag/AgCl, no interferences from endogenous plasma compounds were recorded at the retention times of sildenafil, *N*-desmethyl sildenafil. High resolution was obtained between the analytes and the employed internal standards.

Keywords: HPLC; Sildenafil; Electrochemical; *N*-Desmethyl sildenafil

1. Introduction

Sildenafil citrate (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo-[4,3-d]pyrimidin-5-yl) phenylsulphonyl]-4-methylpiperazine) is widely prescribed for the treatment of impotence and male erectile dysfunction [1]. Sildenafil is rapidly absorbed after oral administration, with absolute bioavailability of about 40%. It is rapidly and extensively metabolized in the liver to the active *N*-desmethyl sildenafil metabolite [1]. Under steady state conditions, the plasma concentrations of *N*-desmethyl sildenafil were approximately 40% of those measured for sildenafil.

Because of its increasing popularity and potential side effects, the need for a procedure to detect both sildenafil and *N*-desmethyl sildenafil in biological samples is becoming increasingly important. The simultaneous determination of sildenafil and the active metabolite *N*-desmethyl sildenafil is also necessary for pharmacokinetic and related studies. Several high-performance liquid chromatographic (HPLC) methods have been reported for the determination of sildenafil and/or *N*-desmethyl sildenafil in biological samples. Gas chromatography–mass spectrometry (GC/MS) [2], micellar electrokinetic chromatography [3], liquid chromatography–mass spectrometry (LC/MS) [4,5] as well as liquid chromatography–tandem mass spectrometry (LC/MS/MS) [6–8] methods have been reported. Liquid chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry (LC/MS/MS) are expensive and thus unavailable in many laboratories. High-performance liquid chromatographic methods with UV detection have been reported for the simultaneous determination of sildenafil and its active metabolite [9–13]. Among others, these methods involved automated sequential

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trace enrichment of dialysate (ASTED), dual and triple column switching, as well as semi microbore, microbore and nanobore HPLC methods to enhance sensitivity and reduce retention time [11]. Some of these methods are not validated on human plasma. To this end several approaches including solid phase extraction-have been suggested as sample preparation and concentration steps [2–6,9].

The above approaches suffered from complicated sample preparation procedures or lack of precision and inferior ruggedness. Although the voltametric behavior of sildenafil citrate using square wave (SW) and adsorptive stripping SW technique has been suggested for the determination of sildenafil in pharmaceutical products, attempts to for utilizing the electrochemical behavior of sildenafil as well as its active metabolite as a detection technique in liquid chromatography have not been reported [14].

The present work demonstrates that significant improvement in sensitivity was achieved by coupling HPLC with electrochemical detection. The simultaneous detection and determination of both analytes was facilitated by the use of two internal standards namely, roxithromycin and clarithromycin, and the use of a one-step liquid–liquid extraction. The developed method was validated for its application to a pharmacokinetic evaluation of sildenafil and *N*-desmethyl sildenafil in 25 healthy human volunteers.

2. Experimental

2.1. Materials

Acetonitrile, methanol (HPLC grade) and *tert*-butyl methyl ether were from Scharlau Chemie SA (Spain). Potassium dihydrogen phosphate, phosphoric acid and potassium hydroxide were from Merck (Darmstadt, Germany). The *tert*-butyl methyl ether was redistilled. Sildenafil citrate (assay 99.67%), its metabolite *N*-desmethyl sildenafil hydrochloride (assay 82.9%), roxithromycin (the internal standard for sildenafil) (assay 97.85%), and clarithromycin (the internal standard for *N*-desmethyl sildenafil) (assay 98.57%) were kindly donated by Tabuk Pharmaceuticals (Saudi Arabia). HPLC grade water was prepared by further distilling and deionizing water after initiating a reversed-osmosis process. An elgacan column (C114) was employed for further water deionization.

2.2. Instrumentation

A high-performance liquid chromatograph (HPLC) was employed in the present study; it consisted of: A solvent delivery system (Spectra System P4000), an isocratic pump using a dual in-line and floating piston design. Chrom Quest software was employed for data collection and handling. A Rheodyne syringe Loading Sample Injector (Model 7125) which is a six-port sample injection valve, in which the sample is loaded into a 100 μl sample loop through a built-in needle port in front of the valve was used. A digital electrochemical Amperometric Detector (Decade), ANTEC LEYDEN (The Netherlands) was used. The electrochemical cell was a VT03 wall jet configuration flow cell with a three-electrode potentiostat, comprised: a glassy carbon working electrode in combination with, a reference electrode (salt bridge Ag/AgCl) and an auxiliary platinum electrode.

2.3. Chromatographic conditions

The following chromatographic conditions were optimized during the method development phase.

The stationary phase was Hypersil C_8 (150 mm × 4.6 mm), 5 μm particle size (Phenomenix, USA). The Guard column was a Hypersil BDS C_8 (10 mm × 4.0 mm), 5 μm particle size (Phenomenix, USA).The mobile phase consisted of acetonitrile, methanol and phosphate buffer (0.05 M) (18.5:34.5:47.0, v/v/v), pH 7.68. A flow rate of 1.3 mL/min was employed.

The following instrumental settings were employed for the amperometric detection.

Applied potential +1.20 V (versus Ag/AgCl), applied time 800 ms, first cleaning potential +1.30 V, cleaning time 360 ms, second cleaning potential −0.65 V, cleaning time at E3 330 ms.

The electrochemical cycle lasted for 1490 ms, with a sampling time (t_s) of 20 ms and a current range of 0.1 μA.

2.4. Preparation of standard solution

A sildenafil stock solution was prepared by dissolving 39.306 mg sildenafil citrate (assay 99.67%) in 25.0 mL mobile phase to make up a stock standard solution (solution A) containing (1122.58 μg/mL) (molar mass of sildenafil = 477.6 g mol^{-1}, molar mass of sildenafil citrate = 666.7 g mol^{-1}). A working standard (solution B) was prepared by diluting 1000 μL of solution A (1122.58 μg/mL) to a final volume 10.0 mL mobile phase to make up a dilute working standard containing 112.26 μg/mL. Another working standard was prepared by diluting 1000 μL of solution B to a final volume 10.0 mL mobile phase to make up a dilute working standard containing 11.23 μg/mL.

N-Desmethyl sildenafil hydrochloride stock standard solution was prepared by dissolving 6.54 mg *N*-desmethyl sildenafil hydrochloride (assay 82.9%) in a 25.0 mL mobile phase to make up the concentration 216.87 μg/mL. A working standard was prepared by diluting 1000 μL of this solution (216.866 μg/mL) to a final volume 10.0 mL mobile phase to make up a dilute working standard containing 21.69 μg/mL *N*-desmethyl sildenafil.

Roxithromycin stock standard solution was prepared by dissolving 50.00 mg roxithromycin (assay 97.85%) in a 50.0 mL aliquot mobile phase to make up the concentration 978.50 μg/mL. A working standard prepared by diluting 320 μL of this solution (978.50 μg/mL) to a final volume 10.0 mL mobile phase to make up a working standard containing 31.31 μg/mL roxithromycin. A clarithromycin stock standard solution was prepared by dissolving 49.99 mg clarithromycin (98.57%) in a 50.0 mL mobile phase to make up the concentration of 985.50 μg/mL. A working standard was prepared by diluting 84 μL of this solution to a final volume 10.0 mL mobile phase, which made up a dilute working standard containing 8.28 μg/mL clarithromycin.

Table 1
Concentrations used for calibration curve for sildenafil and *N*-desmethyl sildenafil

Calibrator number	Sildenafil (ng/mL)	*N*-Desmethyl sildenafil (ng/mL)
Blank	–	–
1	7.858	8.675
2	14.594	13.012
3	28.065	28.193
4	44.903	43.374
5	89.806	75.905
6	112.258	108.435
7	134.710	130.122
8	179.613	162.653
9	202.064	195.183
10	258.193	
11	291.871	
12	381.677	
13	449.032	

2.5. Internal standard solutions and sample preparation

Prior to the extraction, each test sample including all: calibrators, QC samples and authentic samples were spiked with the internal standard (IS). Two internal standards were spiked onto a 1.0 mL plasma sample; roxithromycin (1.566 μg/mL) and clarithromycin (0.414 μg/mL). Each sample was vortexed for 30 s to ensure homogeneous distribution of the IS. Each sample was extracted with *tert*-butyl methyl ether (9.0 mL). The mixture was shaken for (10 min) and centrifuged (1920 × *g*, 5.0 min). An 8.0 mL aliquot of the organic phase was transferred to a clean test tube. The solution was evaporated under a stream of nitrogen (RT). The residue was reconstituted with the mobile phase (500 μL). A 100 μL portion was directly loaded onto the HPLC system.

2.6. Quantification and calibration curve preparation

Calibration curve matrix based standards 7.858–449.032 ng/mL for sildenafil and 8.675–130.122 ng/mL for *N*-desmethyl sildenafil in plasma were prepared (Table 1). Calibrators and blank samples were extracted and analyzed as described above. The peak area ratios were recorded and calibration curves were obtained using the unweighed least-squares regression.

2.7. Validation of the assay method

The developed bioanalytical method for the simultaneous determination of sildenafil and its metabolite, *N*-desmethyl sildenafil, was fully validated. This have included evaluation of the following parameters, specificity, stability, linearity, linear working range, lower limit of quantitation, calibration sensitivity, recovery, intermediate precision and accuracy.

2.8. Sample collection and processing

A pharmacokinetic study (approved by the ethics committee of Al-Essra Hospital) was conducted on 25 subjects who received a single 50 mg dose of sildenafil citrate (Viagra®) with 240 mL of water. A total of 16 samples were harvested at the following time points: 0.00 h (pre-dosing) and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 15.00 and 24.00 h after dosing. Samples were immediately centrifuged, and the plasma was transferred to pre-labelled vials. Samples were then stored frozen at −38 °C until assay.

2.9. Analysis of pharmacokinetic data

All pharmacokinetic analyses were performed using WinNonlin Professional Version 2.1 (Pharsight Co., Mountain View, CA, USA). Pharmacokinetic values for sildenafil and its active metabolite were estimated by noncompartmental methods. Values for peak concentration (C_{max}) and time to C_{max} (t_{max}) were taken directly from the observed data. Individual concentration versus time profiles were plotted, and the terminal disposition rate constant (λ_z) was determined by the log-linear regression of at least three data points judged to be in the terminal phase. The terminal-phase half-life ($t_{1/2}$) was calculated by dividing 0.693 by λ_z. AUCt was determined by the linear trapezoidal rule from time zero to the time of the last observed concentration (Ct). Total AUC was determined as AUC + Ct/λ_z.

3. Results and discussion

3.1. Chromatography

Several stationary phases were investigated. Based on the results; a C_8 column (250 mm × 4.6 mm, 5 μm) was chosen along with the following mobile phase; acetonitrile, methanol and 0.05 M phosphate buffer (18.5:34.5:47.0, v/v/v) (pH 7.68). The applied potentials were carefully chosen and tested to provide the highest possible sensitivity with the lowest background noise. Detector was set at E1, E2 and E3 of +1.2, +1.3 and −0.65 V respectively.

3.2. Specificity

Specificity of the with respect to endogenous plasma components, was determined by analyzing six different sources of blank plasma samples collected under controlled conditions. No interferences were observed at the retention times of sildenafil, *N*-desmethyl sildenafil, roxithromycin and clarithromycin. These were compared with the chromatograms obtained from plasma extracts at the lower limit(s) of quantitation. Figs. 1 and 2 demonistrate method specificity. Figs. 3 and 4 show chromatograms obtained after extraction of spiked plasma and volunteer plasma.

Because of differences in the anticipated maximum concentrations of sildenafil (∼200 ng/mL) and *N*-desmethyl sildenafil (∼80 ng/mL), it was believed that accuracy can be enhanced by using two internal standards, the concentration of which should produce an area ratio of about one in the middle of each calibration curve. Consequently roxithromycin (1.566 μg/mL) and clarithromycin (0.414 μg/mL) were used as internal standards for the simultaneous analysis of sidenafil and its metabolite *N*-desmethyl sildenafil, respectively.

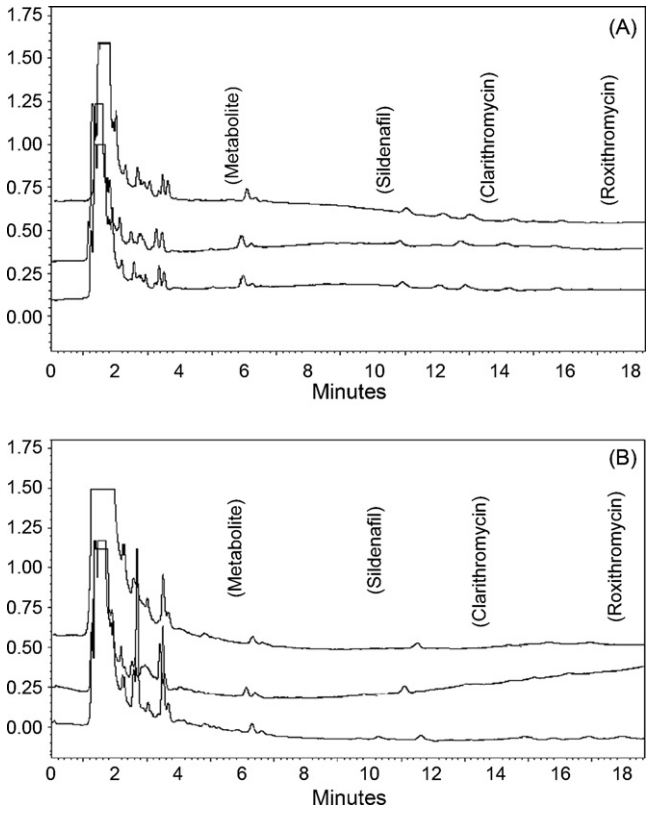

Fig. 1. Representative chromatograms for extracts of six blank plasma samples from different sources (A) and (B).

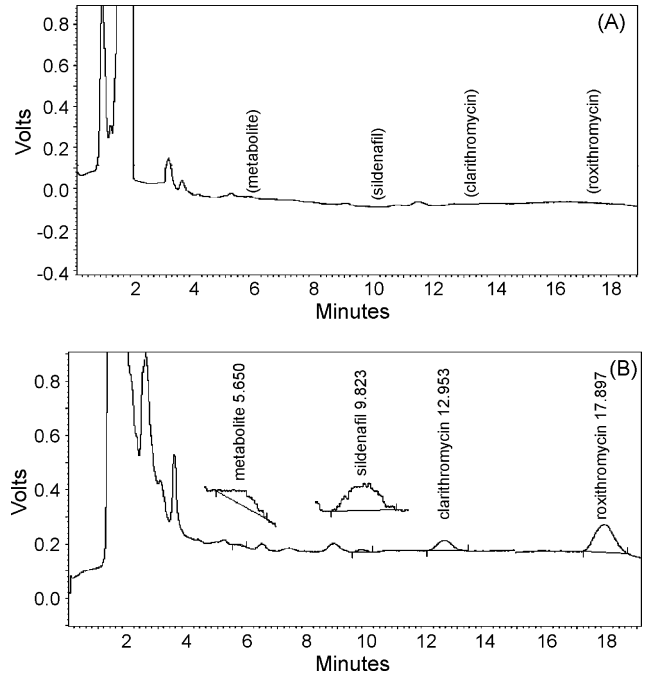

Fig. 2. Representative chromatograms for (A) an extract of a blank plasma Sample. (B) An extract of a spiked plasma sample containing sildenafil at the limit of quantitation (7.858 ng/mL), *N*-desmethyl sildenafil at the limit of quantitation (8.675 ng/mL), clarithromycin (IS) (0.414 μg/mL) and roxithromycin (IS) (1.566 μg/mL).

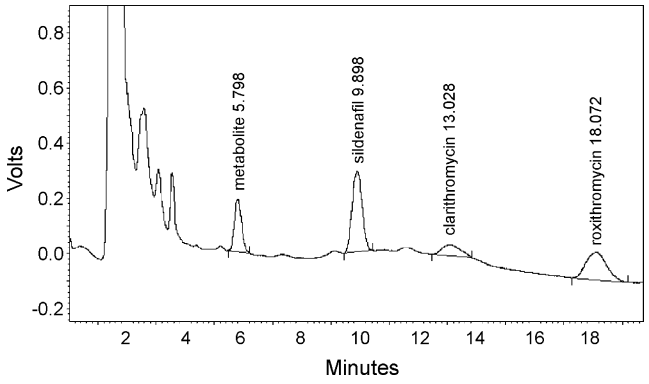

Fig. 3. Representative chromatogram for an extract of plasma containing sildenafil (235.742 ng/mL), *N*-desmethyl sildenafil (173.496 ng/mL), clarithromycin (IS) (0.414 μg g/mL) and roxithromycin (IS) (1.566 μg g/mL).

3.3. Standard curve and linearity

Matrix-based calibration curves which plotted concentrations versus peak area ratios were linear over the range 7.858–449.032 ng/mL for sildenafil, and between 8.675 and 195.183 ng/mL for *N*-desmethyl sildenafil.

The average area ratios from 14 calibration curves, gave the following equation: $Y = 0.0072X + 0.006$, average area ratios for the *N*-desmethyl sildenafil measured from 12 calibration curves gave the following equation: $Y = 0.0169X - 0.0176$. Both curves were linear and produced correlation coefficients of 0.9976 and 0.9973 for sildenafil and *N*-desmethyl sildenafil, respectively.

3.4. Accuracy and precision

Twenty-six individual calibration curves were generated for sildenafil and *N*-desmethyl sildenafil during the assay of analytical batches. Each curve was employed for calculating the corresponding concentrations of sildenafil and *N*-desmethyl sildenafil in authentic samples. Quality control samples were analyzed for each batch of processed authentic samples and with each calibration curve. QC samples were prepared to

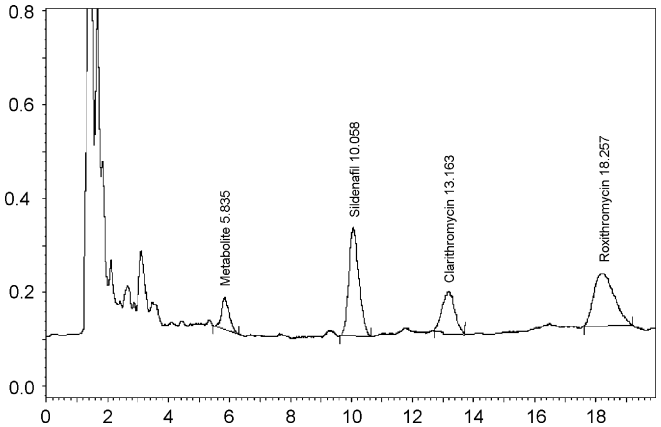

Fig. 4. Representative chromatogram for an extract of a volunteer plasma sample collected 75 min after ingestion of a single 50 mg dose of sildenafil showing sildenafil (130.515 ng/mL), *N*-desmethyl sildenafil (79.756 ng/mL), clarithromycin (IS) (0.414 μg/mL) and roxithromycin (IS) (1.566 μg/mL).

Table 2
Sildenafil Intra-day and inter-day precision and accuracy at four different concentrations of quality control samples

Theoretical concentration (ng/mL)	Intra-day ($n=6$)				Inter-day ($n=18$)			
	Mean recovered concentration (ng/mL)	S.D.	Accuracy	CV (%)	Mean recovered concentration (ng/mL)	S.D.	Accuracy	CV (%)
21.329	20.193	0.810	94.674	4.01	20.893	1.107	97.956	5.298
78.581	83.703	2.259	106.518	2.70	83.129	3.986	105.788	4.795
157.161	165.332	4.039	105.199	2.44	166.363	3.754	105.855	2.257
235.742	252.160	7.853	106.964	3.11	250.767	8.402	106.374	3.351

cover low (21.33 ng/mL), medium 1 (78.58 ng/mL) medium 2 (151.81 ng/mL) and high (235.7 ng/mL) sildenafil concentrations in the plasma matrix. *N*-Desmethyl sildenafil QC concentrations were 21.69, 86.75, 151.81 and 173.50 ng/mL for the low, two medium, and high QC samples, respectively.

Accuracy and precision were determined during a 3-day validation period at the four QC samples ($n=6$ at each concentration). Tables 2 and 3 illustrate that the method was precise and accurate. For sildenafil and *N*-desmethyl sildenafil the accuracy was above a relative error of 94% and the precision was less than 11%, CV.

3.5. Limit of quantification (LOQ) and limit of detection (LOD)

The LOD was calculated based on the standard deviation of the response and the slope of the average calibration graph:

$$\text{detection limit} = \frac{3.3\sigma}{S},$$

where σ is the standard deviation of the response at the anticipated LOD and S is the slope of the calibration graph.

Limit of detection for slidenafil and *N*-desmethyl sildenafil were 3.448 and 4.590 ng/mL, respectively. The limits of quantitation were 7.858 ng/mL (CV 12.20%) for sildenafil and 8.675 ng/mL (CV 17.53%) for *N*-desmethyl sildenafil.

3.6. Recovery

Recoveries of both analytes were calculated by comparing the peak areas obtained after extraction with those obtained from spiked analytes in the mobile phase containing the same concentrations.

The recoveries, measured over 3 days from four quality control samples are summarized in Tables 2 and 3. Sildenafil recovery averaged: 97.956, 105.788, 105.855 and 106.374% for low QC, medium 1 QC, medium 2 QC, and high QC samples, respectively. The recoveries for *N*-desmethyl sildenafil averaged: 105.275, 99.281, 97.381 and 96.52% for low QC, medium 1 QC, medium 2 QC and high QC samples, respectively. The recovery of clarithromycin (0.414 μg/mL) and roxithromycin (1.566 μg/mL) averaged 105.93 and 101.40%, respectively.

3.7. Stability

The stabilities of sildenafil was evaluated for two quality control samples: the low (21.33 ng/mL) and the high (235.74 ng/mL) levels, $N=5$ at each concentration. The same was also evaluated for *N*-desmethyl sildenafil at the low (21.69 ng/mL) and the high (173.50 ng/mL) levels ($N=5$ at each concentration). In addition the stability of the internal standards at their nominal concentrations was evaluated. The stability studies have included the following: (1) short-term stability (for up to 48 h) as well as stability in the plasma matrix at room temperature (RT). (2) Stability of the analytes during sample collection, storage and preparation, including auto-sampler stability. (3) Long-term stability (for up to 50 days) of all analytes when kept frozen (in the mobile phase or in the plasma matrix, at −38 °C). (4) Stability after five freeze and thaw cycles.

Sildenafil and *N*-desmethyl sildenafil in addition to clarithromycin and roxithromycin were found to be stable during sampling, storage, short term, long term and freeze-thaw stability experiments in both mobile phase and plasma samples at both low and high concentrations. Percentage stability ranged from 95 to 106% (CV% of 0.5–10%).

3.8. Robustness

No significant changes in the analytical signals were observed upon changing analysts, columns, sources of chemicals and/or solvents. Robustness experiments indicated that the method generated data of acceptable accuracy and precision.

Table 3
N-Desmethyl sildenafil intra-day and inter-day precision and accuracy at four different concentrations of quality control samples

Theoretical concentration (ng/mL)	Mean recovered concentration (ng/mL)	S.D.	Accuracy	CV (%)	Mean recovered concentration (ng/mL)	S.D.	Accuracy	CV (%)
21.687	23.312	1.134	107.493	4.864	22.831	1.716	105.275	7.516
86.748	77.151	2.299	88.937	2.980	86.124	9.059	99.281	10.519
151.809	141.443	2.810	93.172	1.987	147.833	9.224	97.381	6.239
173.496	165.325	3.128	95.290	1.892	167.458	10.234	96.520	6.111

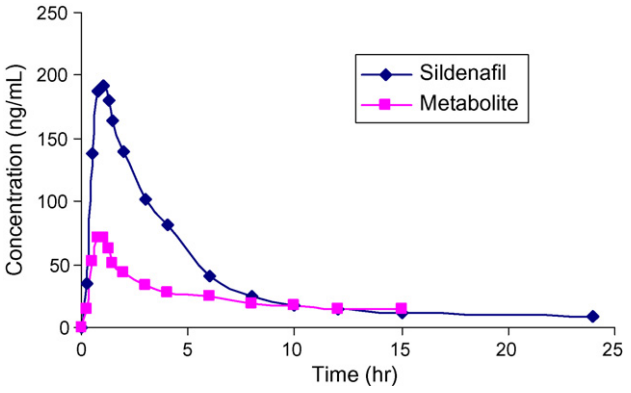

Fig. 5. Concentration–time profile for sildenafil and *N*-desmethyl sildenafil for 25 volunteers who took a single oral dose of 50 mg sildenafil.

3.9. Pharmacokinetic study

Method suitability was demonstrated during method application on a pharmacokinetic study of sildenafil and its active metabolite after a single oral dose of sildenafil (50 mg) to 26 healthy adult male Jordanian subjects. Fig. 5 shows mean plasma concentration profiles for sildenafil and *N*-desmethyl sildenafil. The evaluated pharmacokinetic parameters are summarized in Table 4.

AUC%Extrap was calculated to evaluate the importance of measuring the last concentration using WinNonlin Professional version 2.1 (Pharsight Co., Mountain View, CA, USA) and the following equation: $AUC\%Extrap = (AUC_{\infty} - AUC_t)/AUC_{\infty} \times 100$. The AUC%Extrap value for the 50 mg dose was 6.94 ± 3.51 (mean $\pm$ S.D., $n = 26$). These results indicated that the described method is suitable for purposes of evaluating pharmacokinetic parameters and consequently for bioavailability and bioequivalence studies.

Table 4
Pharmacokinetic parameters (mean and standard deviation)

Parameter	Sildenafil		*N*-Desmethyl sildenafil	
	Mean	S.D.	Mean	S.D.
Terminal rate constant (h^{-1})	0.25	0.08	0.21	0.18
Half-life (h)	3.23	1.40	5.21	3.15
Lag time (h)	0.08	0.14	0.11	0.16
T_{max} (h)	0.96	0.31	0.85	0.26
C_{max} (ng/mL)	206.39	80.94	83.42	45.99
AUC_t (ng h/mL)	740.59	288.59	288.82	188.16
AUC_{∞} (ng h/mL)	794.05	299.38	381.50	228.49
MRT (h)	4.54	1.10	7.47	4.08

4. Conclusions

An HPLC method coupled with an electrochemical detector was developed and validated for its application to a pharmacokinetic study of 50 mg sidenafil tablet in healthy male volunteers. The simultaneous method demonstrated good sensitivity, robustness and high precision and accuracy.

The method was successfully applied to a pharmacokinetic study on sildenafil and its active metabolite.

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