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Original article

Development and validation of LC-MS/MS assay for the determination of Butoconazole in human plasma: Evaluation of systemic absorption following topical application in healthy volunteers

Eman G. Nouman [a], Medhat A. Al-Ghobashy [b,c,*], Hayam M. Lotfy [b,d]

[a] Zi Diligence Research Center, Cairo, Egypt
[b] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Egypt
[c] Bioanalysis Research Group, Faculty of Pharmacy, Cairo University, Egypt
[d] Pharmaceutical Chemistry Department, Faculty of Pharmaceutical Sciences and Pharmaceutical Industries, Future University, Cairo, Egypt

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ABSTRACT

Butoconazole is an imidazole antifungal that is more effective than miconazole and clotrimazole for treatment of vaginal candidiasis. A highly sensitive tandem mass spectrometric assay was developed and validated to evaluate systemic absorption of Butoconazole following intravaginal administration. Chromatographic separation was achieved using Waters Xterra C18 column (3 μm, 3.0 × 50.0 mm). Liquid-liquid extraction using tert-butyl methyl ether was used for preparation of plasma samples. The mobile phase was solvent A: 0.1% formic acid in water and solvent B: acetonitrile: methanol (30:70, v/v), using gradient elution mode at 0.5 mL/min. Detection at positive electrospray ionization in the MRM mode was then employed. Analysis was carried out within 5.5 min over a linear concentration range of 0.10–30.00 ng/mL. Validation was carried out according to US FDA guidelines for bioanalytical method validation. Matrix effect, recovery efficiency and process efficiency have been investigated for the analyte and internal standard in neat solvent, post-extraction matrix and plasma. The mean percentage recoveries were higher than 80%, the accuracy was 93.51–106.85% and the RSD was below 10% throughout the studied concentration range. Results indicated sufficient stability of the target analyte in plasma at the employed experimental conditions. Results of incurred sample re-analysis and incurred sample stability revealed less than 5% variability. The applicability of the assay for monitoring of the systemic absorption of Butoconazole following intra vaginal application to healthy volunteers was demonstrated. Results confirmed that Butoconazole was detected shortly after intra vaginal administration with C_{max} and t_{max} of 30 ng/mL and 6 h, respectively.

1. Introduction

Butoconazole (BUTO) is an antifungal prescription medicine approved by the US Food and Drug Administration (US FDA) for topical treatment of vaginal candidiasis. This opportunistic infection occurs more frequently or is more severe in people with weakened immune systems such as those infected with HIV [1]. Although topically applied, it has been reported that up to 6% of BUTO intravaginal dose is absorbed with C_{max} up to 44 ng/mL [2,3]. This could be attributed to the presence of a dense network of blood vessels that made the vagina an excellent route for drug delivery for both systemic and local effects. However, personal hygiene, formulation factors, vaginal physiology, age of the patient and menstrual cycle could considerably affect the rate and extent of systemic absorption of drugs [4,5].

Recent trends in formulation technologies significantly enhanced the dissolution and release of drugs from topical dosage forms. This could lead to enhanced penetration of drugs through the skin barrier and subsequently more systemic absorption [6,7]. According to the FDA guidelines [8], evaluation of the systemic absorption, following topical application of pharmaceuticals raises the bar for registration of new generic formulations and is more sensitive in determining a "bio-inequivalent" product than any other current methodologies. By measuring the concentrations of drugs in body fluids or tissues at different times after the

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* Corresponding author at: Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo 11562, Egypt.
E-mail address: medhat.alghobashy@cu.edu.eg (M.A. Al-Ghobashy).

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administration, the necessary information on drug absorption, passage of drug molecules between blood and tissues and finally drug elimination can be evaluated. Such recent trend raised the need for validated assays that could determine drugs at ultra-low concentration levels.

Chemically, Butoconazole (BUTO) is 1-[4-(4-Chlorophenyl)-2-(2,6-dichlorophenyl)sulfanylbutyl] imidazole (Fig. 1) [9]. Very few analytical methods have been reported for the routine quantitative analysis of BUTO either in its dosage forms [10,11] or in plasma [12,13]. The latter methods were either of low sensitivity or lacked proper validation. In particular, using an internal standard that is not structurally related to the target analyte without in depth investigation of possible differences in extraction recovery and matrix effect raised a concern about the validity of the reported assay. Moreover, the reported plasma concentration levels for BUTO were not consistent with either the administered dose or data in previously published work.

In this study, a sensitive and precise LC-MS/MS bioanalytical assay for the determination of BUTO in human plasma was developed and validated. The matrix effect was thoroughly investigated along with process efficiency using a set of calibration curves prepared in neat solvent, matrix matched standards and fortified plasma at the same concentration levels. The assay was applied for monitoring of BUTO concentration in plasma following intravaginal application of generic formulations. Results of this study help both formulators and regulatory authorities evaluating the bioequivalence and therapeutic efficacy of topical formulations.

2. Experimental

2.1. Materials

Butoconazole reference standard (100.39 ± 0.76% [14]) was obtained from USBiological Life Sciences (USA), whereas Atorvastatin was used as the internal standard (IS) and was provided by (Zi Diligence laboratory, Egypt). Human blank plasma was obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Egypt). All other chemicals and solvents were of HPLC grade and were obtained from Sigma Aldrich (Germany). Ultrapure water was obtained using a MilliQ UF-Plus system (Millipore, Germany), with a resistivity of at least 18.2 MμΩ/cm at 25 °C and Total organic carbon (TOC) value below 5 ppb.

2.2. Instruments

Chromatographic analysis was carried out using an Agilent 1200 HPLC system (Agilent Technologies, USA). Mass spectrometric detection was carried out using a triple quadrupole API 4000 (ABSciex, Canada) operated in positive electrospray ionization and multiple reaction monitoring (MRM) mode. Hardware control and data acquisition were carried out using Analyst 1.5.2 Software with Hotfixes to February 2011 (ABSciex, Canada).

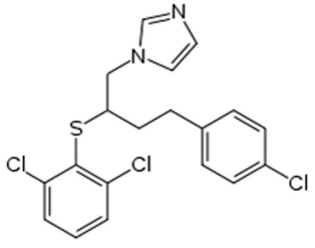

Fig. 1. Chemical structure of Butoconazole.

2.3. Liquid chromatographic and mass spectrometric conditions

Separations were carried out using a Waters Xterra C18 column (3 μm, 3.0 × 50.0 mm) and gradient elution at a flow rate of 0.5 mL/min. The mobile phase was solvent A: 0.1% formic acid in water and solvent B: acetonitrile: methanol (30:70, v/v). Run time was 5.5 min with gradient elution: 0.0–0.5 min (50% B); 0.6–3.7 min (80% B) and 3.8–5.5 min (50% B) and the injection volume was 10 μL. A mixture standard solution of BUTO and IS (200.00 ng/mL each in methanol) was directly infused into the mass spectrometer, and the operating conditions were optimized. The following transitions m/z 413.4 > 164.9 and 559.3 > 440.3 were used to monitor butoconazole and IS, respectively, as summarized in Table 1. The nebulizer gas was air (zero grade), whereas nitrogen was used as the auxiliary, curtain and collision gas. The source/gas-dependent parameters for the BUTO determination were as follows: curtain gas, 10 psi; collision gas, 4 psi; medium temperature, 400 °C; ion spray voltage, 4200 V; ion source gas one and gas two, 25 psi.

2.4. Standard solutions

Stock solutions of BUTO and IS (30.00 μg/mL each) were prepared in methanol and stored at 2–8 °C away from light. Quality control samples of the analyte were prepared in blank plasma at three levels: low (QCL), medium (QCM) and high (QCH) with concentrations of 0.30, 15.00 and 25.00 ng/mL, respectively. For evaluation of matrix effect and recovery, three sets of calibration curves were prepared in neat solvent, post-extraction plasma and plasma at five concentration levels including QCL, QCM and QCH as will be described in detail below. All prepared samples were stored in aliquots of 1.0–2.0 mL at −86 °C until analysis.

2.5. Preparation of fortified samples

Plasma samples were stored frozen at −86 °C, and all analytical procedures were carried out at room temperature. Aliquots of 900 μL plasma were fortified with 100 μL BUTO standard, 100 μL of IS and 100 μL 20% glacial acetic acid and then vortex mixed for 2 min. Liquid-Liquid extraction was then carried out by adding 4 mL tert-butyl methyl ether. Samples were vortexed for 2 min and then centrifuged at 1789g at 4–8 °C for another 2 min. The upper clear layer was carefully separated, evaporated, reconstituted with 100 μL mobile phase and finally 10 μL was injected onto the LC-MS/MS system for the determination of BUTO concentration.

2.6. Assay validation

2.6.1. Selectivity, linearity, accuracy and precision

The selectivity was assessed via analysis of six different batches of blank human plasma randomly selected from different sources. The linearity was evaluated using a blank sample, a zero concentration sample containing the IS, and nine non-zero samples covering a range of 0.10–30.00 ng/mL and analyzed in six replicates. Peak area ratios of each drug to the IS were plotted against the standard concentrations. The linearity was evaluated by least-squares regression analysis and the lower limit of quantitation (LLOQ) was determined using the average of six determinations at nine concentration levels. The accuracy and precision of the assay were determined via analyzing fortified samples at four concentration levels (LLOQ, QCL, QCM and QCH) using six replicates. Within-run precision was determined via analysis of the samples in six replicates in the same day, whereas the between-run precision was evaluated via repeating analysis over 3 days. Calibration curves prepared and constructed in the same batch were employed in all determinations.

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Table 1
Optimized parameters selected for the quantification of Butoconazole.

Analyte	Q1[a](*m/z*)	Q3[b](*m/z*)	DP[c] (V)	EP[d](V)	CE[e](V)	CEP[f](V)
Butoconazole	413.4	164.9	80.00	10.00	25.00	9.90
IS	559.3	440.3	89.00	10.00	30.00	30.00

[a] Q1 Precursor ion.
[b] Q3 Product ion.
[c] DP Declustering potential.
[d] EP Entrance potential.
[e] CE Collision Energy.
[f] CEP Cell exit potential.

2.6.2. Matrix effect and recovery

Three sets of standard solutions of BUTO and IS were prepared at five concentration levels including QCL, QCM and QCH in order to evaluate the matrix effect and recovery: i) the first set (Set 1) was prepared in the mobile phase, the second set (Set 2) was prepared in extracted plasma originating from five different sources and fortified after extraction and iii) the third set (Set 3) was prepared in plasma from the same source but fortified before extraction. Mean peak areas (n = 5) of BUTO and IS were plotted against the standard concentrations for each set and the obtained regression lines were compared. The absolute peak areas in Set 1, Set 2 and Set 3 were used to calculate matrix effect (ME), Recovery efficiency (RE), and process efficiency (PE) values for BUTO and IS, as previously described [15] using the following equations:

$$\text{ME } (\%) = \text{Set 2/Set 1} \times 100 \quad (1)$$

$$\text{RE } (\%) = \text{Set 3/Set 2} \times 100 \quad (2)$$

$$\text{PE } (\%) = \text{Set 3/Set 1} \times 100 = \text{ME} \times \text{RE}/100 \quad (3)$$

2.6.3. Stability

Initially, stock solution stability during short-term storage at 4–8 °C was assessed over 12 h and the response from the LC-MS/MS was compared to that of the freshly prepared solution. QC samples at three concentration levels (QCL, QCM and QCH) were stored at −86 °C for 24 h and thawed unassisted at room temperature. The process was repeated for three cycles. The analysis was carried out in triplicate, and the results were compared to those of the zero cycle. Benchtop stability was evaluated using the described samples after storage at room temperature for a period that exceeded the preparation time of the samples (around 24 h). Long-term stability was evaluated at the same concentration levels at −86 °C for a period of 30 days. QC samples were processed and stored in an autosampler at 2–8 °C for 12 h to determine whether an occasional delay in injection or re-injection of extracted samples could lead to degradation of the analytes.

2.7. Application to incurred samples

A generic vaginal ovule formulation labeled to contain 40 mg BUTO/ovule was administered in a single dose to three healthy female volunteers (age 28–35). Blood samples were withdrawn from a forearm vein at zero time, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 24.0 and 48.0 h into heparinized tubes. Samples were prepared as described, and the concentrations of BUTO were determined. The plasma concentration-time curves were constructed for BUTO and pharmacokinetic parameters were calculated.

2.8. Incurred sample re-analysis and stability

In this study, incurred plasma samples collected at 3, 6, 10 h were re-analyzed in triplicate within the same day and over three different days over a storage period of two weeks. Concentration of BUTO was determined using the corresponding regression equation. The benchtop stability of incurred samples was also assessed following 24 h storage period at room temperature. The analysis was carried out as described and the results were compared to those obtained using a control sample that has not been subjected to the indicated stress condition.

3. Results and discussion

3.1. Liquid chromatography and mass spectrometric conditions

A highly sensitive, accurate and selective LC-LC-MS/MS assay for the determination is required as discussed earlier. Owing to the expected low target plasma concentration levels of BUTO, the method should exhibit high degree of sensitivity. Initially, several trials using different columns and mobile phases with different compositions and ratios were tried. Optimum performance was obtained using Waters Xterra C_{18} column and water, acetonitrile and formic acid as the mobile phase using gradient elution mode. At such conditions, the retention times of both of the drug and IS were very close. This should help compensate for matrix effect more efficiently since both of the Drug and IS enters the mass spectrometer at close retention times (Fig. 2). The mass spectrometric conditions were optimized (Table 1) and the following LC-MS/MS transitions were selected *m/z* 413.4 > 164.9 and 559.3 > 440.3 for the determination of BUTO and IS, respectively, as shown in (Fig. 2).

3.2. Sample preparation

Initially, various sample preparation techniques were tried such as protein precipitation using methanol, acetonitrile and mixtures of both, liquid-liquid extraction using diethyl ether, dichloromethane and ethyl acetate. Results indicated that liquid-liquid extraction using tert-butyl methyl ether was the optimum approach in terms of sensitivity, repeatability and recovery.

3.3. Assay validation

Assay validation was carried out according to the FDA guidelines for bioanalytical method validation [16]. Careful evaluation of the magnitude and direction of matrix effects (positive or negative) and recovery have also been thoroughly discussed [15,17,18].

3.3.1. Selectivity

Results obtained from the analysis of six batches of blank plasma were compared to those of the fortified plasma samples

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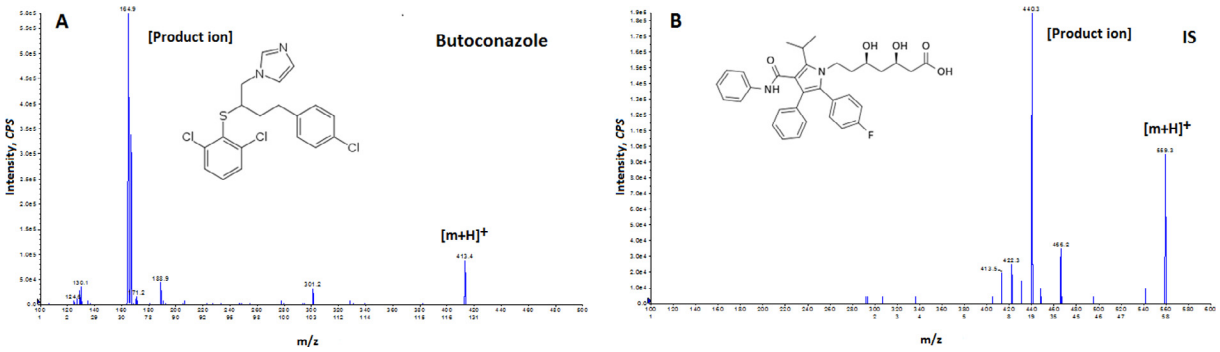

Fig. 2. Mass spectra and product ion scan for A) Butoconazole and B) internal standard.

at LLOQ. Results indicated the absence of endogenous interference at the retention times of the studied drug and IS. Representative chromatograms showing the results of analysis of blank plasma are shown in Fig. 3. This confirmed the high selectivity of the assay in the presence of matrix components.

3.3.2. Linearity and lower limit of quantitation

Linearity was evaluated using the average of six determinations at nine concentration levels, covering the range of 0.10–30.00 ng/mL for BUTO. The mean regression equation was Y = 0.1513X + 0.0275, R^2 = 0.9994, RSD 2.09%, where Y was the peak area ratio of the analyte to the IS and X was the concentration of the analyte in ng/mL. Blank and zero samples were included in the analysis in order to verify the absence of interference but not in the regression analysis. The LLOQ was determined as the lowest concentration of analyte that could be quantitatively determined with acceptable precision and accuracy as described and was included while studying various assay validation parameters.

3.3.3. Accuracy and precision

Analysis of the fortified plasma samples at four concentration levels (LLOQ, QCL, QCM and QCH) revealed that the within-run accuracy of the assay was 98.70–106.85% with a precision of 2.49–6.55% (Table 2). The between-run accuracy was within 93.51–100.97% with a precision of 5.22–8.32% (Table 2). The deviation of the mean from the nominal value served as the measure of accuracy. Obtained results clearly showed that the assay possesses adequate accuracy and precision.

Table 2
Accuracy and precision for the determination of Butoconazole.

	Within-run	Between-run
	Mean recovery% ± RSD*	
	Butoconazole	Butoconazole
LLOQ	98.70 ± 2.49	100.86 ± 5.98
QCL	104.96 ± 2.79	100.97 ± 5.22
QCM	106.65 ± 6.55	95.94 ± 8.32
QCH	106.85 ± 4.47	93.51 ± 7.53
N	6	6

* Mean percentage recovery and RSD were calculated using six determinations.

3.3.4. Matrix effect

The evaluation of the matrix effect and assay reliability is crucial for assay validation. In this study, stable isotope-labeled (SIL) internal standard has not been used and careful assessment of the effects of endogenous matrix components on quantitative determination of BUTO and the selected IS in plasma was thus carried out. The matrix effect and the possibility of ionization suppression or enhancement was evaluated by comparing the results of analysis of three sets of samples (Set 1, Set 2 and Set 3) prepared as described above. In Set 1, standards of BUTO and IS were prepared in the mobile phase and analyzed directly at five concentration levels covering QCL, QCM and QCH, five times at each concentration level (25 samples). Mean peak areas (Table 3) were plotted versus the nominal concentration of either BUTO or the IS (Fig. 4) in order to provide a good insight into the overall assay performance in neat solvent. In Set 2, plasma samples from five different sources were first extracted and fortified after extraction

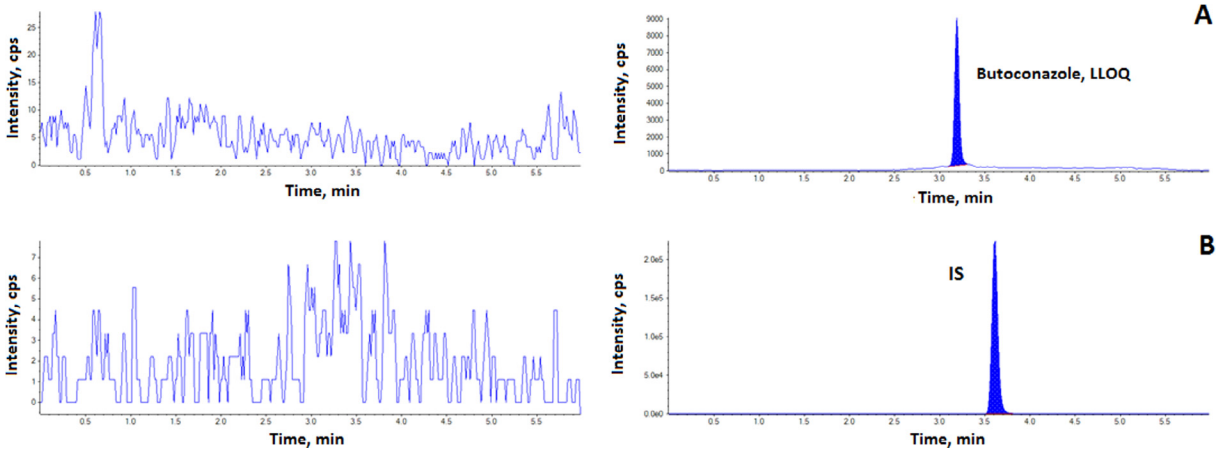

Fig. 3. Mass chromatograms of blank human plasma, fortified with A) Butoconazole at LLOQ (0.10 ng/mL) and B) internal standard.

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Table 3
Matrix effect (ME), recovery (RE) and process efficiency (PE) data for Butoconazole in five different lots of human plasma.

Nominal concentration (ng/mL)	Butoconazole (Mean peak area, n = 5)					
	Set 1	Set 2	Set 3	ME (%)*	RE (%)**	PE (%)***
	Neat solvent	Matrix matched	Plasma			
0.30	131,000	141,000	112,000	107.63	79.43	85.50
1.00	209,000	235,000	192,000	112.44	81.70	91.87
5.00	677,000	757,000	613,000	111.82	80.98	90.55
10.00	1,270,000	1,360,000	1,090,000	107.09	80.15	85.83
15.00	1,770,000	1,960,000	1,580,000	110.73	80.61	89.27
25.00	2,870,000	3,280,000	2,690,000	114.29	82.01	93.73
Mean ± RSD				**110.67 ± 2.57**	**80.81 ± 0.96**	**89.45 ± 3.29**
Nominal concentration (ng/mL)	IS (Mean peak area, n = 5)					
	Set 1	Set 2	Set 3	ME (%)*	RE (%)**	PE (%)***
	Neat solvent	Matrix matched	Plasma			
0.30	41,900	46,100	45,700	110.02	99.13	109.07
1.00	96,700	104,000	103,000	107.55	99.04	106.51
5.00	246,000	281,000	278,000	114.23	98.93	113.01
10.00	501,000	582,000	562,000	116.17	96.56	112.18
15.00	752,000	842,000	819,000	111.97	97.27	108.91
25.00	1,230,000	1,380,000	1,390,000	112.20	100.72	113.01
Mean ± RSD				**112.02 ± 2.77**	**98.61 ± 1.48**	**110.45 ± 2.68**

* Matrix effect (ME) expressed as the ratio of the mean peak area of an analyte fortified postextraction (Set 2, Matrix matched) to the mean peak area of the same analyte standards (Set 1, Neat solvent) multiplied by 100.
** Recovery (RE) calculated as the ratio of the mean peak area of an analyte fortified before extraction (Set 3, Plasma) to the mean peak area of an analyte fortified postextraction (Set 2, Matrix matched) multiplied by 100.
*** Process efficiency (PE) expressed as the ratio of the mean peak area of an analyte fortified before extraction (Set 3, Plasma) to the mean peak area of the same analyte standards (Set 1, Neat solvent) multiplied by 100.

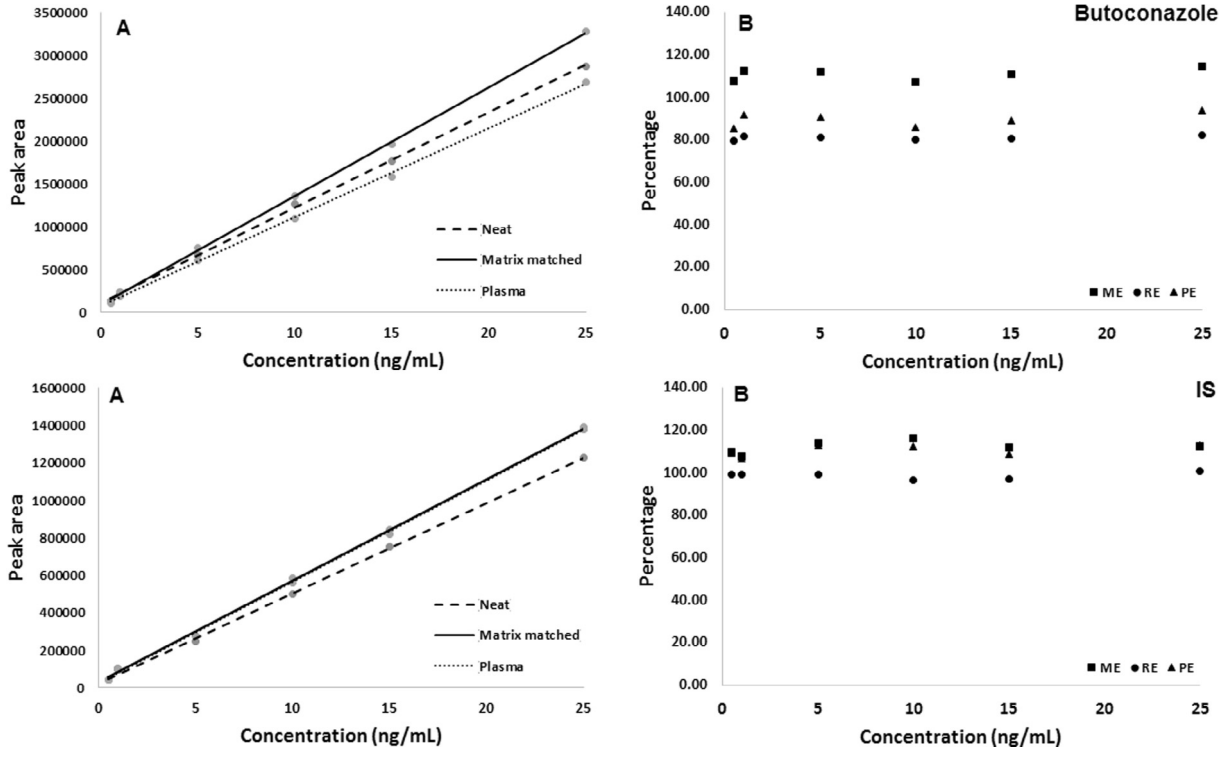

Fig. 4. Calibration curves (A) and scatter plots (B) for Butoconazole and IS prepared in neat solvent, post-extraction plasma and plasma samples. ME, matrix effect; RE, recovery efficiency and PE, process efficiency.

with either BUTO or IS at the same concentration levels used in Set 1. Matrix matched calibration curves were plotted using mean peak area (Table 3) versus concentration of either BUTO or IS (Fig. 4). Results indicated a clear matrix enhancement as indicated by the positive difference in the slope of matrix matched calibration for BUTO (+13.84%) and IS (+12.32%), when compared to calibration curves prepared in neat solvent. In set 3, BUTO and IS were fortified into plasma samples originating from five different sources before extraction. The difference in mean peak areas in this case reflected the combined effects of the sample matrix and recovery of analytes (Table 3). Calibration curves were constructed for either BUTO or IS (Fig. 4) and results were compared to those

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obtained for neat and matrix matched standards. Slope differences of −6.83% and +12.51% were obtained for BUTO and IS, respectively. These results indicated that the extraction recovery for BUTO was lower than that of the IS. However, it was hard to evaluate the recovery based on results of analyzed sets as will be discussed in more details.

Quantitative analysis of the obtained results was then carried out via calculation of the ME, RE and PE as described above and results were summarized in Table 3. Results indicated a homogeneous matrix effect (ME) across the studied concentration range for both BUTO (110.67 ± 2.57) and IS (112.02 ± 2.77), Fig. 4. The ME values were further compared using analysis of variance (ANOVA) and no significant difference was noted between BUTO and IS (P = 0.44). Such results confirmed the suitability of the employed IS to compensate for the matrix enhancement noted with BUTO. Several reports indicated that SIL internal standard is the first choice but is usually quite expensive and not always commercially available. In addition, differences in retention times and recoveries, from the analyte have also been reported [19,20]. We recommend that analysis of the described sets for the analyte and IS should be implemented for assessment of matrix effect in bioanalytical assays, even those employing stable isotope labeled IS [21,22].

3.3.5. Recovery

According to FDA Guidelines [16] for bioanalytical method validation, the recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent. Here, liquid-liquid extraction using tertiary butyl methyl ether was found to be the optimum approach to achieve consistent recovery of the analyte and IS. Results of analysis of fortified plasma samples (Set 3) indicated the combined matrix effect and recovery efficiency. Process efficiency (PE%) calculated using equation 3 (PE (%) = Set 3/Set 1 × 100) would be equivalent to the recovery as per FDA Guidelines. However, this may not be correct since it does take into account the matrix effect that may significantly influence this ratio (Fig. 4). Therefore, the recovery efficiency (RE%) was determined as the ratio of Set 3/Set 2 × 100 (Eq. (2)). The RE% value in this case represented the true recovery value that was not affected by the matrix while PE% represented the overall process efficiency. Results in Table 3 showed RE% of 80.81 ± 0.96 and 98.61 ± 1.48 for BUTO and IS, respectively. Although the RE% values for the target analyte and IS were significantly different (one way ANOVA, P < 0.05), the scatter plots Fig. 4B showed clearly that RE % was consistent and random throughout the studied concentration range. These results indicated the suitability of the selected compound as IS to correct for possible variability in dilutions, evaporation, degradation, recovery, adsorption, derivatization, and instrumental parameters such as injection volume.

3.3.6. Stability

Stock solution stability during short-term storage for 12 h at 2–8 °C was assessed. The response obtained using the LC-MS/MS assay was compared to that of the freshly prepared solution that demonstrated adequate stability of the stock solution. Results confirmed also that three freeze–thaw cycles of the QC samples did not affect the quantification of BUTO. Thawing of the frozen samples and keeping them at room temperature for 24 h did not result in notable degradation of the analytes. The QC samples were stored frozen at −86 °C and remained stable for at least 30 days. The extracted samples were also analyzed after storage in the autosampler (2–8 °C) for at least 24 h. Results summarized in Table 4 indicated that human plasma samples containing BUTO can be handled under normal laboratory conditions without any significant degradation of the studied drugs.

Table 4
Summary of stability data of Butoconazole in human plasma.

	Butoconazole		
	QCL (n = 3)	QCM (n = 3)	QCH (n = 3)
Freeze and thaw stability			
Concentration (ng/mL)	0.307	16.13	24.53
Mean recovery% ± RSD*	102.22 ± 8.19	107.53 ± 4.22	98.13 ± 3.61
Benchtop stability			
Concentration (ng/mL)	0.298	15.94	24.48
Mean recovery% ± RSD*	99.33 ± 5.62	106.27 ± 5.14	97.92 ± 4.6
Long-term stability			
Concentration (ng/mL)	0.294	15.14	24.21
Mean recovery% ± RSD*	97.34 ± 7.41	100.93 ± 7.67	96.84 ± 8.53
Processed sample stability			
Concentration (ng/mL)	0.292	15.33	24.47
Mean recovery% ± RSD*	98.00 ± 5.91	102.21 ± 6.35	97.88 ± 7.42

* Mean percentage recovery and RSD were calculated using three determinations

3.4. Application to incurred samples

In order to demonstrate the applicability of the method, the developed assay was applied to incurred samples collected as described (Fig. 5). Results confirmed the following: i) BUTO was detected shortly after administration of BUTO ovules that confirmed the systemic absorption through the vagina (Fig. 6), and ii) the C_{max} and t_{max} were 30 ng/mL and 6 h, respectively that were generally in agreement to the previously reported data on the pharmacokinetics of BUTO. Such results confirmed the need for in-depth evaluation of the safety of medications that are systemically absorbed following topical administration.

3.5. Incurred sample re-analysis and stability

Although the sample is expected to be generally the same in both QC samples and incurred samples, QC samples do not contain various drug metabolites, co-administered medications along with their metabolites. Thus, it has been recommended that assay validation should be extended to incurred samples [16,23].

In this study, incurred sample re-analysis was carried out using plasma samples collected at different time intervals in order to demonstrate the capability of the assay to remain unaffected at various concentration levels of the target analyte in the real matrix. Results obtained in three different days over a storage period of two weeks revealed less than 5% bias over BUTO concentration range of 0.76–28.31 ng/mL (Table 5). Incurred sample stability has also been investigated in order to verify the stability of the target analytes in real matrix and to confirm the validity of the assay. Analysis results of incurred plasma samples stored at room temperature for up to 24 h demonstrated sufficient stability over the study duration when compared to equivalent control samples that were analyzed immediately after collection (Table 5). Results clearly indicated that the developed assay was valid for the determination of BUTO in incurred plasma samples.

4. Conclusion

Fast, accurate and highly sensitive LC-MS/MS assay was developed and validated for the determination of the butoconazole in human plasma using liquid-liquid extraction for sample preparation. Results of the validation studies showed that the developed assay was selective, accurate and precise over quite low concentration levels that covers the target concentration of the drug in

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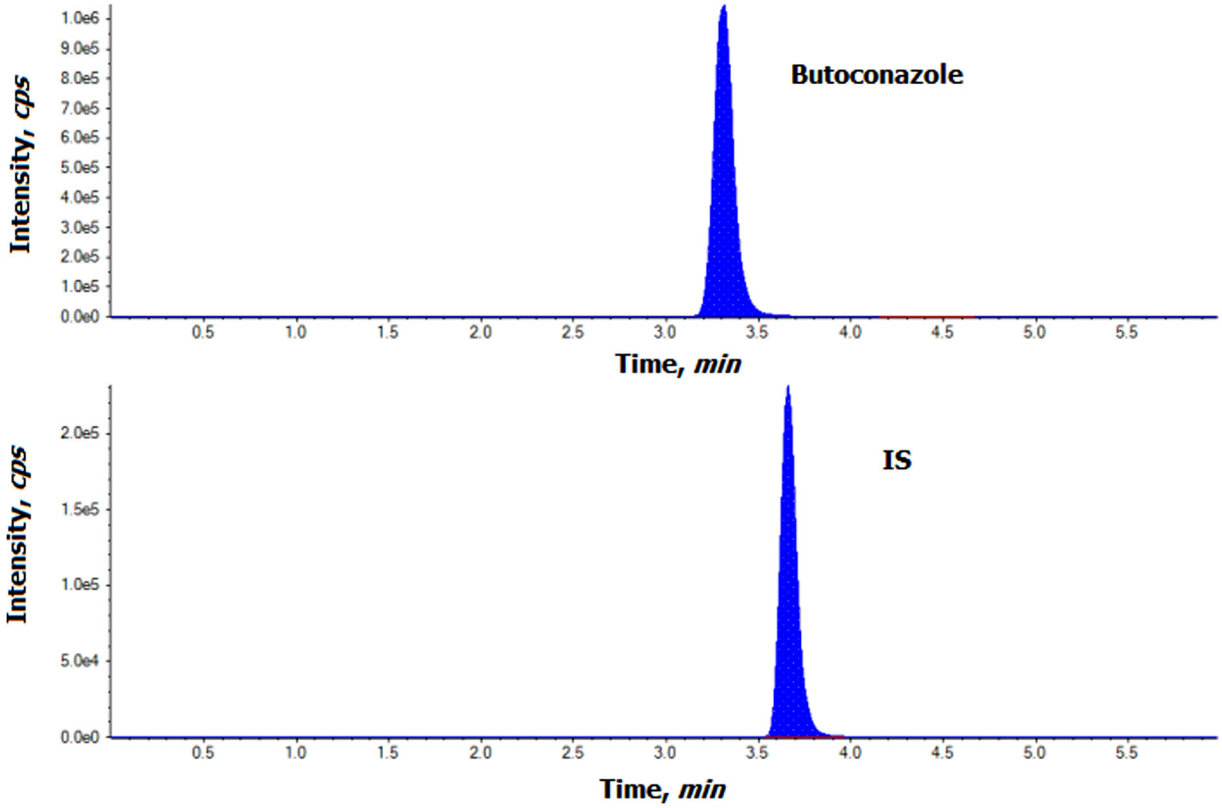

Fig. 5. Mass chromatograms of incurred human plasma samples collected at C_{max} showing analysis results for A) Butoconazole and B) IS following administration of a single dose of intravaginal ovule formulation equivalent to 40 mg Butoconazole/ovule.

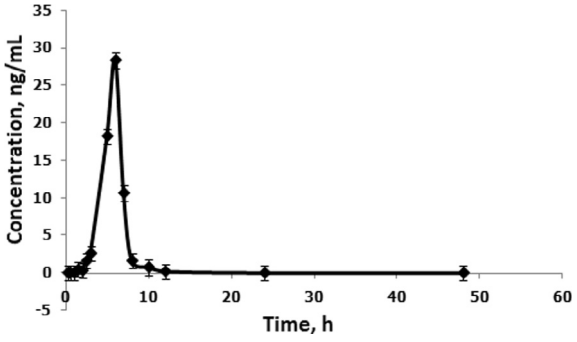

Fig. 6. Butoconazole plasma concentration versus time plot following administration of a single dose of intravaginal ovule formulation equivalent to 40 mg Butoconazole/ovule.

Table 5
Incurred sample reanalysis and stability for determination of Butoconazole in human plasma samples.

	Butoconazole
Incurred sample re-analysis[*]	
Mean concentration ± SD (ng/mL)	
3 h	2.59 ± 1.77
6.0 h (C_{max})	28.31 ± 1.41
10 h	0.76 ± 2.68
N	9
Incurred sample stability[**]	
Concentration ± SD (ng/mL)	9.81 ± 1.95
Mean recovery% ± RSD	95.65 ± 4.64
n	3

[*] Incurred plasma samples collected at different time intervals and analyzed over 3 different days.
[**] Pooled incurred plasma samples kept at room temperature for 24 h.

incurred plasma samples. Results also demonstrated appropriate extraction recovery and lack of matrix interference with the determination which ensured the validity of the method to be used in bioequivalence studies and development of new generic formulations.

Compliance with ethical standards

Authors have no conflict of interest to disclose.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Zi Diligence Research Center, Cairo, Egypt and Ministry of Health, Egypt and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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