

Simple and sensitive method development and validation of Econazole in human plasma by RP-HPLC

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Abstract

A simple and accurate method was developed for the validation of the Econazole using Fluconazole as internal standard with short time of 10 minutes. Optimization of chromatography technique was used during the preparation of this analysis. The method carried out using reversed phase of HPLC. Chromatography using Phenomenex Luna C18 Column (250mm x 4.6mm i.d, 5µm) as the stationary phase and mobile phase of solvent A and B of 0.5% Triethylamine at pH 6.5 and Acetonitrile at pH 3.5. Wavelength was fixed at 260nm and flow rate at 0.6mL/min. Validation studies was achieved by using the fundamental parameters, including accuracy, precision, selectivity, sensitivity, linearity and range, stability studies, limit of detection (LOD) and limit of quantitation (LOQ). Retention time obtained for Econazole and Fluconazole are 7.7 minutes and 5.18 minutes. It shows recovery at 93.5% which is more precise and accurate compared to the other Econazole method. Hence, a simple and accurate method of validation of Econazole in drug free plasma was developed and validated.

Keywords: Econazole, RP-HPLC, Validation, Drug free human plasma.

Introduction

Econazole is an antifungals used to treat infections caused by fungi. It is an imidazole derivative and its chemical name is 1-{2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole.[1,2] Its chemical structure is shown in Figure 1. They work by killing the fungus or preventing its growth. It is therapeutically used for athlete's foot, jock itch, ringworm, and other fungal skin infections (candidiasis). It is also used to treat a skin condition known as pityriasis (tinea versicolor), a fungal infection that causes a lightening or darkening of the skin of the neck, chest, arms, or legs. [3] [4] [5]

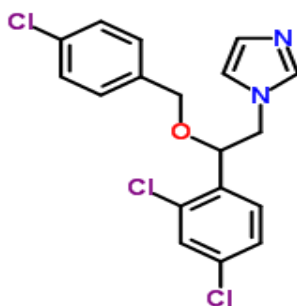


Figure 1: Chemical structure of Econazole.

Econazole interacts with 14- demethylase, a cytochrome P-450 enzyme necessary to convert lanosterol to ergosterol. Econazole inhibits the synthesis of ergosterol which is an essential component of the fungal cell membrane; this will increase the cellular permeability causing leakage of cellular contents. Systemic absorption of Econazole nitrate is low after topical application. Drug concentrations were found in the stratum corneum. Inhibitory concentrations were achieved in the epidermis and as deep as the middle region of the dermis [5].

There are already several bioanalytical methods developed for Econazole. Determination of Econazole with HPLC was performed by A.Mark Dyasand Hugh Delargy and acquired a recovery of 98.5% [6]. This paper describes a new sensitive bioanalytical method for Econazole using RP-HPLC method. By this method, chromatographic conditions have been optimized and validated in accordance to FDA guidelines. This result in a more sensitive, less time consumption and easier method of quantification compared to the other existing methods and it gives better recovery from the human plasma, which is 93.5%.

Experimental

Chemicals and reagents

Acetonitrile and Triethylamine (both HPLC grade) were obtained from Merck, Darmstadt, and West Germany. Ammonium acetate (molecular biology reagent grade) was obtained from System,

Malaysia. Potassium dihydrogen phosphate was obtained from HmBG. Methanol obtained from QREC. And HPLC grade water was used throughout.

Instrumentation and Chromatographic Condition

HPLC chromatographic separation was performed on a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50 μ L loop volume. LC solution version 1.25 was applied for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 0.6mL/min using a mobile phase of 0.5% Triethylamine (pH 6.5) and Acetonitrile (85:15% v/v). The mobile phase was prepared daily, filtered through a 0.45 μ m membrane filter (Millipore) and sonicated before use. A Thermo C18 column (250mm 4.6mm i.d., 5 μ) was used for the separation.

Preparation of Stocks Solution

100mg of Econazole was weighed accurately and dissolved in 100mL of methanol into the 100mL light resistance volumetric flask. The final solution was containing 1mg/mL. It was labeled and the solution was stored in a refrigerator below 8°C.

Sample Preparation (Protein Precipitation)

The blank plasma sample was prepared by adding 0.5mL of plasma and 0.5mL of TCA in Eppendorf tube and vortex for 2 min. Then centrifuge the solution at 4000 RPM for 7min. The supernatant liquid is taken and transferred to another Eppendorf tube. Centrifuge the solution at 4000 RPM for 2 minutes and transferred to HPLC vials. In protein precipitation, Trichloroacetic acid (TCA) is used to remove the protein by denaturation and precipitation [7] (Figure. 2)

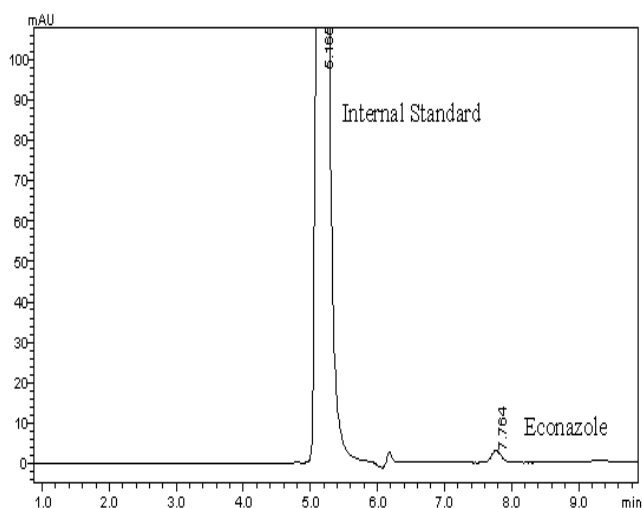


Figure. 2. Typical chromatogram of spiked plasma

Validation

The method was validated in terms of Specificity, Accuracy, Precision, Linearity and Range, Stability, and Recovery.

Specificity

The specificity was established by preparing an Econazole standard and injected 5 times into RP-HPLC system as per the procedure. The specificity method described was investigated by drug free human plasma. Under the proposed assay condition, internal standard and Econazole had a retention time 3.8 minutes and 6 minutes respectively. Rests of the peaks were due to the plasma components. Econazole and internal standard were very well resolved under the proposed chromatographic conditions. None of the drug free human plasma samples studied in this assay in endogenous interference at this retention time.

Accuracy

The accuracy describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by using seven different concentration of Econazole (5, 10, 20, 30, 40, 50 and 70 ng/mL). It should within the range of 80-120%. [8]

$$\%Nominal = \frac{\text{Measured concentration}}{\text{Actual concentration}} \times 100\%$$

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

$$\text{Precision} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100\%$$

Linearity and Range

The linearity of the method was determined at seven different concentration of Econazole (5, 10, 20, 30, 40, 50 and 70 ng/mL).

The calibration curve was constructed by plotting absorbance against different concentration of Econazole (5, 10, 20, 30, 40, 50 and 70 ng/mL). [12]

Linearity, R is found to be 5-70ng/mL. The regression equation was found to be $y = 1.0005 X - 0.4032$ with coefficient of correlation $R^2 = 0.9994$ (Figure. 3 and Table 1).



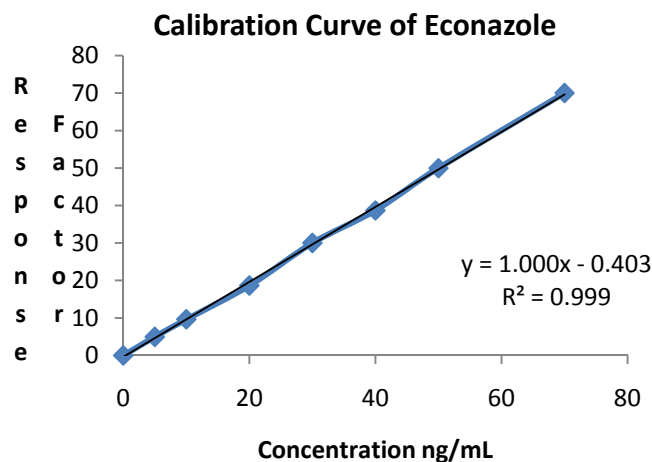


Figure 3. Calibration curve of Econazole

Table 1. Linearity studies

Concentration	Absorbance
0	0
5	4.9540
10	9.6352
20	18.6487
30	29.9954
40	38.6954
50	49.9564
70	69.9924

Stability

Stability test need to be performed in validation analysis of drugs to ensure the products is stable even after a certain time frame to avoid any other interaction, and the reproducibility of the curve should be monitor over the time. In stability studies of analysis of Econazole, an optimum chromatography condition is maintained as such that with the mobile phase of tri-ethylamine and with organic phase of Acetonitrile with ratio of 85:15, and flow rate of 0.6mL/min. The concentration of each sample is 5ng, 10ng, 20ng, 30ng, 40ng, 50ng and 70ng respectively.

5 repeated injections were given to the plasma sample:

$$\frac{\text{mean response of stability sample}}{\text{mean response of comparison sample}} \times 100$$

The results of stability studies are in Table 2.

The accepted limit range of the stability studies should be within 80% - 120%.

Short Term Stability Studies

In short term room temperature stability of drug, the % Nominal was 96.84% for LQC and 98.77% for HQC level. The results of

short term storage at room temperature stability indicated no degradation of Econazole. Results are shown in Table 3.

Table 2. Stability studies

	Nominal Concentration (ng/mL)	
	LQC	HQC
	5.0	70.0
	4.523	68.852
	4.896	69.635
	4.471	69.047
	4.632	68.531
	4.558	67.995
Mean	4.616	68.812
S.D	0.167103261	0.608400362
C.V (%)	3.620087987	0.884148639
% Nominal	92.32	98.30285714
N	5	5

Table 3. Short Term Room Temperature Stability of Drug

	Nominal Concentration (ng/mL)	
	LQC	HQC
	5	70
	4.8941	69.3526
	4.6395	68.7497
	4.9521	69.5214
	4.8837	68.9587
Mean	4.84235	69.1456
S.D	0.138541293	0.353903499
C.V (%)	2.861034281	0.5118236
% Nominal	96.847	98.77942857
N	5	5

Auto Sampler Stability Studies

auto sampler stability of drug in plasma, the % Nominal was 99.16% for LQC and 99.26% for HQC. Results are shown in Table 4.

Table 4. Auto sampler Stability of Drug in Plasma

	Concentration (ng/mL)	
	LQC	HQC
	5	70
	5.0185	69.8547
	4.8897	69.9921
	4.9967	69.0319
	4.8859	68.5974
	5.0014	69.9581
Mean	4.95844	69.48684
S.D	0.065007369	0.634580285
C.V (%)	1.31104478	0.913238082
% Nominal	99.1688	99.26691429
N	5	5

Freeze Thaw Stability Studies

The blood plasma is taken out from the fridge and allowed to defrost in room temperature for 30minutes. 5µl of plasma is taken into Eppendorf tube and 5µl TCA is added for protein precipitation. The result is shown in Table 5.

Table 5. Freeze/Thaw Cycle Stability of Drug

	Concentration (ng/mL)	
	LQC	HQC
	5	70
	4.8964	68.7124
	4.9902	69.1598
	4.8632	69.9231
	4.9921	68.9945
	4.8762	69.0175
Mean	4.92362	69.16146
S.D (±)	0.062774533	0.455618605
C.V (%)	1.274967058	0.658775284
% Nominal	98.4724	98.80208571
N	5	5

Long Term Stability Studies

For long term stability studies, both standard and sample solutions were analyzed over the period of 72 hours. Results are shown in Table 6.

Table 6. Long Term Stability of Drug

	Concentration (ng/mL)	
	LQC	HQC
	5	70
	4.6592	69.0213
	4.8915	68.9972
	5.9978	69.1524
	4.8979	68.0175
	4.9912	69.8513
Mean	5.08752	69.00794
S.D (±)	0.523384378	0.654482622
C.V (%)	10.28761318	0.948416403
% Nominal	101.7504	98.58277143
N	5	5

Recovery

Recovery is a ratio of the detector response of an analyte from an extracted sample to the detector response of the analyte from an unextracted sample containing the same amount of analyte that was added to the extracted sample.

Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

$$\% \text{Recovery} = \frac{\text{Unextracted sample}}{\text{Extracted sample}} \times 100\%$$

The percentage recovery of Econazole was found to be in the range of 97.36% to 99.93% and the results are presented in the Table 7.

Table 7. Recovery studies

Extracted	Unextracted	% Recovery
11524	13526	85.19888
10458	14025	74.56684
11369	13894	81.82669
10895	14008	77.77698
11395	13975	81.53846

LOD and LOQ (limit of detection and limit of quantification)

LOD and LOQ were calculated as $3.3 \sigma / S$ and $10 \sigma / S$ respectively; where σ is the standard deviation of the response (y-intercept) and S is the slope of the calibration plot. The LOD and LOQ for Econazole were predicted basing on the parameters of standard error of estimate and slope, calculated from linearity of the response data of Econazole are 3.0 ng/mL and 8.0 ng/mL respectively.

Conclusion

An HPLC based method of Econazole has been developed and validated in drug free human plasma. The sensitivity and simplicity of the method makes it suitable for pharmacokinetic studies and can be interchangeable in clinical practice.

References

- [1]. Harry GB, et al. Analytical Profiles of Drug Substances and Excipients. New York: Academic Press. 1994, 23:126-149.
- [2]. Gordana P, Mira C, Katarina V, Sote V, Danica A. Comparison of HPTLC and HPLC for Determination of Econazole Nitrate in Topical Dosage Forms. JPC. 2004, 17:109.
- [3]. Pothana S, Nalini S, Sadanandam M. Development And Validation of RP-HPLC And UV Methods of Analysis for Fluconazole in Pharmaceutical Solid Dosage Forms. 2009, 1:1131-1136.
- [4]. Hillis J, Morelli I, Neville D, Fox J, Leary AC. The Validation of a Bioanalytical Method for the Determination of Fluconazole in Human Plasma. Chromatographia. 2004, 59:203-207.



- [5]. Stephen E, Wolverton. Comprehensive Dermatologic Drug Therapy. Elsevier Inc. 2007.
- [6]. Dyas AM, Hugh D. Analytical Profiles of Drug Substances and Excipients. New York: Academic Press. 1994, 23:148.
- [7]. Sekar V, Jayaseelan S, Subash N, Kumar EU, Perumal P, Venkatesh RP. Bioanalytical Method Development and Validation of Letrozole by RP-HPLC Method. International Journal of Pharmaceutical Research and Development. 2009, 1(10):1-7.
- [8]. FDA.Guidance for Industry. Bioanalytical method validation. US department of health and human services. Food and drug administration, Center for Drug Evaluation and Research, 2001.

