Original Article

Estimation and Validation of Etoposide by RP-HPLC Method in Rat Plasma by Liquid-Liquid Extraction

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ABSTRACT

The objective of this article is to develop a new validated RP-HPLC method for the estimation of Etoposide, an anti-cancer drug in rat plasma. Etoposide was estimated by a new RP-HPLC method developed on a phenomenex C18 (4.6 x 250 mm, 5µ i.d) column, by isocratic elution using mobile phase mixture of methanol: pH 6 phosphate buffer in a ratio of 80:20 % v/v, at a flow rate 0.9 ml/min and monitored at 273 nm with an average retention time of 4.7 ± 0.1 min. Extraction of the sample from rat plasma was performed by employing liquid-liquid extraction method using chloroform as a solvent. The evaporated chloroform extract was reconstituted by using methanol exhibits linearity in the concentration range of 2000-5000 µg/ml. The LOD and LOQ were found to be 184 µg/ml and 561 µg/ml, respectively with a correlation coefficient of 0.9989 and slope of 1.3818. The method was proved to be more accurate, simple, precise and rapid by statistical validation, extraction and recovery studies.

Keywords: Etoposide, methanol, phosphate buffer pH 6, rat plasma, extraction

INTRODUCTION

Etoposide[1] is 4'-demethyl-epipodophyllotoxin 9-[4,6-O-(*R*) -ethylidene-beta-D-glucopyranoside], 4' -(dihydrogen phosphate), used in the treatment of cancer such as Kaposi's sarcoma, Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, nonlymphocytic leukemia, glioblastoma multiform and often given in combination with other drugs such as bleomycin in treating testicular cancer and also used in a conditioning regimen prior to a bone marrow or blood stem cell transplant.

The literature survey revealed that, there were spectrofluorimetric[2] methods and RP-LC[3, 4] methods developed for the estimation of etoposide. HPLC coupled with Mass[5] (LC/MS) and LC/MS/MS[6] turned out

highly sensitive investigation which makes the method expensive and was limited for industrial scale. The extraction procedures adopted for the bio-analytical study of etoposide in human serum and human plasma[7] were found to be expensive and tedious. The present study was sprang up using HPLC coupled with UV detector and was depicted to be economical over expensive industrial scale equipments and operated with universal detector. The aim of the present study was to develop a simple, sensitive and economical method for the estimation of etoposide in rat plasma by liquid-liquid extraction process that can be employed for routine laboratory purpose.

MATERIALS AND METHODS

A gift sample of etoposide presented by Aurobindo Pharma Ltd. was used as standard without further purification. Water HPLC grade and methanol HPLC grade were purchased from Rankem Pvt Ltd, Mumbai. Potassium dihydrogen ortho phosphate AR, sodium hydroxide AR grade purchased from Rankem Pvt Ltd, Mumbai were used in the analysis.

Equipment

SHIMADZU LC 20AD system with SPD-20A UV/VIS detector equipped with Spinchrom software was used for method development, double-beam Perkin Elmer (LAMBDA 25) UV-VIS spectrophotometer was used for spectral measurements and ELICO pH meter for pH measurements.

Chromatographic conditions

The separation was achieved on a Phenomenex C_{18} (250 mm x 4.6 mm, 5 µ) column, with a mobile phase of methanol: buffer of pH 6 in the ratio of 80:20 v/v and at a flow rate of 0.9 ml/min. The detection was monitored at 273 nm and at ambient temperatures.

PROCEDURE

Etoposide stock solution

125 mg of etoposide was weighed and transferred into a clean 25 ml volumetric flask and dissolved in HPLC grade methanol and diluted to the mark to produce 5 mg /ml of etoposide in methanol filtered through 0.22 µm membrane filter and sonicated for 10 min before use.

Preparation of mobile phase

Phosphate buffer pH 6.0: To 125 ml of 0.2M potassium dihydrogen ortho phosphate solution, 14 ml of 0.2M sodium hydroxide was added and diluted to 250 ml with water. Methanol and phosphate buffer were filtered through 0.45 µm membrane filter and sonicated for 30 min before use.

Preparation of calibration standards

The collected rat blood was mixed with EDTA to precipitate plasma proteins and centrifuged for 5 min. The supernatant is used further for preparing calibration standards and as blank. Aliquots of etoposide was added to 0.9 ml of plasma into a series of 2 ml eppendroffs so that the volume is maintained to 1ml and concentrations to 2-5 mg/ml, shaken for 5 to 10 min and then add 1 ml of chloroform, vortexed for 10 min and centrifuged at 2,500 rpm for 5 min. Chloroform layer was separated and evaporated to dryness at room temperature using a steam of nitrogen. The residue was reconstituted with 1 ml of mobile phase to produce 2000, 2500, 3000, 3500, 4000, 4500 and 5000 µg/ml, respectively.

Calibration Studies

The calibration study was performed at seven different concentration levels of 2000-5000 µg/ml. All the working standard solutions were filtered though the nylon membrane filters (0.22 µm) and then sonicated for 15 min prior to use. Triplicate

20 µl injections were made for each concentration and chromatogram was recorded under recommended conditions. Calibration graph was plotted for obtained peak area values against corresponding concentrations. The optimized parameters were given in **table 1**.

RESULTS & DISCUSSION

Method development

Detection wavelength was chosen by scanning the sample over a range of wavelengths from 200-350 nm in a spectrophotometer and the suitable wavelength was found to be 273 nm. The UV spectrum of etoposide was given in **fig. 1**. In order to reach proper retention time and sharp peak with enhanced sensitivity under isocratic conditions, the mobile phase composition was optimized by several trails with methanol-water, methanol-buffer, acetonitrile-water and acetonitrile-buffer in various compositions. A satisfactory separation was obtained with a mobile phase consisting of methanol: phosphate buffer pH 6.0 in the ratio of 80:20 % v/v at a flow rate of 0.9 ml/min. This composition removes tailing of peak and delivers good symmetry. To determine the effect of flow rate, the method was performed at different flow rates 0.5 ml/min, 0.7 ml/min, 0.9 ml/min, 1 ml/min and 1.1 ml/min. The optimum flow rate, 0.9 ml/min was chosen finally as it was found ideal with retention time of 4.7 ± 0.1 min which was shown in the **fig. 2**. The change in the wave length of detection between the run was performed to achieve better detector response and 273 nm had shown good response under the calibrated conditions

For extraction of drug in human plasma, chloroform was found as suitable extraction medium, as it showed better recovery and permits determination of the drug with reasonable response with reliable results. The other solvents like DMSO, diethyl ether, ethyl acetate, cyclohexane were tried for extraction but the drug was insoluble or sparingly soluble hence extraction could not be achieved from plasma. Chloroform has low boiling point hence by heating at 50^0 C under a stream of nitrogen the solvent was evaporated and etoposide residue was obtained.

Method validation

In the present study linearity was established by analyzing a series of eight concentrations ranging between 2000-5000 µg/ml and the results were presented in **table 2 and fig 3**. For quantitative applications linear calibration chromatograms were obtained with correlation coefficients near to 0.9989. Intra-day and inter-day precision studies were carried out and the % RSD obtained from the studies was found to be satisfactory. The results were depicted in **table 3**. The precision studies proved the method to be reproducible. The robustness of a method is evaluated by varying method parameters such as percent organic phase, pH of the buffer, flow rate and found no effect on the results of the method and was presented in **table 4**. To determine accuracy standard drug solution was spiked with sample solution to get different concentrations of 50%, 100%, 150%. The mean recovery and % RSD were calculated and depicted in table 5. Good recovery after extraction from rat plasma using chloroform proved the method to be more accurate.

CONCLUSION

The proposed validated HPLC method is simple, selective, precise and accurate for quantification of etoposide in rat plasma. The method may be useful for bio-availability and bio-equivalence studies on the drug.

REFERENCES

1. Government of India. Ministry of Health and family welfare. Indian Pharmacopoeia Vol I. Indian Pharmacopoeial Commission, New Delhi; 2010: 1322 – 1324.
2. Snehalatha M and Saha RN. New sensitive and validated spectrofluorimetric method for the estimation of etoposide in bulk and pharmaceutical formulations. *Pharmazie* 2006; 61: 664-666.
3. Dave RM, Patel RK, Patel JK. RP-HPLC method development and validation of etoposide. *J Pharm. Research* 2012; 5: 3618 – 3620.
4. Hayat MM, Ashraf Md, Nisar-Ur-Rehman, *et.al*. HPLC determination of etoposide in injectable dosage forms. *J. Chil. Chem. Soc* 2011; 56: 881-883.
5. Chen CL, Uckun FM. Highly sensitive liquid chromatography- electro spray mass spectrometry (LC-MS) method for the determination of etoposide levels in human serum and plasma. *J Chromatogr B Biomed Sci Appl* 2000; 744: 91-98.
6. Pang S, Zheng N, Felix CA, Scavuzzo J, Boston R, Blair IA. Simultaneous determination of etoposide and its catechol metabolite in the plasma of pediatric patients by liquid chromatography/tandem mass spectrometry. *J mass spectrum* 2001; 36: 771-778.
7. Ronald A. Fleming, Clinton F. Stewart. High-Performance liquid chromatographic determination of etoposide in plasma. *J.Liquid Chromatogr* 1991; 14(7): 1275-1283.

Table 1. Optical and regression parameters

Parameters	Chromatographic conditions
Calibration range (µg/ml)	2000-5000
Detection limit (µg/ml)	184
Quantitation limit (µg/ml)	561
Slope (b)	1.381848
SD of Slope (S_b)	77.54427
RSD of Slope (%)	0.7
Intercept (a)	-689.16
Correlation coefficient	0.9979
Theoretical plates	15708.444

Table 2. Linearity of Etoposide

Conc µg/ml	Area mV
2000	1355
2500	2338
3000	3255
3500	4099
4000	4978
4500	5763
5000	6554

Table 3. Inter day and intra day precision studies

Inter day		Intra day	
Mean area	6284.333	**Mean area**	6406.667
SD	3390.545	**SD**	3455.743
% RSD	0.6	**% RSD**	0.5

Table 4. Results of Robustness studies

	Flow rate ml/min		Wavelength		Mobile phase ratio	
Result	0.8	1	271	275	82:18	78:22
Area mV	6103	6009	6130	6075	6206	6055

Table 5. Results of Accuracy studies

Spiking level %	% Recovery	% R.S.D
50	98.51	0.6
100	99.01	0.9
150	98.68	0.8

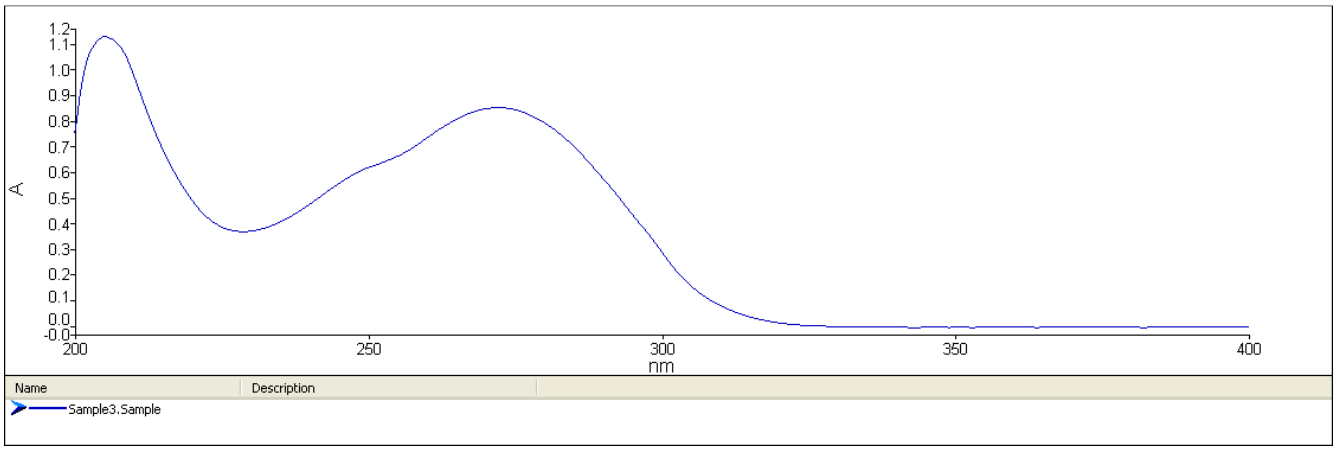

Figure.1. UV absorption plot of etoposide in methanol

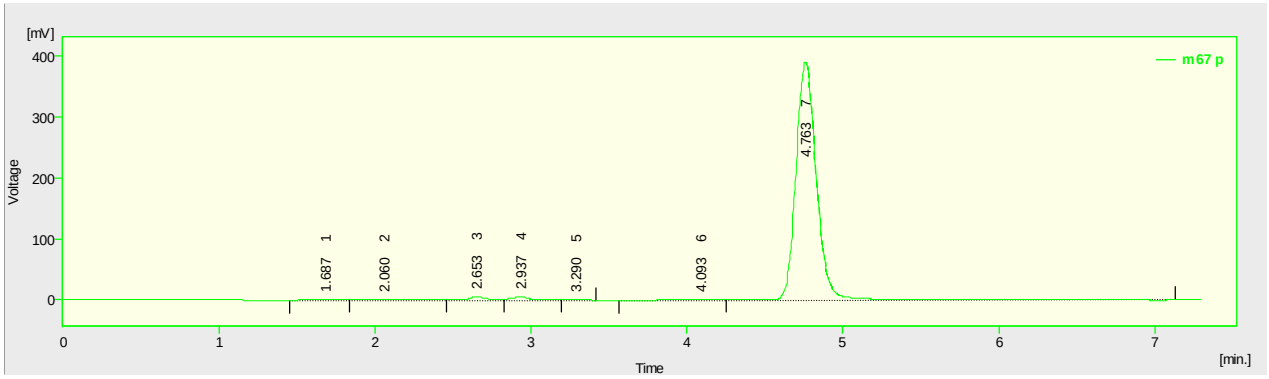

Figure.2. Chromatogram of etoposide in plasma sample by HPLC method

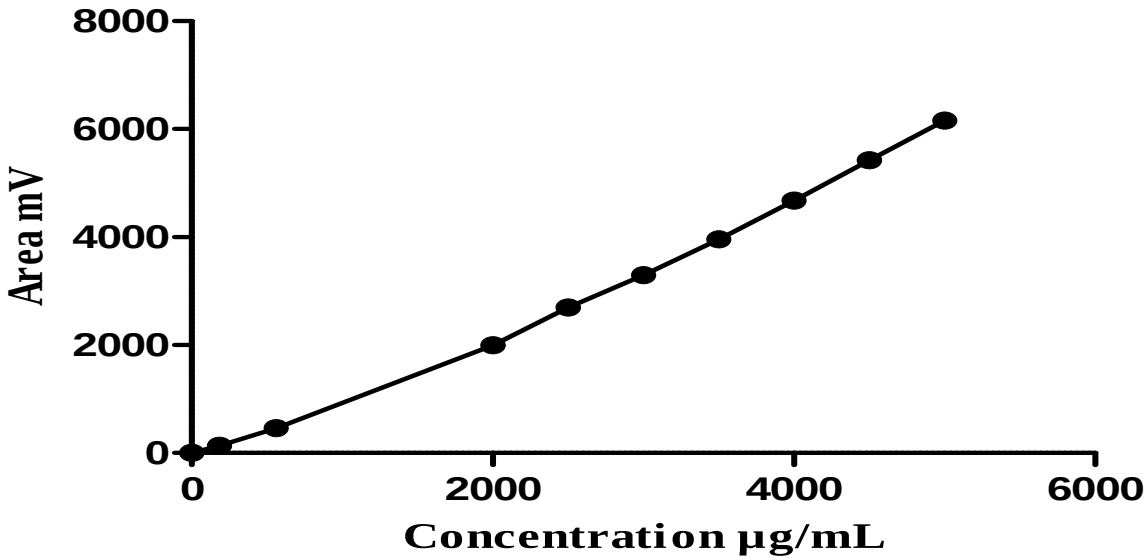

Figure.3. Linearity plot of etoposide