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New, sensitive and validated spectrofluorimetric method for the estimation of etoposide in bulk and pharmaceutical formulations

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A new, simple and sensitive spectrofluorimetric method was developed for the routine estimation of etoposide, an anticancer drug, in bulk and in pharmaceutical formulations. The medium selected for the estimation of etoposide was methanol : phosphate buffer (pH 7.4) in a ratio of 70 : 30 v/v. Excitation and emission wavelengths used were 240 and 324 nm respectively. The method was validated according to ICH and U.S.P guidelines. Linearity range was 200–1000 ng/ml with detection and quantitation limits of 23 ng/ml and 72 ng/ml respectively. Regression equation obtained was Fluorescence Intensity = 6.704 (Concentration in ng/ml) + 115.4. The drug was found to be stable and there was no interference from the excipients present in different formulations of etoposide.

1. Introduction

Etoposide (CAS number: 33419-42-0) is a semisynthetic derivative of podophyllotoxin, a naturally occurring compound, extracted from the roots and rhizomes of plants *Podophyllum peltatum* and *Podophyllum emodi* (Issell et al. 1984). It has broad spectrum of antitumor activity and is preferably used for the treatment of patients with small cell lung cancer, testicular tumors, Kaposi's sarcoma, lymphomas and several types of leukemia. Etoposide is believed to act by the inhibition of the topoisomerase enzyme and/or induction of direct DNA breaks (Wozniak and Ross 1983; Kaul et al. 1996).

Existing methods for the estimation of etoposide are based on high performance liquid chromatography using UV (Beijnen et al. 1988; Strife et al. 1980), fluorescence (Strife et al. 1981) and electrochemical (Mross et al. 1994; McLeod and Relling 1992) detectors. These methods are tedious, expensive, complicated and time consuming. Thus, there is a need for a sensitive, simple and inexpensive method for the determination of etoposide particularly for routine analysis. Fluorimetry, by virtue of its high sensitivity meets their requirements. A survey of the literature has not revealed any spectrofluorimetric method for the estimation of etoposide.

2. Investigations, results and discussion

For optimization of the medium to estimate etoposide, various aqueous media like acetate buffer (pH 5.8), phosphate buffers (pH 6.0 to 8.0) individually or in combination with different ratios of methanol were tried. The spectral characters of etoposide were found to be independent of pH of the solution. Addition of methanol in various proportions with phosphate buffer (pH 7.4) has improved sensitivity of the analysis. The final decision of using mixture of phosphate buffer (pH 7.4) and methanol in 70 : 30 v/v was made based on sensitivity, cost, ease of preparation and applicability of the method to routine analysis and dissolution studies. Emission spectra of different concentrations of etoposide in selected medium are shown in the Fig.

Different drug concentrations and their relative fluorescence intensities are shown in Table 1. At all the concentration levels the standard deviation was low and the relative standard deviation (RSD) did not exceed 1.7%. The predicted concentrations were nearly matching the nominal concentration. The linearity range was found to be 200–1000 ng/ml. The linear regression equation obtained was: Fluorescence Intensity = 6.704 (Concentration in ng/ml) + 115.4; with a regression coefficient of 0.9994. Slope and intercept were 6.704 and 115.4 respectively. Lower values of standard error of slope (± 0.0409), standard error of intercept (± 27.12) and standard deviation of response (± 77.56) indicates high precision of the proposed method. The mean slope and intercept values were within the 95% confidence interval.

2.1. Validation

Analysis of formulations has shown that common excipients used in formulations like benzyl alcohol, ethyl alcohol etc, did not interfere with the estimation of etoposide at the wavelengths used in this method. Presence of excipients did not change the fluorescence intensity confirming specificity and selectivity of λ_{em} and λ_{exc} for the drug.

The results of accuracy for the proposed method are summarized in Table 2. Percentage relative error ranged from −1.3 to 1.2%, which indicates that this method is accurate. High recoveries determined by the standard addition method (100.7, 99.5, 99.8 and 100.5 for the respective pure drug concentration) and low RSD (1.315, 1.518,

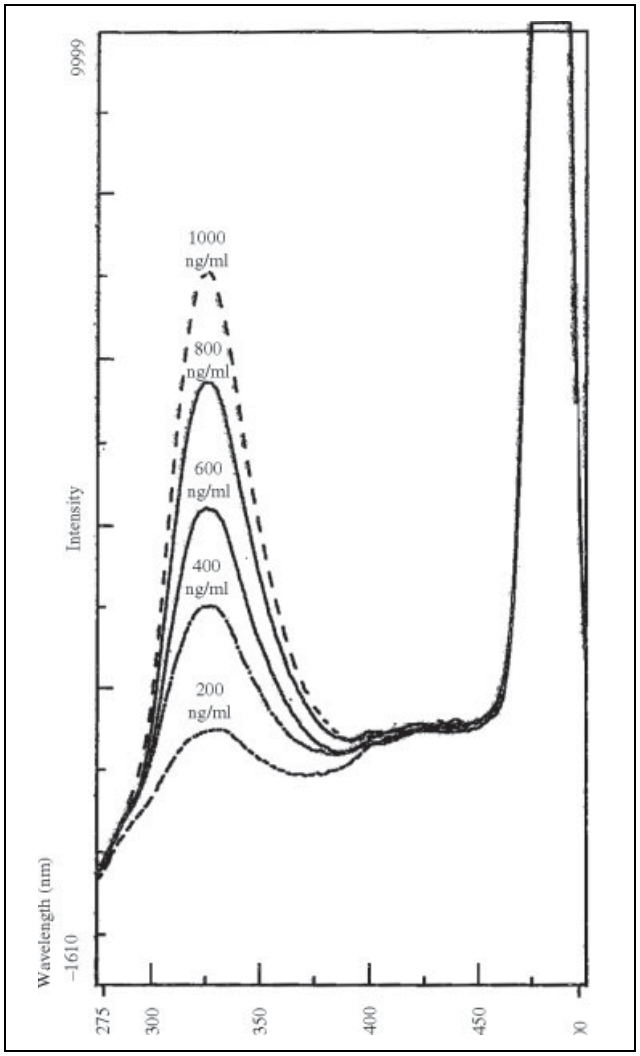

Fig.: Emission spectra of different concentrations of etoposide in selected medium

Table 1: Calibration data of the spectrofluorimetric determination of etoposide

Nominal concentration (ng/ml)	Fluorescence intensity[a, b] (± SD)	RSD (%)[c]	Predicted concentration[d] (ng/ml)
200	1451.8 ± 18.21	1.2540	199.34
400	2746.8 ± 0.67	0.0243	392.50
600	4214.4 ± 55.22	1.3103	611.42
800	5494.6 ± 57.30	1.0428	802.37
1000	6782.0 ± 119.75	1.7658	994.41

[a] average of nine determinations
[b] arbitrary units
[c] RSD (%) of each point from the regression line
[d] predicted concentration calculated from the regression equation

Table 3: Intra-day and inter-day variability

Level	Intra-day repeatability % RSD (n = 3)			Inter-day repeatability (n = 27)	
	Day 1	Day 2	Day 3	Mean % recovery	% RSD
LQC	0.249	2.213	0.179	101.85	0.265
	2.123	0.582	0.176		
	0.930	0.462	1.430		
MQC	0.799	0.046	0.098	99.72	0.372
	1.435	0.097	0.426		
	0.621	0.744	0.446		
HQC	1.729	1.978	0.010	98.62	0.043
	0.747	0.072	0.587		
	1.477	1.529	1.548		

% RSD – percentage relative standard deviation. (each day nine replicates were prepared independently).

0.482 and 1.096 respectively) show that the method can estimate etoposide accurately. Overall precision (RSD) ranged from 1.06 to 1.27%. Intra-day relative standard deviations (RSDs) were found lower than 2.21% and the inter-day RSDs were lower than 0.37% for all concentration levels (Table 3). The observed RSD values were within the limits, indicating ruggedness and intermediate precision of the developed method. DL and QL were found to be 23.92 ng/ml and 72.47 ng/ml respectively. Variation of pH of the medium by ± 0.1 and strength of methanol by ± 1% did not have any significant effect on the relative intensity. The etoposide solution in selected medium exhibited no emission spectral changes when kept at room temperature for a study period of 24 h.

2.2. Estimation of etoposide in formulations

The method was evaluated by estimation of etoposide in pharmaceutical formulations and the results are shown in Table 4. For different formulations recovery ranged from 99.4 to 100.0% with standard deviations of not more than 1.759. The estimated drug content with low values of standard deviation established the precision of the pro-

Table 4: Determination of etoposide in pharmaceutical preparations

Preparation	Assay (± SD)	% Recovery (± SD)
Etosid soft gelatin capsules – 50 mg	50.10 ± 0.880	100.21 ± 1.759
Etosid injection – 100 mg/5 ml	99.40 ± 0.727	99.40 ± 0.727
Fytosid injection – 100 mg/5 ml	99.62 ± 1.273	99.62 ± 1.273

Table 2: Accuracy and precision data for the developed method

Level	Predicted conc. (ng/ml)[a]			Mean % recovery (± SD)	Accuracy (%)[b]
	Range	Mean (±SD)	% RSD		
LQC	296.02–313.20	303.73 ± 3.86	1.272	101.24 ± 1.288	1.243
MQC	483.97–503.68	493.38 ± 5.22	1.057	98.68 ± 1.043	−1.324
HQC	872.03–919.67	890.72 ± 10.66	1.196	98.97 ± 1.184	−1.031

[a] predicted concentration of etoposide was calculated by linear regression equation
[b] accuracy is given in relative error % (= 100 × [(predicted concentration – nominal concentration)/nominal concentration)]. Each determination is result of nine separate determinations

posed method. In all the formulations, etoposide was estimated accurately and precisely by this method.

2.3. Conclusion

The proposed method can be used for the estimation of etoposide in bulk, and pharmaceutical formulations at low level. Common excipients did not interfere with estimation of etoposide. This can be a suitable alternative for the HPLC method as this method is fast, easy, inexpensive and sensitive. The method can also be used for routine dissolution studies from formulations.

3. Experimental

3.1. Materials

Pure etoposide was obtained as gift sample from Dabur Research Foundation, Sahibabad, India. All other chemicals and reagents used were of analytical grade. Formulations of etoposide used for the study were soft gelatin capsules (Etosid, Cipla LTD, India) containing 50 mg etoposide, U.S.P. each capsule and injections (Etosid, Cipla LTD, India and Fytosid, Dabur India LTD, India) containing etoposide U.S.P. 100 mg/5 ml and were procured from the Indian market. Etosid capsules contain excipients like ferric oxide red and titanium dioxide. Etosid and Fytosid injections contain excipients like benzyl alcohol I.P., Ethyl alcohol I.P. in a sterile non-aqueous vehicle.

3.2. Equipment

A spectrofluorimeter (Jasco, FP777, Japan) with a xenon lamp and inbuilt software was used for all measurements using 1 cm quartz cells. Experimental parameters were: excitation band width – 5 nm, emission band width – 10 nm, photo-multiplier tube response – high, slit width – 0.5 nm, $\lambda_{exc} = 240$ nm and $\lambda_{em} = 324$ nm.

3.3. Analytical method development

Different media made of methanol and different buffers individually and in combinations, were investigated to develop a suitable spectrofluorimetric method for the analysis of etoposide. For selection of media the criteria employed were sensitivity, ease of sample preparation, cost and applicability of the method to the routine analysis. A combination of methanol and phosphate buffer pH 7.4 in ratio of 70 : 30% v/v was selected for the spectrofluorimetric estimation of etoposide.

3.4. Calibration curve

A primary stock solution of 200 µg/ml etoposide was prepared in methanol. A secondary stock of 20 µg/ml was prepared by diluting the primary stock with a selected medium. The concentration range of 200–1000 ng/ml was prepared by suitable dilution of the secondary stock by the medium selected. The fluorescence intensity was measured at $\lambda_{em} = 324$ nm, irradiating at $\lambda_{exc} = 240$ nm. Nine replicates of all the solutions were prepared and analysed. The calibration curve was then prepared by plotting the fluorescence intensity versus the concentration of the drug. A calibration equation was developed by least square regression analysis. Stability of stock and bench top solutions in the medium were studied for 24 h.

3.5. Validation

Validation parameters were determined according to standard procedures given in the ICH guidelines and USP. Specificity and selectivity of the method was assessed by preparing a drug concentration of 0.5 µg/ml from pure drug stock and a commercial sample stock in selected medium and analysed ($n = 5$). Solutions of common excipients used in formulations were prepared, with and without drug and analysed for any change in emission and excitation scans of etoposide.

As a part of determining accuracy of the proposed method, different levels of drug concentrations (LQC – 300 ng/ml, MQC – 500 ng/ml and HQC – 900 ng/ml) were prepared independently from stock solution and analysed ($n = 9$). To give additional support to the accuracy of the developed assay method, the standard addition method was used to check recovery. Different concentrations of pure drug solutions (100, 200, 400 and 600 ng/ml) were added to a known preanalysed formulation sample (400 ng/ml) and the total concentration ($n = 5$) was analysed. The recovery of the added pure drug was calculated as % Recovery $= [(Cv - Cu/Ca)] \times 100$, where Cv is the total drug concentration measured after standard addition; Cu is the drug concentration in the formulation and Ca the drug concentration added to the formulation.

As a part of precision, repeatability was calculated by taking different levels of drug concentrations (same as accuracy) independently the from stock solution which were analysed ($n = 9$) (Table 2). Inter day and intra day variation were carried out to determine in intermediate precision of the method (Table 3).

To establish linearity of the proposed method, separate series of five concentrations (200, 400, 600, 800 and 1000 ng/ml) were prepared and analyzed. Least square regression analysis was done for the obtained data. The detection limit (DL) and quantitation limit (QL) of etoposide for the proposed method were determined using calibration standards. DL and QL were calculated as $3.3\ \sigma/S$ and $10\ \sigma/S$ respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation. Robustness of the method was determined by changing pH of the phosphate buffer by ± 0.1 and composition of methanol by $\pm 1\%$. Stability of the etoposide in the selected medium was observed by spectral changes at room temperature for 24 h.

3.6. Estimation of etoposide in commercial formulations

Contents of 10 capsules of etoposide was mixed properly. A quantity equivalent to 2 mg of etoposide was taken and dissolved in 30 ml of methanol. Phosphate buffer pH 7.4 was added to make up the volume to 100 ml. Final dilution was made with same medium in order to obtain concentrations within the linearity range. For injections, a volume equivalent to 2 mg etoposide was taken and dissolved in methanol and the same steps were repeated to obtain concentrations in the linearity range. Five replicates were prepared for all formulations.

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