

## DEVELOPMENT AND VALIDATION OF SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF CYCLOPHOSPHAMIDE IN BULK DRUG AND ITS PHARMACEUTICAL DOSAGE FORM

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### ABSTRACT

**Objective:** To develop a simple, sensitive and reproducible spectrophotometric method for the determination of Cyclophosphamide (CP) in bulk and pharmaceutical formulations.

**Method:** The proposed method is based on the formation of a blue colored complex of the drug with ammonium molybdate, which is measured spectrophotometrically at 722 nm. The color was stable for an hour.

**Results:** Beer's law is obeyed over a concentration range from 20-45 µg/ml. The apparent molar absorptivity and Sandell sensitivity values are  $0.90948 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$  and  $0.02870 \mu\text{g}/\text{cm}^2$  respectively. The LOD and LOQ of the proposed method were calculated 0.02559 and 0.08521 µg/ml respectively. All the variables were studied to optimize the reaction conditions. No interference was observed in the presence of common pharmaceutical excipients. The validity of the method was tested by analyzing CP in its pharmaceutical formulations and critically tested for its accuracy by statistical tests.

**Conclusion:** Good recoveries were obtained by the developed method; the obtained results were critically analyzed and successfully employed for the determination of CP in its pharmaceutical dosage forms.

**Keywords:** UV-Spectrophotometry, Cyclophosphamide, Cytotoxic, Anti-cancer, Validation.

### INTRODUCTION

Cyclophosphamide (CP), 2-(bis(2-chloroethyl)amino)-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide, is one of the most commonly used antineoplastic alkylating agents (Figure.1). This drug has been usually administered in combination with other antineoplastic agents like Ifosfamide (IF) for the treatment of malignant diseases like leukemia, lymphomas and a variety of bone and soft tissue sarcomas [1]. It has also been used due to its immune suppressive properties in the treatment of autoimmune diseases as well as in renal and bone marrow transplants. CP is a pro-drug that requires metabolic activation to phosphoramidate mustard, the cytotoxic antineoplastic agent. This metabolic activation initially starts in the liver by cytochrome P450 enzymes [2].

The efficacy and toxicity of many anticancer drugs are related to parent drug and/ or metabolite concentrations. Therefore, a profound knowledge of the pharmacokinetic properties of the drug is essential. Furthermore, since CP has a narrow therapeutic window, drug monitoring routines in clinical settings may be required for safe and efficacious therapeutic use [3].

By the extensive literature survey, we found that there are numerous methods, such as gas chromatography with mass detector (GC-MS) [4], high performance liquid chromatography (HPLC) [5-9], UV-spectrophotometric [10, 11], liquid chromatography with mass detector (LC-MS) [12-17], UPLC-MS/MS [18], have been used to measure the CP in formulations and biological samples. However, these methods are involved with sophistication, skills, extraction, and more expensive than proposed method [19, 20].

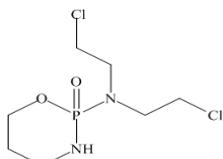


Fig. 1: Structure of Cyclophosphamide

In the proposed method, we have used spectrophotometer, which is a method of choice still in many pharmaceutical industries for the routine analysis. It serves to be one of the quickest, promising and

most reliable techniques. The aim of the present study is to develop and validate a new simple, sensitive, rapid, reliable and precise spectrophotometric method for the analysis of CP in its dosage forms.

### MATERIALS AND METHOD

#### Apparatus

Spectral and absorbance measurements were carried out by using double beam UV- Spectrophotometer ELICO-SL-244 with 1 cm matched quartz cell.

#### Materials and reagents

All the chemicals used were of analytical grade. All the solutions were prepared freshly and the deionized water is used throughout the experiment.

- Ammonium molybdate (0.005M): Weighed exactly 0.61795g ( $\pm 0.0001\text{g}$ ) of ammonium molybdate in 100 ml volumetric flask it was dissolved and diluted to the mark using deionized water.
- Ferric chloride solution (0.01M): Weighed exactly 0.22560g ( $\pm 0.0001\text{g}$ ) of ferric chloride in 100 ml volumetric flask it was dissolved and diluted to the mark using double distilled alcohol.
- Stannous chloride (0.01M): Weighed exactly 0.16221g ( $\pm 0.0001\text{g}$ ) of stannous chloride in 100 ml volumetric flask it was dissolved and diluted to the mark with deionized water.

Pharmaceutical grade Cyclophosphamide (CP) claimed to be 99.7 % pure was received from the Cipla India Ltd. Mumbai, which is used as a reference standard for the analysis without any further purifications. Endoxan<sup>®</sup>237 mg, (labeled to contain 50 mg of active ingredient) tablets were produced and marketed by Zydus cedilla. This drug is used as a sample for the proposed method, which is obtained from the pharmaceutical store.

#### Standard drug solution

Stock solution of CP (1 mg/ml) was prepared by using 10 mg of reference standard drug in a 10 ml volumetric flask, added 5 ml methanol to dissolve the content and brought up to the mark with methanol, mixed thoroughly for homogeneity. Working solution of

lower concentration (100 µg/ml) was prepared by further dilution of the above standard stock solution with methanol.

#### General procedure for the determination of Cyclophosphamide

Variable aliquots of working standard solution containing 20-45 µg/ml of CP were transferred into a series of test tubes. To each test tube 1 ml of ferric chloride solution was added, test tubes are heated for 5 min. filtered the contents to 10 ml standard volumetric flasks added 2 ml of ammonium molybdate solution followed by this added 2 ml of hot stannous chloride solution resulting in the formation of a deep blue colored phosphomolybdate complex, then the flasks are allowed to cool to room temperature and the solutions made up to the mark with water. The colored species absorbance was measured at 722 nm using reagent as a blank. The calibration graph was prepared by plotting absorbance versus concentration of drug and the concentration of unknown was read from the calibration graph or computed from the regression equation derived from the Beer's law data.

#### Assay procedure for pharmaceutical tablets

For the analysis of CP in tablet formulation, commercially available tablets (10) were weighed and ground into a fine powder, mixed the pulverized tablet powder for homogeneity. An accurately weighed portion of the powder equivalent to 100 mg of CP was transferred into a 100 ml beaker containing small volume of water and the solution was shaken thoroughly for 10-15 min. and filtered through a Whatman filter paper no.41 to remove the insoluble matter. The filtrates were collected in a 100 ml volumetric flask and filter paper was washed thoroughly with water and the washings are also collected, the final volume (100 ml) was made with methanol. A suitable aliquot of this solution in the working range of CP was treated as per procedure described in the above determination of pure CP. The nominal content of CP in the tablets was calculated either from a previously plotted calibration graph or using the regression equation.

### RESULT AND DISCUSSION

#### Determination of absorption maxima ( $\lambda_{max}$ )

To determine the  $\lambda_{max}$  of the colored species, 1 ml of 100 µg/ml of the CP was added to a test tube and 1 ml of ferric chloride solution, heated the contents for 5 min. filtered the contents to 10 ml standard volumetric flask add 2 ml of ammonium molybdate solution followed by this add 2 ml of hot stannous chloride solution resulting in the formation of a deep blue colored phosphomolybdate complex then the flasks are allowed to cool to room temperature and the solution made up to the mark with water. The colored species was measured against reagent blank in the range of 500 nm to 800 nm. The  $\lambda_{max}$  of the complex was found to be 722 nm. Absorption spectrum of the proposed method was shown in Fig.2. Under the experimental conditions each reagent blank showed a negligible absorbance at the corresponding  $\lambda_{max}$ .

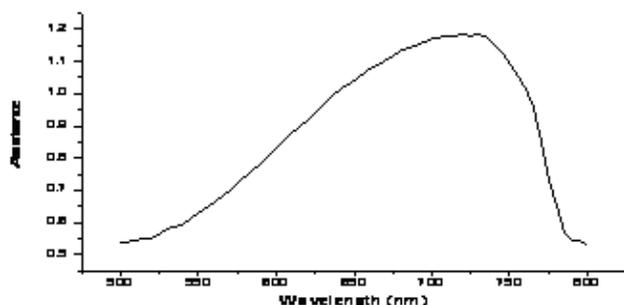


Fig. 2: Absorption spectra of the colored species

#### Investigation of assay parameters

Optimum reagent concentrations required for the formation of sensitive and quantitative colored products were determined by varying one reagent concentration and fixing the concentrations of other reagents and its effect on absorbance was measured at 722 nm.

#### Effect of heating time

To study the effect of heating time for the development of maximum color, the contents of the mixture were heated for up to 30 min. at  $100 \pm 1^\circ\text{C}$ . The intensity of the color developed was measured at room temperature after the dilution to 10 ml with deionized water. It is apparent from the investigation that the maximum intensity of color was obtained after 5 min of heating and remained constant up to an hour. Therefore the optimum heating time was fixed to 5 min.

#### Effect of reagent concentration

The effect of amounts of ammonium molybdate, ferric chloride and stannous chloride were studied on the related absorbance values. Volumes of 0.5–3.0 ml of ammonium molybdate (0.005M), 0.5–3.0 ml of ferric chloride (0.01M) and 0.5–3.0 ml of stannous chloride (0.01M) were examined. The investigations showed that 2.0 ml of ammonium molybdate, 1.0 ml of ferric chloride and 2.0 ml of stannous chloride gave maximum absorbance. There is no change in intensity of the color any further with the increasing amounts of ammonium molybdate, ferric chloride and stannous chloride. So the 2.0 ml of ammonium molybdate, 1.0 ml of ferric chloride and 2.0 ml of stannous chloride were chosen throughout the experiment.

#### Interference studies

To study the potential interference from the commonly used excipients and other additives such as glucose, lactose, starch, sodium starch glycolate, cellulose, magnesium stearate and ascorbic acid recovery studies were carried out. Under the experimental conditions employed, to a known amount of drug. The recovery studies suggest that there was no significant interference from the excipients on the assay of the drug.

#### Validation of the method

##### Detection and Quantification limits

According to the Analytical Methods Committee [21, 22] the detection limit (LOD) is the concentration of drug corresponding to a signal equal to the blank mean ( $Y_B$ ) plus three times the standard deviation of the blank ( $S_B$ ). Quantification limit (LOQ) is the concentration of drug corresponds to the blank mean plus ten times the standard deviation of the blank. The LOD and LOQ values for CP were found to be 0.02559 and 0.08521 µg/ml respectively.

#### Quantification

The optical characteristics such as Beer's law limits, Sandell sensitivity and molar absorptivity were calculated for the proposed method and the results are summarized in Table 1. Regression analysis of the Beer's law plot at their  $\lambda_{max}$  revealed a good correlation as shown in Fig. 3. For the regression analysis we have selected different concentration sets but the best fit curve was obtained in the concentration range 20 to 45 µg/ml of standard CP. For the verification of Beer's law we have taken a series of 10 ml volumetric flasks and added the working standard solution (100 µg/ml) serially from 2.0 ml to 4.5 ml followed by added all the reagents as mentioned in the assay procedure. Graph of absorbance versus concentration showed zero intercept and are described by the regression equation  $Y = bx + a$  (where, 'Y' is the absorbance, 'b' is the slope, 'x' is the concentration of the drug in µg/ml and 'a' is the intercept) obtained by least squares method. The results were summarized in Table 1.

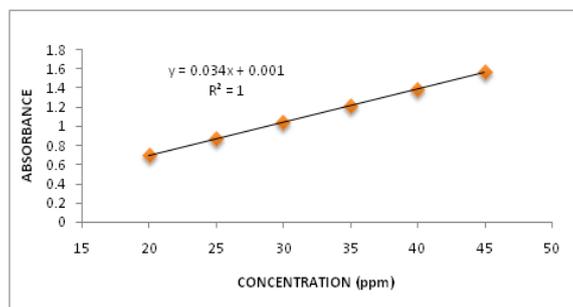


Fig. 3: Beer's law calibration curve

**Table 1: Optical and Regression characteristics, precision and accuracy of the proposed method**

S. No.	Parameters	Value
1	$\lambda_{\max}$ (nm)	722
2	Beer's law limit ( $\mu\text{g/ml}$ )	20-45
3	Sandell sensitivity ( $\mu\text{g/cm}^2/0.001$ abs. unit)	0.02870
4	Molar absorptivity ( $\text{L mole}^{-1}\text{cm}^{-1}$ )	$0.90948 \times 10^4$
5	Stability of Color (hours)	1
6	Regression equation	$y = 0.034x + 0.001$
7	Correlation coefficient	1.00
8	% Relative standard deviation	0.02361
9	% Range of errors	
	0.05 %	$1.2193 \pm 0.267 \times 10^{-3}$
	0.01%	$1.2193 \pm 0.469 \times 10^{-3}$
10	Limit of detection ( $\mu\text{g/ml}$ )	0.02559
11	Limit of quantification ( $\mu\text{g/ml}$ )	0.08521

**Accuracy precision and recovery studies**

The accuracy and precision of the proposed method was evaluated by performing five replicate determination of CP in pure form at three different concentrations (20, 30 and 40  $\mu\text{g/ml}$ ) by short term (intra-day) precisions as shown in Table 2. The standard analytical errors, relative standard deviations (%RSD) and recoveries obtained in the intra-day analysis for the proposed method were found to be acceptable. Thus the proposed method is effective for the determination of CP.

The accuracy of the proposed method was further checked by performing recovery experiments through standard addition technique. For this purpose, a known amount of pure CP was added to pre-analyzed dosage forms and then determined by the recommended procedure. The results are as shown in Table 3. The values of mean recovery and relative standard deviation (%RSD) were in the range of 99.98-100.0 % and 0.016-0.017 % respectively. This indicates the reproducibility of the method. No interference was observed from the common excipients of tablet.

**Table 2: Evaluation of the accuracy and precision of the proposed method by intra-day assay**

Observed concentration of CP ( $\mu\text{g/ml}$ )	Intra-day			
	Mean*	Error (%)	RSD (%)	Recovery (%)
20	19.97	0.005	0.016	99.85
30	30.00	0.010	0.021	100.0
40	40.03	0.007	0.011	100.1

\*For five determinations

**Table 3: Determination of CP in pharmaceutical formulation by standard addition technique**

Amount of drug before addition ( $\mu\text{g}$ )	Amount of drug added ( $\mu\text{g}$ )	Theoretical amount ( $\mu\text{g}$ )	Mean amount recovered ( $\mu\text{g}$ ) (n=5)	Mean % of recovery (n=5)	RSD%
20	20	40	39.99	99.98	0.017
40	20	60	60.01	100.0	0.016

**Table 4: Results of analysis of tablet formulation containing CP**

Formulation	% Found $\pm$ SD				t-test**
	Labeled amount(mg)	Reference method	Proposed method*	%Recovery of proposed method	
Endoxan® 50	50	50.60	$49.88 \pm 0.1146$	99.8	2.343

\* Recovery amount was the average of five determinations

\*\*The t-value from table is 2.776 at 95% level.

**Applicability of the Method**

The proposed method applied to the analysis of CP in pharmaceutical dosage forms and the results were statistically compared with reference method by calculating the student's t-values. The evaluated t-values were less than the tabulated values at the 95% confidence level for five degrees of freedom, as revealed by the results compiled in Table 4. This actually suggests that the proposed method is accurate and precise as the reference method[10].

**CONCLUSIONS**

The proposed method was quite simple and does not require any pretreatment of the drug and tedious extraction procedure. The method has a wider range with good accuracy and precision. Hence, the data presented in the manuscript demonstrate that the proposed

method was accurate, precise, linear, selective and offers advantages of reagent availability and stability, less time consumption and highly sensitive. Thus it can be extended for routine analysis of CP in pharmaceutical industries and hospitals and research laboratories. Unlike the LC/MS procedure and HPLC procedures, the UV-visible spectrophotometer instrument is simple and not of highly expensive on the other hand in simplicity and user friendly the method could be considered superior in comparison with the previously reported methods. Moreover the method is free from interferences by common additives and excipients.

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## REFERENCES

1. Sanson AL, Silva SCR, Martins MCG, Paiva AG, Maia PP and Martins I. Liquid-liquid extraction combined with high performance liquid chromatography-diode array-ultra-violet for simultaneous determination of antineoplastic drugs in plasma. *Brazilian J Pharm Sci* 2011; 47 Suppl.2: 363-371.
2. Hymer CB and Cheever KL. Evaluation of a procedure for the simultaneous quantification of 4-ketocyclophosphamide, Cyclophosphamide and Ifosfamide in human urine. *J Chromatogr Sci* 2010; 48: 328-333.
3. Said R, Hassan Z, Hassan M and Rehim MA. Rapid and Sensitive Method for Determination of Cyclophosphamide in Patients Plasma Samples Utilizing Microextraction by Packed Sorbent Online with Liquid Chromatography - Tandem Mass Spectrometry. *J Liq Chromatogr and Rel Technol* 2008; 31: 683-694.
4. Jardine J, Fenselau C, Appler M, Kan MN, Brundrett RB and Colvin M. Quantitation by gas chromatography-chemical ionization mass spectrometry of cyclophosphamide, 3phosphoroamide mustard and nornitrogen mustard in the plasma and urine of patients receiving cyclophosphamide therapy. *Canc Res* 1978; 38: 408-415.
5. Rustum AM and Hoffman NE. Determination of cyclophosphamide in whole blood and plasma by reversed-phase high-performance liquid chromatography. *J Chromatogr* 1985; 422: 125-134.
6. Kennedy R, Groepper D, Tagen M, Christensen R, Navid F, Gajjar A and Stewart CF. Stability of cyclophosphamide in extemporaneous oral suspensions. *Ann pharmacother* 2010; 44; Suppl 2: 295-301.
7. Malothu N, Veldandi UK and Devarakonda RK. Development of rapid and simple HPLC method for the determination of cyclophosphamide in human serum. *Asian J Pharm Cli Res* 2009; 2: Supp 3: 34-38.
8. Ahmad M, Usman M, Madni A, Zubair M, Zaman Q, Qureshi M, et al. A fast and simple HPLC-UV method for simultaneous determination of three anti-cancer agents in plasma of breast cancer patients and its application to clinical pharmacokinetics. *African J Pharm Pharmacol* 2011; 7: 915-922.
9. Dhakane VD and Ubale MB. Development and validation of a reverse phase high performance liquid chromatographic method for the estimation of cyclophosphamide in bulk drug. *Int J Pharm Pharm Sci* 2013; 5: Suppl 2: 184-187.
10. Amer MM, Hassan SS, Abd El-Fattah SA and El-Kosasy AM. Spectrophotometric and spectrofluorimetric determination of cyclophosphamide and its isomer Ifosfamide. *Anal Lett* 1998; 31, Suppl 14: 2411-2430.
11. Dierickx KME, Journe F, Gerbaux P, Morandini R, Kauffmann JM and Ghanem GE. Improving the spectrophotometric determination of the alkylating activity of anticancer agents: A new insight into the mechanism of the NBP method. *Talanta* 2009; 77:1370-1375.
12. Baumann F and Preiss R. Application of liquid chromatography-mass spectrometry in the determination of oxazaphosphorines and their metabolites. *Anal Chim Acta* 2003; 492: 147-156.
13. Silvertand LHH, Vazvaei F, Weigl P, Rosing H, Hillebrand MJX, van Maanen MJ et al. Simultaneous quantification of fludarabine and cyclophosphamide in human plasma by high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rap Comm Mass Spectr* 2005; 19: 3673-3680.
14. Stokvis E, Rosing H and Beijnen JH. Liquid chromatography-mass spectrometry for the quantitative bioanalysis of anticancer drugs. *Mass Spectr Rev* 2005; 24: 887-917.
15. Rehim AA and Rehim MA. Screening and determination of drugs in human saliva utilizing Microextraction by packed sorbent and liquid chromatography-tandem mass spectrometry. *Biomed chromat* 2013; PMID: 23629973: PubMed - as supplied by publisher.
16. Negreira N, Alda ML and Barcelo D. On-line solid phase extraction-liquid chromatography-tandem mass spectrometry for the determination of 17 cytostatics and metabolites in waste, surface and ground water samples. *J chromatogr A* 2013; 1280: 64-74.
17. Seria J, Claparols C, Cassan CJ, Albasi C, Vignoles MM and Sablayrolles C. Optimization of pressurized liquid extraction using a multivariate chemometric approach for the determination of anticancer drugs in sludge by ultra high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2013; 1283: 27-38.
18. Climent LF, Mozaz SR and Barcelo D. Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. *Anal Bioanal Chem* 2013; DOI 10.1007/s00216-013-6794-4.
19. Odraska P, Dolezalova L, Kuta J, Oravec M, Piler P and Blaha L. Evaluation of the efficacy of additional measures introduced for the protection of healthcare personnel handling antineoplastic drugs. *Ann Occup Hygiene* 2013; 57; Suppl. 2: 240-250.
20. Caerhati T and Szogyi M. Chromatography of anticancer drugs. *European Chem Bull* 2013; 2; Suppl 10: 715-721.
21. Analytical Method Committee, 1987, Recommendations for the definition, estimation and use of the detection limit. *Analyst* 1987.
22. Balasekaran C and Prameela rani A. Development and validation of spectrophotometric method for the determination of DDP-4 inhibitor, sitagliptin, in its pharmaceutical dosage forms. *Int J Pharm Pharm Sci* 2010; 2; Suppl 2: 138-142.