

**Research Article****DEVELOPMENT OF RAPID AND SIMPLE HPLC METHOD FOR THE DETERMINATION OF CYCLOPHOSPHAMIDE IN HUMAN SERUM**

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**ABSTRACT**

Cyclophosphamide belongs to the group of alkylating agents which bind to DNA-usually via the binding of alkyl groups. As a result, a reactive intermediate is formed which binds to DNA and causes single or double stranded DNA breaks and may crosslink the chains of DNA thus disturbing the fundamental mechanisms concerned with the cell growth, mitotic activity, differentiation and function. This study describes a simple and fast high-performance liquid chromatography method for the determination of Cyclophosphamide in human serum. Samples were collected from adult cancer patients at Mahatma Gandhi Memorial hospital (Warangal, AP, India) at various time intervals after the end of each infusion. Serum was deproteinized with the acetonitrile and the supernatant was injected into a 250×4.6 mm ODS column. Mobile phase was made up of water and acetonitrile (70:30) with a flow rate of 1.2ml/min. Ultraviolet detection was done at 197 nm and at ambient temperature. Para aminoacetophenone was used as internal standard. Cyclophosphamide and internal standard retention times were 4.6 and 9.5 minutes, respectively. Results showed that reproducibility (precision) of method within a day was 2.6 to 6 percent and between days was 5.6 to 8.5 percent. The recovery of the method was between 98.8 and 97.01 percent. The quantitation limit of the method for cyclophosphamide was 0.1µM. This method is suitable for quantitation of cyclophosphamide after infusion of doses of this drug and has good accuracy, precision and quantitation limit.

**KEY WORDS** Cyclophosphamide; HPLC; Serum Concentration.

**INTRODUCTION**

The oxazaphosphorine cyclophosphamide (CPH) is commonly used DNA alkylating agent in cancer chemotherapy. Cyclophosphamide is a prodrug that is activated *via* 4-hydroxylation by cytochrome P450s such as CYP2B6 and CYP3A4 to generate alkylating nitrogen mustards (phosphoramidate mustard and the byproduct acrolein). The resultant mustards can alkylate DNA to form DNA-DNA cross-links, leading to inhibition of DNA synthesis and cell apoptosis<sup>1,3</sup>. Pharmacokinetics of CPH is markedly influenced by route of administration and duration of treatment, age, co medication, liver and renal function. Large interpatient variability in pharmacokinetics, clinical response rate and toxicity has been observed in cancer patients treated with CPH. Resistance to CPH occurs due to

decreased activation by CYP3A4 and CYP2B6, increased deactivation of the agents, decreased entry into or increased efflux from tumor cells, increased cellular thiol level, increased DNA repair capacity, and/or deficient apoptotic response to DNA damage<sup>11,2</sup>. A full understanding of factors affecting the pharmacokinetics, pharmacodynamics, toxicology and pharmacogenetics of CPH is important to optimize the dose and regimens of CPH in cancer chemotherapy.

CPH concentration in plasma and other biological fluids is determined to study its pharmacokinetics and also to predict and prevent its toxicity when administered in high dose intravenous infusion of the drug. CPH disposition has been described by other investigators employing <sup>14</sup>C-Cyclophosphamide<sup>5,9</sup>, bioassays<sup>7,10</sup>, alkylating activity<sup>5</sup> gas chromatography<sup>9</sup>. The first 3 assay techniques are non specific

making the results of these studies difficult to interpret. The last 3 techniques have greater specificity but are cumbersome, technically complex and prone unsatisfactory reproducibility between laboratories. The high pressure liquid chromatography techniques which have developed is a simple sensitive highly specific and rapid assay method for CPH with a high degree of reproducibility.

The aim of our study is to describe a simple, fast, accurate and precise method for the determination of cyclophosphamide in serum for pharmacokinetic studies and routine therapeutic drug monitoring in high-dose intravenous infusion of this drug.

## MATERIALS & METHODS

### Chemicals

CPH (>98.5%purity for HPLC) and P-aminoacetophenone were purchased from Sigma Aldrich (USA). Methanol and acetonitrile (Merck, Germany), and ethyl acetate were of HPLC grade and were obtained from Merck Laboratory Supplies (Germany). Distilled and deionized water was obtained by passage through ELGA® (a trade name of Vivendi Water Systems Ltd., Wycombe, Bucks, UK) Stock solutions were prepared by dissolving the compounds in water. The standard solutions were prepared every day.

### Chromatographic conditions

A Shimadzu liquid chromatography system equipped with a LT 10AT VP pump, a SPD 10A VP variable wavelength UV visible spectrophotometric detector and a Rheodyne 20 microliter loop injector system was used (Shimadzu, Kyoto, Japan). INERTSIL ODS-3V C-18, 4.6x250mm [GL sciences Inc, Japan] chromatography column was used for analysis. The mobile phase consisted of water acetonitrile, with the ratio of 70:30 respectively. The flow rate was 1.2ml/minute and the eluent were

monitored spectrophotometrically at 197 nm at room temperature.

### Solutions of external and internal standards

A stock standard solution of CPH (10000µM/ml) was prepared using distilled water. Stock solutions of internal standard (p-aminoacetophenone) were also prepared in the methanol at the concentrations of 5µg/ml. Further dilution of stock solution of cyclophosphamide was done with drug free plasma to prepare different concentrations of cyclophosphamide (5, 10, 30, 50 and 70µM/ml).

### Sample collection and preparation

Received cyclophosphamide at doses of 600mg/m<sup>2</sup> as small infusions, as part of protocols for the treatment of various cancer diseases at MGM Hospital, Warangal, India. Blood samples were collected at various time points after the end of each infusion. To each 200µL of patient's or standard sample, 10µL stock solutions of 5µg/mL internal standard was added to samples with cyclophosphamide concentrations. After complete mixing of samples with internal standard, 5 ml of ethyl acetate .10 µL of NaOH and vortex mixed for 2 minutes, then centrifuged at 3000 rpm for 15 minutes. 20µL aliquots of the supernatant were directly injected into the chromatography column. Each sample was analyzed in duplicate. All samples or standard solutions were stored at -80°C until analyzed.

### Recovery and precision

The recovery was studied by preparation of the various amounts of cyclophosphamide in blank serum (spiked blank). Cyclophosphamide was determined according to the described method. The recovery was calculated by comparison of the found amounts with the added ones. The reproducibility (precision) of method within a day was 2.6 to 6 percent and between days was 5.6 to 8.5 percent. The

recovery was between 98.8 and 97.01 percent.

## RESULTS

Under the conditions used for the chromatography, the retention times for CPH and the internal standard were  $5.6 \pm 0.03$  and  $6.5 \pm 0.98$  minutes, respectively. FIG. 2 shows the chromatograms of human blank serum used for the preparation of different concentrations of CPH standard solution. The chromatographic condition employed

serum samples containing methotrexate and analyzed by chromatography.

According to the previous reports, the major and most important metabolite of CPH has identified but its pharmacological effect is not significant<sup>6,7,9</sup>. We did not have any pure standard of this metabolite and unable to obtain it from elsewhere, therefore it was not possible to identify its retention time positively and to quantitate its concentration in serum.

In order to determine serum concentration of CPH, internal standardization method was used. After preparation of various

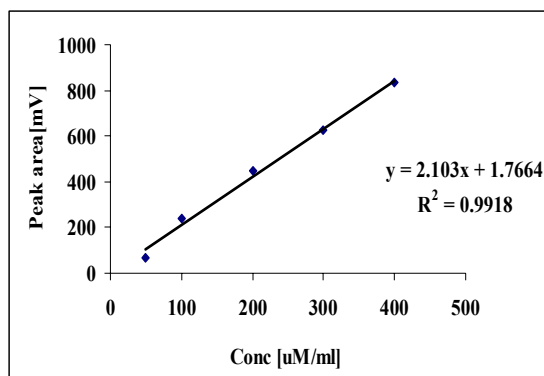
Spiked concentration ( $\mu\text{m}/\text{ml}$ )	Day	Measured concentration		
		Mean( $\mu\text{m}/\text{ml}$ )	Sd	RSd
<b>Interday Variation</b>				
50	1	49.13	0.376	0.765317
	2	49.18	0.100	0.203335
	3	49.54	0.096	0.193783
	4	49.79	0.030	0.060253
200	1	198.98	0.650	0.326666
	2	199.31	0.120	0.060208
	3	199.04	0.050	0.025121
	4	199.40	0.960	0.481444
400	1	398.99	0.960	0.240608
	2	399.05	0.069	0.017291
	3	398.75	0.870	0.218182
	4	398.65	0.050	0.012542
<b>Intraday variation</b>				
50		49.90	49.8	49.7
200		199.80	199.56	197.98
400		399.81	398.79	399.56

**Table 1. Inter and intra-day precision of determination of Cyclophosphamide in human serum**

was quite specific for CPH and p-aminoacetophenone. FIG. 1 shows the standard graph of CPH, FIG. 3 and 4 shows the standard and patient serum chromatograms.

Other drugs that might be administered concomitantly with Cyclophosphamide such as methotrexate, 5-fluorouracil, dexamethasone, ondansetran could not interfere with CPH Peaks because either they have no significant absorption at 197 nm or their retention times are quite different. This fact was proved in this study by adding each of these drugs to

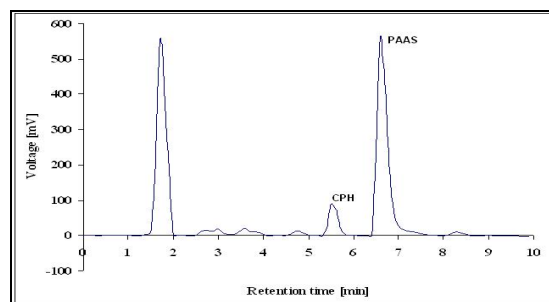
concentrations of CPH and analyzing chromatography each standard solution, two standard curves were prepared by plotting the ratio of peak area of CPH to internal standard (p-aminoacetophenone) versus concentration of CPH. A good linearity was seen for both the standard curves (FIG. 1). To assess the accuracy of the method, recovery of Cyclophosphamide from plasma samples with known concentrations was compared with the solutions of at the same concentrations as shown in Table 1.



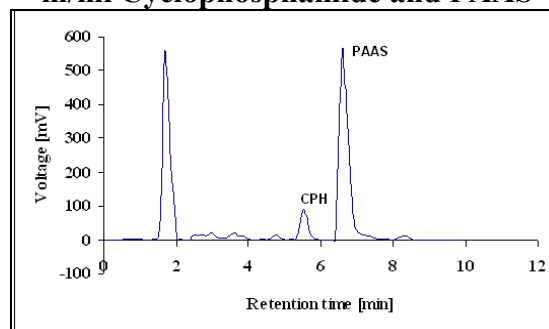
**FIG 1. Standard graph of cyclophosphamide in serum**

For the assessment of method precision, reproducibility of the results obtained for different concentrations of CPH was determined at 5 different days and 5 times in one day. The results of reproducibility study are shown in Table 2 as recovery and accuracy determination.

The limit of quantitation of CPH in plasma with the above sample pretreatment method was  $0.1\mu\text{M}$ .



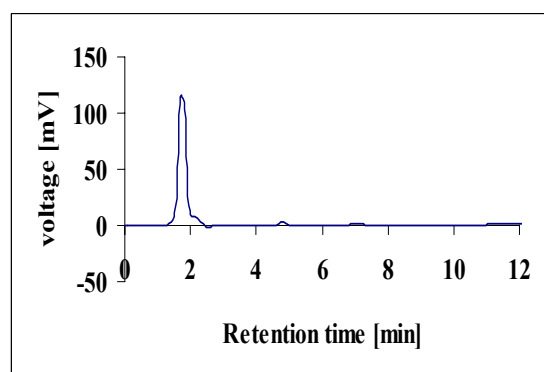
**FIG 3 . Serum sample spiked with  $10\mu\text{m/ml}$  Cyclophosphamide and PAAS**



**FIG 4. Cyclophosphamide and its PAAS in patient serum sample, at 10 min after 4h infusion**

Substance	Concentration	Recovery (%) Mean±sd	Accuracy (%) Mean±sd
CPH	50( $\mu\text{M}$ )	98.82±0.62	99.88±0.5
CPH	200( $\mu\text{M}$ )	99.59±0.1	100.05±0.08
CPH	400( $\mu\text{M}$ )	99.71±0.04	100.01±0.04
Internal Standard	50( $\mu\text{g/ml}$ )	97.01±0.02	95.69±1

**Table 2. Absolute recovery and accuracy of determination of Cyclophosphamide in human serum**



**FIG 2. Human Blank Serum**

## DISCUSSION

Various methods of high-performance liquid chromatography for the determination of CPH in biological fluids have been described so far which differ in chromatography type (reverse phase or ion-pair chromatography) or detection system (UV or fluorescence). Reverse-phase high performance liquid chromatography with UV detection has been most recommended<sup>6,8</sup>. But, an

important point is that most of them are tedious and expensive because they use more material and have many stages of experiment. Besides, they are not suitable for a routine and quick therapeutic drug monitoring (TDM) test which is necessary for a child cancer patient in a high dose infusion therapy at hospital, or reference laboratories.

In this article a simple and fast method for the determination of CPH in serum is described that has equal precision and accuracy to other similar methods. A full chromatography takes 10 minutes. To include sample preparation time it may need 25 minutes for the whole of each analysis, which is comparatively a short time. The short duration of assay time is of quite important in routine monitoring of the drug in serum to predict and prevent future toxicity in high-dose of CPH intravenous infusion. On the other hand this method has a satisfactory quantitation limit that makes it ideal for pharmacokinetic studies and therapeutic drug monitoring of CPH after the administration of high doses of this drug. To improve the quantitation limit further we could use solid phase extraction technique along with fluorescence detection after post column derivitization of the CPH compounds. So that the method become suitable for determination of CPH ratio of lower doses of the drug but such methods are more tedious, time consuming and expensive.

## CONCLUSION

A one step extraction procedure for CPH from serum and an improved method for determination of CPH are reported. Compared to previously published methods, the suggested extraction procedure is considerably more simple, rapid, reliable and sensitive. The HPLC technique based on UV detection is suitable for determination of small amounts of CPH with good accuracy and

reproducibility. Simple sample preparation procedure and a relatively short chromatographic time make this method suitable for processing of multiple samples in a limited amount of time for pharmacokinetic studies

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