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Colorimetric determination of cyclophosphamide and ifosphamide

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Abstract: A simple colorimetric method for the determination of cyclophosphamide and ifosphamide in pure and in dosage forms is suggested. It depends on the reaction of the secondary amino group in both, with nitrous acid, thus forming a nitroso derivative which can be measured at 325 nm for cyclophosphamide and 335 nm for ifosphamide. Beer's law is obeyed with concentrations from 20 to 90 μg ml^{-1} for cyclophosphamide and from 5 to 100 μg ml^{-1} for ifosphamide, with repeatability of 99.83 ± 0.256% and 99.72 ± 0.649%, respectively. Application to different pharmaceutical preparations has shown no significant difference when compared with the B.P. 1988 method.

Keywords: *Cyclophosphamide; ifosphamide; colorimetry; nitrosation.*

Introduction

Cyclophosphamide has been used clinically as a useful anticancer drug for more than 30 years, and the discovery of new drugs that might replace it appears unlikely to occur in the near future [1]. Ifosphamide is an investigational anticancer drug isomeric with cyclophosphamide [2].

A variety of methods have been used to determine cyclophosphamide, ifosphamide and their metabolites. Original studies used nitrobenzylpyridine for measuring the alkylating activity of cyclophosphamide [3]. Other methods were infra red spectroscopy [4], nuclear magnetic resonance spectroscopy [5], mass spectroscopy [6], UV spectrophotometry and polarography [7] fluorimetry [8] and flow injection [9]. More recently, high-performance liquid chromatography [10–15], thin layer chromatography [16, 17], and gas chromatography [18, 19] have been recommended. Colorimetric methods are based on the measurement of the intensity of its cobalt–thiocyanate ternary complex [20], and on the colour produced with nicotinic acid and benzidine [21]. Analyses depending on determination of its chloride content [22] or its phosphorous content [23–26] have been also reported.

In the present study, a colorimetric method is described based on measurement of the nitroso derivative of cyclophosphamide or ifosphamide. This method is simple, accurate and useful for the determination of many samples in parallel. In addition, it needs no preliminary treatment, such as hydrolysis or ashing and needs no sophisticated or costly instruments.

Experimental

Apparatus

UV and visible Beckman Spectrophotometer, Vacuum Rota Vapour.

Reagents

All chemicals were of analytical grade and were used without further purification: 5% v/v hydrochloric acid; 10% v/v hydrochloric acid; 20% w/v sodium nitrite; 20% w/v sodium hydroxide; 15% w/v sulphamic acid.

Reference samples of cyclophosphamide and ifosphamide were purchased from Asta Pharma AG company and assayed by the B.P. (1988) method to contain 99.9 ± 1% and 99.6 ± 0.9% respectively, of $C_7H_{15}N_2O_2Cl_2P$.

Procedures

Bulk powder

For cyclophosphamide. In a 20-ml test tube, weigh accurately, 2–9 mg of the powder and dissolve in about 1 ml of water. Add 1 ml of

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5% hydrochloric acid followed by 1 ml of 20% sodium nitrite and insert in a water bath at 60–65°C for about 20 min. Cool under tap water, then add, dropwise, 5 ml of 15% sulphamic acid while stirring, and cool under tap water. Add 2 ml of 20% sodium hydroxide. Transfer the resultant solution into a 100-ml volumetric flask and complete to the mark with water. Determine the absorbance of the resultant yellow solution at 325 nm against a reagent blank. Calculate the concentration of cyclophosphamide from a calibration curve (Fig. 1a) prepared from 20 to 90 μg ml^{-1}, or from the regression equation $A = 0.0125C$; where A = absorbance and C = concentration in μg ml^{-1}.

For ifosphamide. Apply the above procedure, but using 10% instead of 5% hydrochloric acid, allowing the reaction to proceed for 40 min instead of 20 min, heating the reaction mixture in a water bath at 70–75°C, and measuring the absorbance of the yellow solution at 335 nm against a reagent blank.

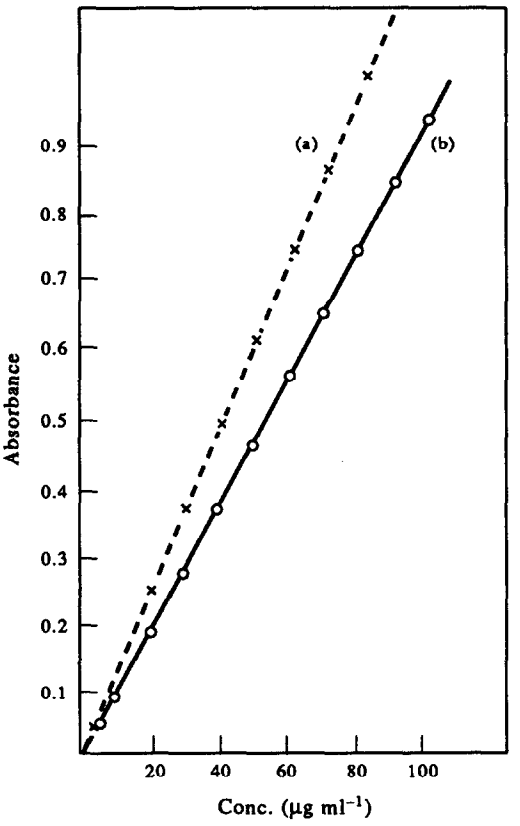

Figure 1
Linearities of the absorbances at 325 and 335 nm to the concentrations of authentic samples of cyclophosphamide (a) and ifosphamide (b) respectively.

Calculate the concentration of ifosphamide from a calibration curve (Fig. 1b) prepared from 5 to 100 μg ml^{-1}, or from the regression equation $A = 0.0095\ C$; where A = absorbance and C = concentration in μg ml^{-1}.

Pharmaceutical formulations

Endoxan injection. Mix and weigh the contents of 10 vials and calculate the average weight of one vial. Transfer a weight of the mixed powder equivalent to about 100 mg of cyclophosphamide, dissolve in about 5 ml of water, and dilute to volume in a 10-ml volumetric flask with water. Take an aliquot volume of the solution corresponding to 2–9 mg of cyclophosphamide and continue as for bulk powder procedures above, from "Add 1 ml of 5% hydrochloric acid . . .".

Endoxan tablet. Weigh and powder 20 tablets and calculate the average weight of one tablet. Transfer a quantity of the powder equivalent to about 100 mg of cyclophosphamide, accurately weighed, into a 100-ml volumetric flask. Add 70 ml of chloroform, shake vigorously for 15 min, then add sufficient chloroform to produce 100 ml. Filter through a dry filter paper, remove the chloroform at a temperature not exceeding 40°C, in a rotary evaporator, and dissolve the residue in about 10 ml of water, then dilute to volume with water in a 10-ml volumetric flask. Take an aliquot volume of the aqueous solution corresponding to about 2–9 mg of cyclophosphamide and continue as for bulk powder procedures above, from "Add 1 ml 5% hydrochloric acid . . .".

Holoxan injection. Mix and weigh the contents of 10 vials and calculate the average weight of one vial. Transfer a weight corresponding to about 100 mg of ifosphamide and dissolve in about 10 ml of water, then dilute to volume with water in a 10-ml volumetric flask. Measure an aliquot volume of the solution corresponding to 0.5–10 mg of ifosphamide and continue the procedure as before.

Results and Discussion

It has been found that the nitroso derivative of cyclophosphamide absorbs maximally at 325 nm, while that of ifosphamide absorbs at 335 nm (Fig. 2). The yellow colour produced upon nitrosation is thus suggested for the

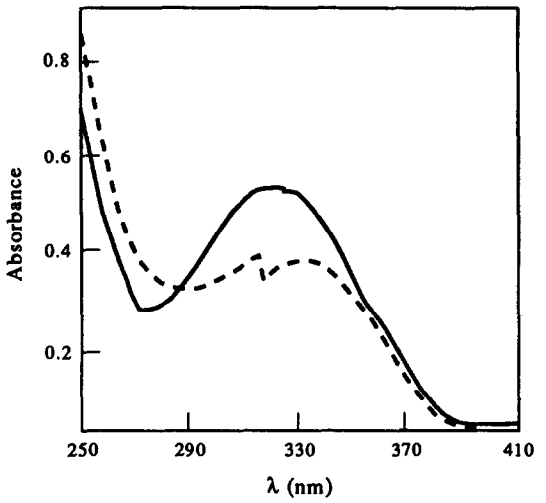

Figure 2
Absorption curves of nitroso cyclophosphamide (——) and nitroso ifosphamide (---); each 40 μg ml^{-1}.

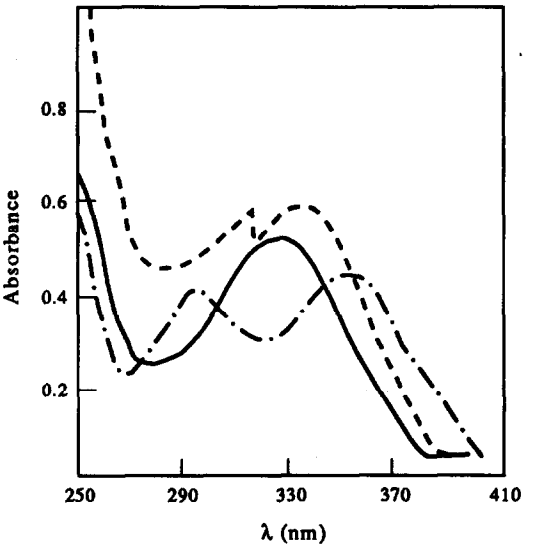

Figure 3
Absorption spectrum of nitroso cyclophosphamide (——) and nitroso ifosphamide (. . .), and reagent blank without sulphamic acid (-·-·).

colorimetric determination of the two compounds. Attempting to obtain the most intense and stable colour, the variables of the experimental conditions were critically investigated as follows.

Effect of sulphamic acid

It was necessary to get rid of the residual blank reagent prior to measurement of the yellow nitroso-derivative. Sodium nitrite, under the same experimental conditions and in the absence of cyclophosphamide or ifosphamide, shows, with water as a blank, a spectrum possessing two maxima, one at about 352 nm and another at 298 nm; the former maximum thus overlapping with the spectrum of the nitroso derivatives of cyclophosphamide or ifosphamide at 325 nm or 335 nm, respectively (Fig. 3).

Some authors [7] reported measurement of the nitroso derivative of cyclophosphamide at about 350 nm against a reagent blank. According to the present investigation, it is likely that these authors were measuring the residual sodium nitrite at 350 nm. Thus, if the amount of sodium nitrite added in the blank was exactly the same as that of the experiment, the absorbance of sodium nitrite at 350 nm in the blank would be higher than of the experiment and the difference would correspond to cyclophosphamide; the authors would not be measuring the nitroso derivative itself. Addition of 5 ml of 15% sulphamic acid, after complete nitrosation was found suitable to

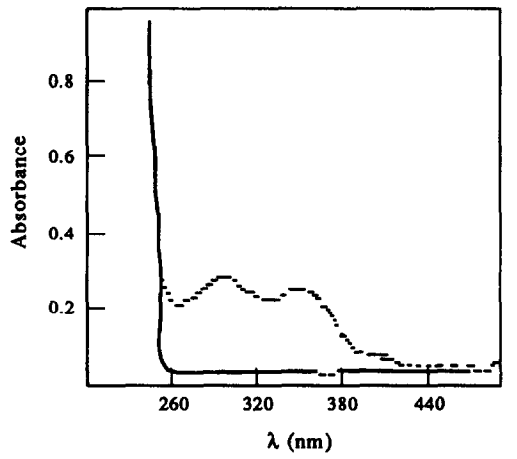

Figure 4
Reagent blank, without addition of sulphamic acid (---), and after addition of sulphamic acid (——).

obtain a reagent blank with a plain spectrum between 260 and 470 nm (Fig. 4).

Effect of temperature and heating time

The reaction was found to proceed very slowly at room temperature. However, raising the temperature of the reaction mixture was found to accelerate the process of nitrosation. A temperature of 60–65°C for 20 min and 70–75°C for 40 min in a water bath were found optimal for cyclophosphamide and ifosphamide, respectively. Increasing either the temperature or the time over the optimal led to a gradual decrease in the maximum absorbance

of the nitroso-derivatives. Furthermore, heating above 90°C led to disappearance of the yellow colour and consequently, of the peaks of maximum absorbance (Fig. 5). The only way accurate results can be achieved is by treating samples in exactly the same manner, heating them for the same time at the same temperature.

Effect of acid strength

The acidity of the reaction mixture was found to affect colour production. Final concentrations of 0.05% and of 0.1% hydrochloric acid (v/v) were the best concentrations for cyclophosphamide and ifosphamide, respectively. Higher concentrations of hydrochloric acid were found to distort the absorbance curve (Fig. 6). The resultant yellow colour was stable up to 40 min after which it started to fade (Fig. 7).

The observed differences in reaction parameters proposed for cyclophosphamide and ifosphamide are due to the secondary amino group in ifosphamide which is more hindered than that in cyclophosphamide. While the amino group of cyclophosphamide is typical having an available lone pair of electrons, the amino group of ifosphamide is associated with the P = O group which attracts the nitrogen lone pair of electrons, thus behaving more like an amino group.

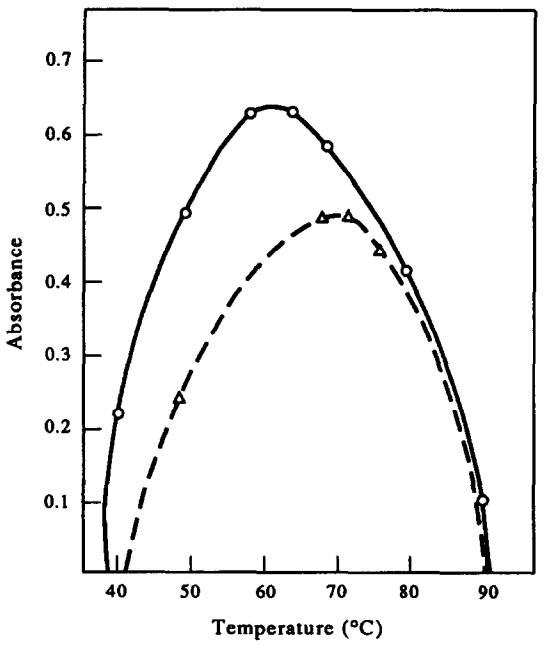

Figure 5
Effect of temperature on nitrosation of cyclophosphamide (——) and ifosphamide (---).

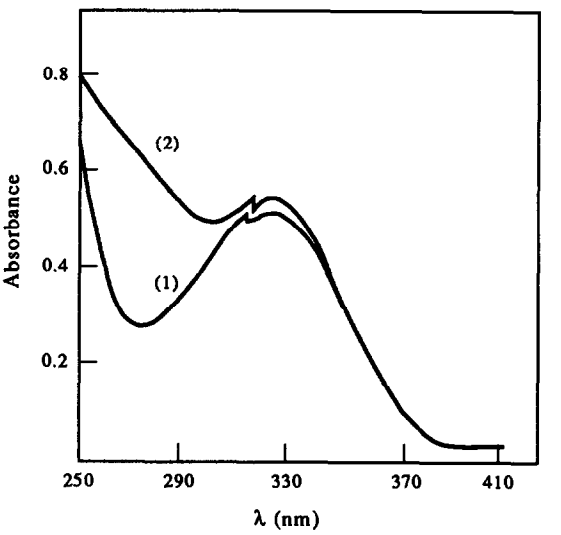

Figure 6
Absorption spectrum of nitroso cyclophosphamide, in 0.05% hydrochloric acid (1) and in 1% hydrochloric acid (2).

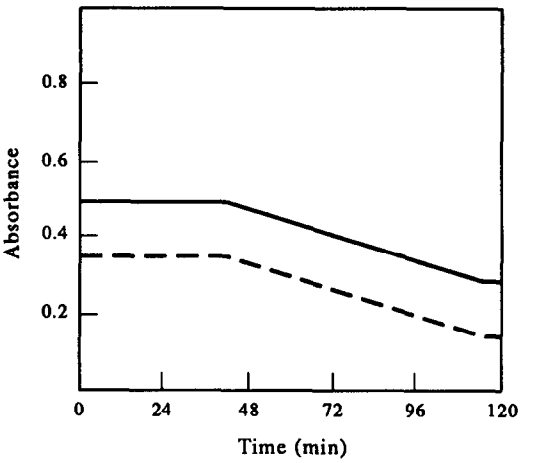

Figure 7
Stability of the colour of nitroso cyclophosphamide (——) and nitroso ifosphamide (---); each in a concentration of 40 μg ml^{-1}.

For application of the proposed method to Endoxan tablet, it was found essential to separate the active ingredient cyclophosphamide from the other additives present in the tablet; extraction with chloroform [26] or with ether [27] were reported to be the most suitable.

Applying the proposed procedure to 20–90 μg ml^{-1} of a reference sample of cyclophosphamide, and to 5–100 μg ml^{-1} of a reference sample of ifosphamide, the procedure proved to have an accuracy of 99.83 ± 0.256% for cyclophosphamide and 98.72 ± 0.649% for ifosphamide (Table 1).

Table 1
Determination of cyclophosphamide and ifosphamide by the proposed nitrosation procedure as applied to reference samples

	Cyclophosphamide		Ifosphamide	
Amount taken ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Accuracy (%)	Found ($\mu g\ ml^{-1}$)	Accuracy (%)
5	—	—	4.9	98.00
10	—	—	10.0	100.00
20	19.9	99.50	20.1	100.50
30	29.9	99.67	29.9	99.67
40	39.8	99.50	39.7	99.25
50	50.0	100.00	49.9	99.80
60	60.0	100.00	60.0	100.00
70	69.9	99.86	70.0	100.00
80	79.9	99.88	79.8	99.75
90	90.2	100.22	90.0	100.00
100	—	—	100.0	100.00
Mean		99.83		99.72
RSD		0.256		0.649

* Relative standard deviation (coefficient of variation).

Table 2
Determination of cyclophosphamide and ifosphamide in pharmaceutical formulations by the suggested nitrosation procedure, and assessing the accuracy of the results by standard addition

	Found		Control experiments	
Pharmaceutical preparations*	(mg)	(%)	Standard added	Recovery of added standard (%)
Endoxan vial 200 mg	199.0	99.5	3	100.18
Cyclophosphamide	199.5	99.8	4	100.02
anhydrous per vial (Batch 1)	199.8	99.9	5	100.02
Mean		99.7		100.07
RSD		±0.21		0.092
Endoxan vial 200 mg	197.3	98.6	3	99.97
Cyclophosphamide	198.0	99.0	4	99.98
anhydrous per vial (Batch 2)	198.2	99.1	5	100.10
Mean		98.9		100.01
RSD		±0.27		±0.072
Endoxan tablet 50 mg	49.6	99.2	3	100.05
Cyclophsophamide	49.2	98.4	4	100.02
anhydrous per tablet (Batch 1)	50.3	100.6	5	99.88
Mean		99.4		99.98
RSD		±1.12		±0.091
Endoxan tablet 50 mg	48.5	97.0	3	100.11
Cyclophosphamide	48.7	97.4	4	100.71
anhydrous per tablet (Batch 2)	49.1	98.2	5	100.03
Mean		97.5		100.28
RSD		±0.63		±0.371
Holoxan vial 2 g	2071.3	103.57	3	102.8
ifosphamide per vial (Batch 1)	1996.1	99.81	4	100.3
	2028.5	101.43	5	97.8
Mean		101.6		100.3
RSD		±1.27		±2.49
Holoxan vial 2 g	2030.1	101.51	3	100.3
ifosphamide per vial (Batch 2)	1979.6	98.98	4	100.6
	2000.1	100.01	5	101.8
Mean		100.17		100.9
RSD		±1.270		±0.787

* All produced by Asta Pharma AG Company.

Table 3
Statistical comparison between the results of the proposed method and those of the pharmacopoeial method, for determination of cyclophosphamide and ifosphamide

	Cyclophosphamide		Ifosphamide	
	Proposed method	Pharmacopoeial method	Proposed method	Pharmacopoeial method
Mean	99.83	99.9	99.72	99.6
±SD	0.3	1%	0.6	0.9%
n	8	5	11	5
Student's *t* (2.167)*	0.067	—	0.11	—
F.L.†	99.83 ± 0.07	—	99.6 ± 0.02	—

*Figure between parentheses represents theoretical figure.
†Fiducial limits (confidence intervals).

The procedures were applied to the determination of some pharmaceutical formulations of cyclophosphamide and ifosphamide and the results are given in Table 2. Control experiments were performed to evaluate the accuracy of the procedure when applied to the pharmaceutical formulations, by adding known concentrations of cyclophosphamide or ifosphamide to a suitable aliquot of the pharmaceutical preparation and the recovery of the added cyclophosphamide or ifsophamide was computed. The mean recoveries for pharmaceutical preparations proved to be almost the same as for the pure reference samples; indicating that common additives do not interfere with the suggested procedure. A statistical comparison between the results of the proposed method and those of the pharmacopoeial method for determination of cyclophosphamide and ifosphamide is given in Table 3.

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