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Short communication

Quality control and stability study using HPTLC: applications to cyclophosphamide in various pharmaceutical products

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Abstract

Cyclophosphamide is an alkylating agent widely used from cancer chemotherapy to immunotherapy purposes. In paediatrics oncology, oral cyclophosphamide prescribed at low dosages for a long time treatment is currently investigated. This treatment is a putative well tolerated regimen for children treated for a wide variety of recurrent solid tumours. For these purposes, new oral formulations more convenient for children than cyclophosphamide 50 mg tablets are needed. Thus, we present a rapid method for the assay of cyclophosphamide in various pharmaceutical preparations using high-performance thin-layer chromatography (HPTLC) and derivatization with phosphomolybdic acid. This method is accurate and precise and allows quantitation of cyclophosphamide in aqueous solutions from 400 to 1200 μg/mL. It is suitable for quantitation and stability studies of cyclophosphamide in pharmaceutical products, i.e. capsules and infusion bags prepared in a hospital pharmacy. According to pharmaceutical guidelines, we demonstrated that low dose cyclophosphamide capsules, extemporaneously prepared for children, are stable at least for 70 days.

Keywords: Cyclophosphamide; HPTLC; Stability study; Quality control

1. Introduction

Cyclophosphamide ($C_7H_{15}C_{l2}N_2O_2P{\cdot}H_20$, CAS 6055-19-2, CPM) is used in a wide variety of therapeutic indications from cancer to inflammatory and immune diseases. This drug is registered to both European [1] and US pharmacopoeia [2]. CPM is an inactive drug and its activity is provided through the metabolism by cytochromes P450 [3] leading to the formation of a phosphoramide mustard, the active compound. CPM is administered at doses ranging from 20 mg/m^2 to 1.2 g/m^2. CPM is mostly administered by intravenous route because this formulation allows avoiding variability due to oral route. Oral use is usually preferred when protracted daily administration is considered. In hematology, CPM is a component of high dose chemotherapy regimens to prepare allogeneic hematopoietic stem cell transplantation [4]. At conventional doses, CPM is a major drug for the treatment of leukemias, especially in children, and non-Hodgkin lymphoma. This drug is used at several dosages for the treatment of a variety of solid malignancies such as breast cancer [5], in adults as well as in children. Mesna (UROMITEXAN®) is a drug available to reduce the vesical toxicity due to the acrolein metabolite of CPM given at high doses.

An oral formulation of CPM has been developed a long time ago; which is currently indicated for a limited number of therapeutics applications, such as autoimmunes diseases [6].

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Oncology is one of the few areas of medicine where most patients are treated intravenously rather than receiving oral drugs. Oral chemotherapy is attractive because of its convenience and ease of administration at home [7]. In paediatrics oncology, oral CPM is currently investigated for its putative efficiency as a well tolerated regimen against a wide variety of recurrent solid tumours [8]. The availability of an oral formulation could make possible to use it during a long lasting low dose maintenance treatment (25 mg/m^2 daily). For this purpose, a new paediatric formulation more convenient than CPM tablets set at 50 mg is needed for the oral route. When considering the development of a new formulation it is necessary to perform a validated analytical method. Thus, we developed a rapid and suitable analytical method for identifying and quantifying CPM in pharmaceutical preparations, i.e. infusions bags or capsules. Analytical methods to assay CPM in pharmaceutical products already exist [9,10] and most of these methods are HPLC ones [11,12] such as the standard USP method [2]; moreover most available methods were performed for pharmacokinetics and not for pharmaceutical purpose [13]. CPM and metabolites pharmacokinetics were determined in children [14] using a sensitive high-performance thin-layer chromatography (HPTLC) including derivatization with 4-nitrobenzylpyridine; since it is a rapid method, we developed another HPTLC method to assay CPM not in biological samples but in various pharmaceutical products. This new method uses HPTLC before a derivatization procedure using phosphomolybdic acid. The range of concentration, set from 400 to 1200 μg/mL, is adequate for pharmaceutical applications with high concentrations of CPM in a simple medium composed of pharmaceutical adjuvants. This method was helpful to perform a stability study of CPM capsules at two low dosages, 10 and 25 mg. These capsules were developed for children receiving oral CPM at the daily dose of 25 mg/m^2 during 28 days. This work is part of the quality control program for pharmaceutical products prepared in the Department of Clinical Pharmacy at the Institut Gustave Roussy [15].

2. Materials and methods

2.1. Materials and chemicals

2.1.1. Chemicals and solvents

Cyclophosphamide monohydrate ENDOXAN® and mesna UROMITEXAN® were pharmaceutical products from Baxter Oncology (Maurepas, France). Phosphomolybdic acid was purchased from Sigma-Aldrich (Lyon, France). Acetic acid and ethanol were analysis grade from Carlo Erba (Rodano, Italy). Methanol and dichloromethane were HPLC grade from Carlo Erba (Rodano, Italy). Carmin, lactose and capsules n°4 were purchased from Cooper (Melun, France). Injection grade water, isotonic saline solution and 5% dextrose were purchased from Macopharma (Tourcoing, France).

2.1.2. HPTLC CAMAG® analytical station

HPTLC CAMAG® is an high-performance thin-layer chromatography platform provided by Camag (Muttenz, Switzerland). The informations relative on the HPTLC analytical platform were published previously [13,15].

2.1.3. Stationary phase

The stationary phases (Lichrospher® Si60 F254 nm), manufactured by Merck, were made of uniform 0.2 mm thin silica layers that were placed on a glass surface of 20 × 10 cm. The granulometry phase (2–10 μm) is guaranteed by the manufacturer.

2.2. Methods

2.2.1. Qualitative and quantitative HPTLC densitometric assay

2.2.1.1. Samples, standards and quality controls (QCs) preparation. Cyclophosphamide stock solutions (10 mg/mL) were prepared using commercially available cyclophosphamide 500 mg ENDOXAN® vials dissolved in 50 mL injection water, as specified by the manufacturer. This stock solution was diluted 1/5 in injectable water to obtain a 2 mg/mL working solution. Cyclophosphamide standards were made by diluting working solution in methanol/water (1:1). Standard concentrations were set at 400, 600, 800, 1000, 1200 μg/mL. To validate the method, three QCs were prepared at 500, 700 and 1100 μg/mL from another stock solution, according to the same procedure. For routine purpose, only the lower and upper QCs are used (QC_L and QC_H). All these solutions were transposed in glass snap-ring clipped vials which were arranged on the autosampler's rack.

2.2.1.2. Sample application. Automated TLC sampler III® devices take into account defined parameters such as the volume, size of the sprayed band and accurate positioning on the chromatography plate. These parameters were computerized by ATS III® software. The system is washed with an isopropanol/methanol/water mixture (33:33:33, v/v/v) between each deposition. Two microliters of solution are sprayed onto the plate to form 2 mm width and 4 mm long bands, 6 mm apart. Bands were sprayed at 15 mm from the left edge and the delivery speed was 200 nL/s. During the same run, it is possible to assay five standards, two QCs and 24 samples placed within the QCs, i.e. low and high QCs.

2.2.1.3. Mobile phase and migration. Chromatographic development was standardized with a 5 min horizontal sandwich migration method, using a dichloromethane/methanol/acetic acid mixture (97:3:2, v/v/v).

2.2.1.4. Derivatization procedure. After chromatographic development, the HPTLC plate was dried with a hair dryer. Derivatization was performed by automated soaking of the plate in a phosphomolybdic acid ethanolic solution (1.25%,

w/v). After drying, the HPTLC plate was heated at a temperature of 190 °C for 10 min on a TLC plate heater. Previously published HPTLC method used 4-nitrobenzylpyridine for derivatization of busulfan and a triethylamine/ethanol solution for revelation [16]. Cyclophosphamide was used as an internal standard because of the unstable nature of the chromophore as shown previously by Tasso et al. [14]. A new method for cyclophosphamide derivatization was developed using a one step procedure without the need of an internal standard.

2.2.1.5. Densitometric analysis. The plate was analysed on a TLC scanner 3® densitometer driven by the CATS 4® software (4.05 version) as previously reported [13,15,16]. The densitometric measurement was performed at the maximum of absorbance of the derivatized product wavelength, i.e. 700 nm, as shown in Fig. 1.

2.2.1.6. Identification and quantitation procedure. In TLC, each compound can be characterized with its retention factor (R_f) which is the ratio between substance migration and solvent migration distances. With this parameter, cyclophosphamide can be qualitatively assayed in pharmaceutical preparations. The cyclophosphamide peak surface was automatically measured for each sample, QCs or standards. QCs and samples were calculated according to their response and the calibration curve equation.

2.2.2. Validation of the method

2.2.2.1. Selectivity. Cyclophosphamide was co-eluted with five products which were included in its pharmaceuticals forms, i.e. carmin, lactose, gelatin, dextrose and mesna.

2.2.2.2. Calibration. The calibration function, i.e. relationship between cyclophosphamide peak areas and its applied amount was determined by linear regression over a defined range from 400 to 1200 μg/mL. For routine use, each calibration curve is validated using two QCs prepared at 500 and 1100 μg/mL. Samples are placed on the rack within the QCs. Six calibration curves (tested with three QCs, i.e. 500, 700 and 1100 μg/mL) were done to ensure that the linear regression model was the most accurate for quantitation purposes.

2.2.2.3. Accuracy. Accuracy provides information about the recovery of the analyte from the sample through the analysis of in-system calibration of sample solutions of known substance content. The solutions were spiked with three different known concentrations of cyclophosphamide, i.e. 500, 700 and 1100 μg/mL, respectively. These low, medium and high QCs were analysed individually six times. Means and relative standard deviations (R.S.D.) were calculated.

2.2.2.4. Precision. In accordance with International Conference of Harmonisation guidelines (ICH Q2A and Q2B), precision includes three components: repeatability, intermediate precision and reproducibility. Here, reproducibility was not studied. We thus analysed repeatability and intermediate precision as we previously reported for other HPTLC analytical methods [13,16].

3. Results and discussion

3.1. Validation of the method

3.1.1. Selectivity, specificity

Cyclophosphamide is separated from the other chemical compounds detected by the process as shown in Fig. 2. The CPM retention factors was 0.55. For the infusion bag assay,

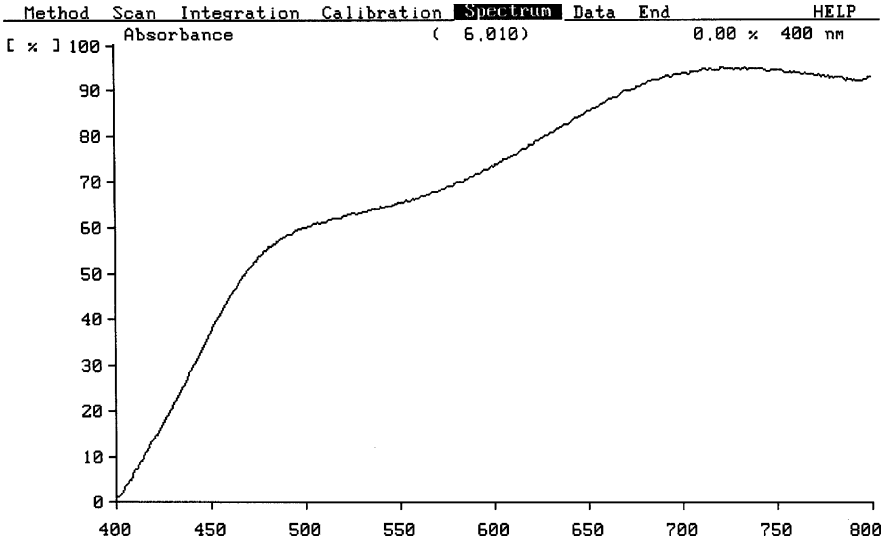

Fig. 1. Visible spectrum of derivatized cyclophosphamide. This figure shows a spectrum of cyclophosphamide after derivatization with phosphomolybdic acid. Absorbances (in % of the maximum value) are plotted on *Y*-axis while wavelength in nanometer from 400 to 800 nm are plotted on *X*-axis. Absorbance wavelength was set at 700 nm for quantitative purpose.

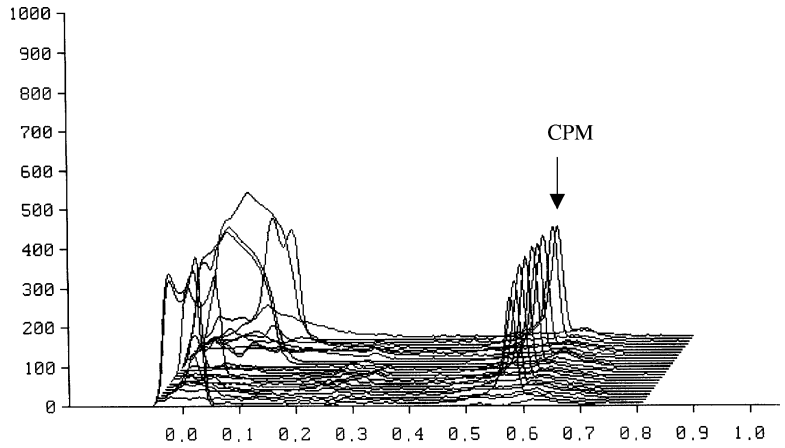

Fig. 2. Specificity – HPTLC analysis of CPM with various chemical adjuvants. CPM was mixed with lactose, carmin, gelatin, sodium chloride, dextrose and mesna. These mixtures were thus analysed by HPTLC. This figure shows the superposition of 26 chromatograms with adjuvants alone or mixed with cyclophosphamide after chromatographic development, derivatization procedure and densitometric analysis (700 nm). Intensity (arbitrary unit) are plotted on *Y*-axis and R_f are plotted on *X*-axis.

we verified that there was no analytical interference with dextrose, sodium chloride and mesna. For the capsules, the lack of interference with lactose, gelatin and carmin was checked. All adjuvants tested presented R_f very close to 0 and thus there were no analytical interference with CPM (Fig. 2).

3.1.2. Calibration

The calibration function was determined by linear regression over 400–1200 μg/mL, with a $r^2 = 0.9921$ (six determinations). The slope of the mean equation was $\beta = 0.1547 \pm 0.0080$ (mean ± S.D.) and the *Y*-intercept was $\alpha = 29.62 \pm 6.76$ (mean ± S.D.). Thus, the mean equation according to linear regression was $Y = 0.155X + 29.62$; *Y* is the response (arbitrary unit) and *X* the concentration (μg/mL).

3.1.3. Accuracy

The results are summarized in Table 1. Means and relative standard deviation (R.S.D.) values were calculated from the six determinations of each QC (500, 700 and 1100 μg/mL). The method was considered accurate according to the values which were below 2.2%.

3.1.4. Precision

The CV values for repeatability (CV_r) and for intermediate precision (CV_i) are summarized in Table 2. For quality control of pharmaceutical products, the method can be considered precise enough since these values were below 6.3%.

Table 1
Results of the accuracy study

	Mean	R.S.D.	Bias (%)
QC_L 500 μg/mL	510.6	28.9	2.13
QC_M 700 μg/mL	698.9	16.7	−0.15
QC_H 1100 μg/mL	1101.0	45.5	0.1

Values calculated were based on six different measurements.

3.2. Application

3.2.1. Development of cyclophosphamide capsules

In our institution, a clinical trial with CPM 25 mg/m^2/day during 28 days is ongoing. Since previously reported stability studies of CPM solutions [11,12] had shown that the drug in water solution was stable at 4 °C no more than 14 days, we decided to develop dry forms of CPM. For each children, oral capsules set at 10 or 25 mg will be prepared extemporaneously. These oral formulations are prepared with cyclophosphamide monohydrate (ENDOXAN® 500 mg), lactose, carmin and gelatin capsules. To prepare capsules at the correct dosage given in mg of cyclophosphamide base, we have to take into account a conversion factor, i.e. 500 mg of cyclophosphamide base is corresponding to 534.5 mg of monohydrate cyclophosphamide (weighted powder).

3.2.1.1. Quality control – cyclophosphamide capsules. Mass uniformity of 20 randomized capsules in all batches was verified as specified by the European Pharmacopoeia (4th edition) [17]. To validate the manufacturing procedure, we checked the amount of CPM in capsules prepared as specified by the European Pharmacopoeia (4th edition) [18]. The results of such a test on capsules set at 10 mg are presented in Table 3. The calculated amounts were easily included in the acceptance criteria defined by the European Pharmacopoeia, i.e. no more than one capsule outside the 85–115% limit but within the 75–125% limit of the target value. To assay CPM

Table 2
Validation of the repeatability and intermediate precision study

	CV_r repeatability (%)	CV_i intermediate precision (%)
QC_L 500 μg/mL	5.7	5.6
QC_M 700 μg/mL	2.4	4.4
QC_H 1100 μg/mL	4.1	6.3

Values calculated were based on six different measurements.

Table 3
Dose in 10 CPM capsules with 10 mg as the theoretical value

Capsules	CPM (mg)	Amount μg/mL in water	Dilution factor	Results μg/mL	Bias %
1	10	1000	0.7	0.651	−7
2	10	1000	0.7	0.670	−4
3	10	1000	0.7	0.683	−2
4	10	1000	0.7	0.706	1
5	10	1000	0.7	0.715	2
6	10	1000	0.7	0.684	−2
7	10	1000	0.7	0.689	−2
8	10	1000	0.7	0.692	−1
9	10	1000	0.7	0.712	2
10	10	1000	0.7	0.681	−3

The powder content in each capsule is poured into 10 mL of a methanol/water mixture (1:1, v/v). This CPM solution is thus diluted in a hydroalcoholic solution.

content, capsules were opened and the capsule and its powdered content, i.e. monohydrate cyclophosphamide, lactose and carmin, were poured into 10 mL of methanol/water (1:1) mixture. This hydroalcoholic solution was correctly diluted to obtain a concentration between 400 and 1200 μg/mL. The sample was then assayed by HPTLC.

3.2.1.2. Stability study – cyclophosphamide capsules. Because the formulation was performed for patients who will receive the treatment during 28 days, we decided to perform a stability study by assaying the exact dose of CPM in capsules set at 10 and 25 mg during a 70 days period. Two batches of 50 capsules were manufactured for each dose. The dose was checked in six capsules of each batch at different times point along the study (day 0 and once a week up to 70 days). Results are presented in Fig. 3. During the study period, there was no evidence of degradation of CPM in the preparation. All capsules tested were fulfilling acceptance criteria defined by European Pharmacopoeia (4th edition) [17,18]. Thus, we are able to warrant the stability of these extemporaneous preparations during the treatment duration (28 days).

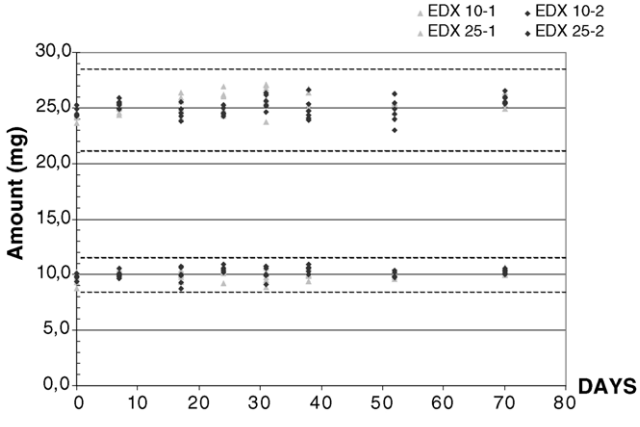

Fig. 3. Stability of CPM capsules set at 10 and 25 mg. Two batches of 50 capsules set at 10 mg (EDX 10-1 and EDX 10-2) and two batches of 50 capsules set at 25 mg (EDX 25-1 and EDX 25-2) were prepared. The exact amounts of CPM were measured at different time-points along a 70 days period. On this figure, calculated amounts of CPM are plotted on the *Y*-axis and days are plotted on the *X*-axis. Dash lines represent limit of acceptance for 10 and 25 mg capsules (±15%). All tested capsules were conforms and there was no trend of CPM degradation.

3.2.2. Quality control – infusion bags

We demonstrated that there was no analytical interference with sodium chloride, dextrose and mesna. Mesna is added to some infusion bags according to the manufacturer. Infusion bags contain cyclophosphamide from 1 to 15 g/L and thus our method is adequate to check the dose of cyclophosphamide in infusion bags after an appropriate diluting step. Since July 2001, the HPTLC platform has strongly contributed to operators training and involvement in the product process. It has resulted in reduction in the non-conformity rate. This quality management tool is a part of ISO 9001 certification procedure [15].

Acknowledgements

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