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Simultaneous Determination of Cyclophosphamide and Ifosfamide in Plasma Using SPE-HPLC-UV Method

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SUMMARY. A reproducible and selective method for the simultaneous determination of cyclophosphamide (CP) and ifosfamide (IF) in plasma has been developed and validated using isocratic elution. The assay is performed by HPLC-UV, with a C18 column (5μm, 150 x 4 mm) and detection in 195 nm. The mobile phase was constituted by phosphate buffer 10 mM pH 6.0: acetonitrile (77.25:22.75), with a flow of 1 mL/min. SPE was used for sample clean-up in a range from 3 (LOQ) to 540 μM. The repeatability coefficients of variation (CV) ranged from 0.5 to 7.8% and the intermediate precision CVs varied from 0.9 to 5.7%. Extraction efficacy and accuracy varied from 94 to 115%. The simple method may permit the determination of cyclophosphamide and ifosfamide in plasma, simultaneously, to pharmacokinetics and bioequivalence studies.

INTRODUCTION

The use of the so-called "chemotherapy" in the treatment of cancer has been constantly increasing over the last decades. Unfortunately, well-known are the side effects of antineoplastic drugs in cancer patients, due to the fact that these agents cannot distinguish between healthy cells and cancerous cells [1].

The optimal dose should give a maximal anti-tumour effect with acceptable levels of toxicity. In clinical practice, these drugs frequently cause toxic effects that would be considered unacceptable. Although toxicity is commonly regarded as the most important effect to control, partly because it is easier to measure, the risk of under-dosing and reduced efficacy must also be considered [2].

Cyclophosphamide (CP), 2-bis(2-chloroethyl) aminotetrahydro-2H-1,3,2-oxazophosphorine-2-oxide, is one of the most commonly used of the antineoplastic alkylating agents. It has also been used for its immunosuppressive properties in the treatment of autoimmune diseases as well as in renal and bone marrow transplants [1,3].

Ifosfamide (IF), 3-(2-chloroethyl)-2-[(2-chloro-ethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorin-2-oxide, is a structural analogue of cyclophosphamide (CP) having alkylating activity after enzymatic activation to cytotoxic compounds. IF differs from CP in the transfer of one 2-chloroethyl group from the extracyclic to endocyclic nitrogen. This results in differences in pharmacological and toxicological properties [1].

Both cyclophosphamide and ifosfamide are widely used in the treatment of non-Hodgkin lymphomas and a variety of bone and soft tissue sarcomas. In comparison with many other anticancer drugs, cyclophosphamide exhibits relatively little non-hematopoeitic toxicity. The pharmacological profile of these drugs is important and in order to achieve this information reliable methods for determination of oxazaphosphorines and their metabolites are necessary when determining optimal dosages [4].

Cyclophosphamide (CP) and ifosfamide (IF) are chemical analogues and therefore the analytical methods for their determination are similar.

KEY WORDS: Antineoplastic drugs; HPLC, Cyclophosphamide, Ifosfamide, Simultaneous determination.

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In the past 10 years these oxazaphosphorines have been determined by gas chromatography (GC) with nitrogen-phosphorus detector (NPD). The advantages of NPD are its high selectivity and sensitivity, a relatively large linear range therefore, it has been demonstrated that thermal decomposition of underivatized CP and IF occurs [5].

Determination of oxazaphosphorines in serum, blood or plasma has been demonstrated using HPLC with detection at low UV wavelengths [6-11]. The clean-up procedure plays an important role in determination of drugs in various biological materials. The choice of clean-up procedure depends on the material being extracted, *e.g.* plasma or urine, and the substances being determined. In this case the removal of interfering substances from the oxazaphosphorines is the main problem and the pH-value plays an important role [12]. Both drugs have similar properties, when pKa and solubility are considered [13].

In this paper, we presented an optimized and validated method for the quantitative simultaneous determination of cyclophosphamide and ifosfamide in human plasma using liquid chromatographic method, with a previous sample clean-up by solid phase extraction (SPE).

MATERIAL AND METHODS

Chemicals

Cyclophosphamide, Aldrich (purity > 99%), ifosfamide (Holoxan®) ASTA MEDICA. Stock solutions were prepared by dissolving the compounds in water. The standard solutions were prepared every day, stored in the dark and refrigerated. Acetonitrile, methanol, ethyl acetate (HPLC grade) were obtained from MALLINCRODT. Cartridges C18 (200 mg) were purchased from Supelco.

HPLC apparatus and conditions

The HPLC system consisted of a Shimadzu LC-10ATvp (Kyoto, Japan) gradient system equipped with a Shimadzu SIL-10AF (Kyoto, Japan) auto-injector with a 50 µL loop. The column oven used was a Shimadzu CTO-10ASvp (Kyoto, Japan) operated in 40 °C. The detection was, firstly, performed with a Shimadzu SPD-M10Avp (Kyoto, Japan) diode array detector. After that the wavelength was selected and the analysis was performed at 195 nm in a Shimadzu SPD-10Avp (Kyoto, Japan) UV detector. Separation was achieved by isocratic elution carried out with a mobile phase constituted of 10 mM phosphate buffer (pH 6)- acetonitrile (77.25:22.75) delivered at flow rate of 1.0 mL/min at ambient temperature through a Supelcosil™ LC-18 (150 mm x 4.6 mm, 5mm) protected by a similar guard-column (4 mm x 4.6 mm). Data acquisition and treatment was performed by a Class-VP software (Shimadzu).

Preparation of samples

Liquid-Liquid Extraction

Different mass of IF and CP were first added to blank plasma samples (1.0 mL), which were vortex-mixed for 1 min. Then, 2.0 mL of acetonitrile were added to the plasma and it was mixed for 30 seconds. After this, 1 mL of phosphate buffer (pH 7) and 5 mL of ethyl acetate, as an extracted solvent, were transferred into the tube. Samples were vortex-mixed for 5 min and then centrifuged (580 g, 10 min).

The organic layer (5.0 mL) was evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 µL mobile phase and 20 µL were injected into the HPLC system.

Solid Phase extraction

The cartridges C18 (200 mg- Supelco®) for solid phase extraction (SPE) were pretreated two times with 2 mL of methanol and 2 mL of water. After, 1 mL of plasma samples, spiked with different mass of IF and CP, containing 1 mL of phosphate buffer (pH 7) were transferred into the cartridge which was washed with 3 mL of phosphate buffer (pH 5). Then the analytes were eluted with 3 mL of ethyl acetate. After evaporation, to dryness under a gentle stream of nitrogen, the residue was reconstituted in 200 µL mobile phase and 20 µL were injected in the HPLC system.

Assay validation

Validation of this study was in compliance with a current FDA and IUPAC guidelines [14-16]. The following parameters were assayed: linearity, lower limit of quantification (LOQ), extraction efficacy, accuracy, precision and selectivity.

The selectivity of the analytical method was evaluated by analyzing drug-free blank plasma from six different samples. Since the two drugs quite close to each other antineoplastic drugs, possible interference, 5-fluoruracil, methotrexate, doxorrubicin and paclitaxel were evaluated.

Linearity was tested by examination of a plot of residuals produced by linear regression of the responses on the amounts of the analytes in a calibration set, between 3 to 540 µM, for both

analytes, in six replicates for each level. An analytical curve using the optimized method was obtained spiked plasma samples (n=3 for five levels) over the expected range.

LOQ was determined by comparing the measured signals of samples with known low concentrations of analytes to those of blank samples, ten lots, thereby establishing the minimum amount of compounds that could be reliably quantified, with a signal-to-noise ratio of 10:1. The LOQ was defined as the lowest concentration that could be determined with accuracy and precision below 20%.

Extraction efficacy was determined by five replicate analyses of samples after the addition (spiking) of a known mass of the drugs in the middle (72 µM for CP and IF) and high level (540 µM for CP and IF). The results were compared with those obtained when the analytes were spiked after the clean-up procedures of the sample. The accuracy was expressed as the ratio of the compounds added to the measured (mean value/ nominal value) x 100, using the middle and high level.

Precision was determined with five replicate analyses of samples containing known amounts of the analytes, using the LOQ, middle and high level, during a single analytical run (repeatability) and between-runs (intermediate precision). These test was expressed as relative standard deviation (R.S.D. %).

Stability of analytes was investigated for samples kept at auto-sampler and long-term. To perform the auto-sampler stability assay, the LOQ, middle and high level, post processed, were assessed after 12 hours in auto-sampler. The long-term stability was investigated for samples kept at -20 °C for 2 weeks. For all stability tests the samples analyses were compared with freshly prepared samples and the Student's t-test was applied.

Method application

To assess the applicability of the validated method, the IF and CP plasma levels were measured after administration of this drugs in rats. Male Wistar rats weighing 300 ± 20 g were kept under standardized conditions, according to institutional guidelines. Blood samples were collected 0.5 hour after oral administration of vehicle or IF plus CP at dose of 10 mg/kg (n= 5). The samples were centrifuged for 15 min at 2000 g and the plasma was stored at -20ºC until analyzed for IF and CP.

RESULTS AND DISCUSSION

Direct analysis of oxazaphosphorines by HPLC has been successfully carried out. The HPLC method appears to be most feasible for attaining the lower limit of detection and quantification when used for detection of multiple antineoplastics [9,17]. As a result of this information, this identification technique was selected for the method to be developed. However, the retention times of CP and IF are very close, making the quantification of these analytes difficult. We developed a fast and selective assay to accurately measure the compounds.

The HPLC behavior of CP and IF extracted from human plasma was isocratically examined using a C-18 analytical column. We have tested several mobile phases and found that 10 mM phosphate buffer, pH 6- acetonitrile (77.25: 22.75) in a flow rate of 1.0 mL/min gave a satisfactory separation of the compounds of interest. With these conditions was possible detected both analytes in a run time of 10 min, which can be considered an advantage for the routine application. The selected chromatographic conditions provided optimum resolution of IF and CP which eluted at approximately 7.0 and 7.9 min, respectively.

In the literature, a number of different UV wavelengths have been used for detection of cyclophosphamide and ifosfamide, with a range from 193 to 207 [18,19]. However, in this study the standard solutions were evaluated in a DAD detector, after this was possible verify that 195 nm is a satisfactory wavelength to detect both compounds (Fig. 1).

Before method validation, a system suitability test was performed. The following parameters were evaluated: plate count (N), resolution (Rs) and tailing factor (T) and these results are presented in Table 1. A system suitability test was performed because this test provides assurance that a system's performance is appropriate for the intended use. The results were considered satisfactory, according to Shabir [20], who reported an acceptable range of plate count > 2000, resolution > 2.0 and tailing factor between 0.5 and 2.0.

For clean-up of samples, a number of experiments were then conducted to optimize the extraction efficacy. In order to find the most efficient extraction procedure for both analytes, using SPE, the conditioning, sample application, wash and elution conditions were compared. The pH influence and solubility were consid-

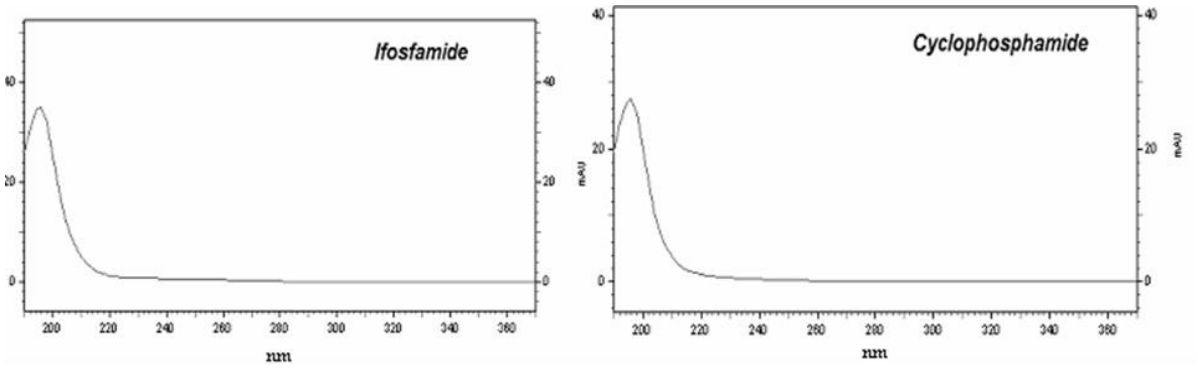

Figure 1. Detection with a Shimadzu SPD-M10Avp (Kyoto, Japan) diode array detector, for standard solutions of cyclophosphamide and ifosfamide (20 µg/ mL).

Parameters	Ifosfamide	Cyclophosphamide
Plate count	4550	4039
Resolution* (Rs)	0	2.0
Tailing factor (T)	1.5	1.9
Migration time	7.04	7.92

Table 1. System suitability parameters for HPLC-UV method evaluated for simultaneous determination of cyclophosphamide and ifosfamide. * Resolution was calculated between cyclophosphamide and ifosfamide.

ered, for LLE and SPE. According to Sottani *et al.* [21], the optimum pH for liquid–liquid extraction of cyclophosphamide is pH 7.0 (extraction yield over 90%) and it was used in present study.

Analysis of blank plasma of six different individuals did not show any interference. None of other co-administered drugs tested interfered with the analysis.

Figure 2 shows typical chromatograms of human plasma without spiking the compounds, extracted by LLE (A) and SPE (B), an extracted of human plasma spiked with 15 µM of cyclophosphamide and ifosfamide when SPE was used (C). Ethyl acetate is the solvent of choice for these analytes in LLE for surfaces and urine [21] however, for plasma it is possible observed, also in Figure 2, that there is a possible interference peak which eluted between 6.0 and 8.5 minutes. A comparison of the chromatograms presented in Figure 2 demonstrates that no interference from endogenous components in the retention time of the drugs, when was utilized SPE.

So, solid phase extraction was chosen for purification of CP and IF in this study and when it was compared with liquid-liquid extraction, with ethyl acetate, it offered several advantages: lesser amount of solvent required, no disposal

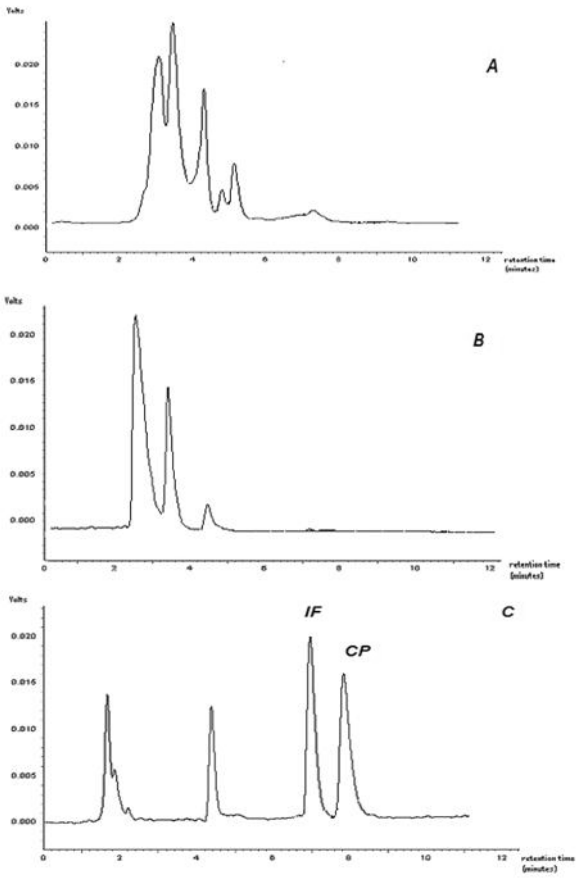

Figure 2. Typical HPLC chromatograms, in optimal conditions evaluated: (**A**) blank sample extracted with liquid-liquid; (**B**) blank sample extracted with solid phase; (**C**) plasma spiked (15 µM) with ifosfamide- IF and cyclophosphamide- CP.

of large quantities of organic solvents, higher concentration factor, no problem with the miscibility of solvent, no emulsion formation. Interferences had not been observed in chromatographic run, specially, in the retention time of the analytes, in the conditions evaluated, which demonstrates selectivity.

Parameter	Result	
	IF	*CP*
Limit of Quantification (LOQ)	3 µM	3 µM
Dynamic Range	3-540 µM	3-540 µM
- correlation coefficient	0.9962	0.9985
- mean regression equation	9131.6x-22987	8074.8x- 32610
Extraction efficacy (%)		
middle level	111	94
high level	97	115
Accuracy (%)		
middle level	114	94
high level	97	101

Table 2. Linearity, limit of quantification, extraction and accuracy of the proposed method, using solid-phase extraction, for simultaneous determination of ifosfamide (IF) and cyclophosphamide (CP) in plasma by HPLC-UV.

Calibration curves obtained by plotting the peak area resulted in linear response in the range studied. The results of linearity test are presented in Table 2. The calibration curves were linear from 3-540 µM for CP and IF (1 to 150 µg/mL). The mean correlation coefficients for six replicates calibration curves constructed using 1.0 mL of plasma spiked with IF and CP was 0.9962 and 0.9985, respectively. Analysis of variance of the correlation coefficients indicated non-significant differences ($p > 0.05$) confirming the linearity of the standard curves in the range studied.

The LOQ for this method was attained with plasma samples containing 3 µM of the analytes, corresponding to 1 µg/mL. These values are sufficient for the carried out pharmacokinetic studies. Our HPLC system was equipped with a 50 µL injection loop and from the method evaluated was possible quantify a minimum mass equivalent to 40 ng/ analyte.

Results obtained for extraction efficacy and accuracy using solid phase extraction are summarized in Table 2. Extraction efficacy was also acceptable, within the range studied, according the guidelines adopted [14-16]. The percentage obtained varied from 97 to 111%, for ifosfamide and from 94 to 115%, for cyclophosphamide.

Accuracy was calculated by comparing the concentrations obtained from plasma spiked to the actual added concentrations. The results varied from 97 to 114%, for ifosfamide and from 94 to 101%, for cyclophosphamide. Both results were considering acceptable [14-16].

IF	(Rep) (Int) (R.S.D %)*	CP	(Rep) (Int) (R.S.D %)*
Low level (3 µM)	5.4 5.7	Low level (3 µM)	2.7 5.5
Medium level (72 µM)	3.8 0.9	Medium level (72 µM)	7.8 5.3
High level (540 µM)	0.5 3.6	High level (540 µM)	1.9 2.9

Table 3. Repeatability (Rep) and intermediate precision (Int), (n=6), of the proposed method, using solid phase extraction, for simultaneous determination of ifosfamide (IF) and cyclophosphamide (CP) in plasma by HPLC-UV.* R.S.D= relative standard deviation.

Analyte	Plasma concentration range (µM)	R.S.D*
IF	19.0- 20.6	3.9
CP	24.5- 30.1	10.4

Table 4. Plasma concentration range of ifosfamide (IF) and cyclophosphamide (CP) after administration of 10 mg/kg of the drugs, in rats Male Wistar (n = 5), analysed by the proposed method. * R.S.D: relative standard deviation.

Repeatability and intermediate precision can be observed in Table 3. Coefficient of variation from repeatability varied from 0.5 to 5.4%, for IF and from 1.9 to 7.8 for CP, in three levels assayed. Intermediate precision produced satisfactory CVs for ifosfamide, between 0.9 to 5.7% and for cyclophosphamide, ranged from 2.9 to 5.5%. According to the guidelines adopted, 10 to 15% are acceptable values, for biological samples [14-16].

The stability study showed no statistically difference between 12 h in auto-sampler, long-term (samples kept at -20 °C for 2 weeks) and freshly prepared samples, for the three levels assessed, with *p*-values > 0.05.

The results reported above suggested that the LOQ obtained in the study is sufficient to quantify plasmatic concentration around typical doses. The concentrations of IF and CP after administration of these drugs at dose of 10 mg/kg are presented in Table 4. With oral administration of the substances the plasma level can be detected after 0.5 h. With the limited sampling we could not perform pharmacokinetic estimates however we could assess the applicability of the validated method. The results ranged between 19.0 to 20.6 µM, for IF, and between 24.5 to 30.1 µM, for CP.

According to Moore *et al.* [22], following IV dose of 600 mg/m², in patient with breast cancer, the cyclophosphamide maximum plasmatic concentration was 121 ± 21 µM and Kurowski *et al.* [23], related that the maximum plasmatic concentration for ifosfamide was 203 (168-232) µM, after IV dose of 1.5 g/cm².

In study conducted by Batey *et al.* [23], patients were scheduled to be treated with cyclophosphamide (600 mg/m²). The plasma concentration time profile was traced by analysis performed with a HPLC instruments equipped with a UV detector but using a mobile phase constituted by water/ acetonitrile 75:25 v/v, with results between 10 to 45 µg/ mL.

CONCLUSIONS

For simultaneous quantification of cyclophosphamide and its analogue ifosfamide in human plasma, a fast, reproducible and selective SPE-HPLC-UV method was developed and validated. This method is reliable, precise and linear in the range evaluated. Solid phase extraction was effective, with no interfering peaks from matrix compounds. Finally, the reported method was sensitive to detect a low concentration, for the two drugs, in rat plasma samples, which has a proven viability for quantitative analysis, such as pharmacokinetics and bioequivalence studies and to evaluate the plasmatic concentration in patients in therapy with these antineoplastic drugs.

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