Fast Analysis of Chemotherapy Drugs

with the Agilent Pursuit XRs Ultra Diphenyl Column

Application Note

Pharmaceutical

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Abstract

Many intravenous chemotherapy drugs are made up on the day of use for cancer patients going to outpatient clinics. Several drugs can be contained in a single chemotherapy bag, and it may be necessary to assess the quality of the made-up preparation before it is given to the patient.

Undergoing chemotherapy can be a stressful experience, exacerbated by delays in beginning the process. Therefore, time spent on drug analysis should be kept to a minimum. Using an HPLC system with Agilent Polaris and Agilent Pursuit columns, researchers at the l'Hôpital Européen Georges Pompidou validated a method that differentiated cancer drugs in less than three minutes. This is a major step in developing an analytical verification procedure to improve the quality of drug delivery for cancer patients, without compromising patient experience.

Agilent Technologies

Introduction

In French hospitals, the preparation of anticancer chemotherapies is under pharmaceutical control and must be managed in a centralized way in a specialized unit by the PUI (les Pharmacies à Usage Intérieur). The chemotherapies are sterile preparations, formulated in-house as the need arises.

The preparations must be formulated aseptically. Sterility is assessed indirectly by microbiological verification of the laboratory environment. However, there is no obligation for physico-chemical verification. At the l'Hôpital Européen Georges Pompidou, in an attempt to improve quality, an analytical method was developed that quantitatively and qualitatively verified preparations formulated in the hospital. In addition, verification was done before the chemotherapy was administered, to permit pharmaceutical authorization of use in real time. Fast analysis was required to accomplish these tasks.

Flow Injection Analysis

The usual analytical method involves flow injection analysis (FIA), employing an HPLC pump, injector, and diode detector to analyze these preparations by direct injection into the detector without the need for a chromatography column. FIA is appropriate if there is only one active ingredient in the solute. The main advantage of FIA is speed, with an analysis time of 30 seconds. Fast analysis is essential to obtain authorization for use of preparations such as these that are usually administered to outpatients on a daily basis.

However, before using FIA, it is necessary to distinguish the different active ingredients by their spectra (200-400 mm) and to quantify the content of the concentration range used in therapy. To assess these parameters, an analytical validation of the dosing technique must be executed for each molecule.

When the dosing technique is validated, the chemotherapeutic bags are considered to be verified. Their pharmaceutical validation rests on two criteria: a quantitative validation that corresponds with an acceptance of approximately 15% of the measured concentration and a qualitative validation of the active component that rests on the spectral recognition of at least 95% of the spectra available in the spectral library of the control laboratory. Nevertheless, there are limits to FIA:

- An excipient can interfere if it exhibits a UV spectrum similar to that of the drug and therefore disturbs spectral quantification and recognition of the active ingredient.
- It is difficult to recognize drugs with similar molecular structures that have indistinguishable UV spectra, such as cyclophosphamide/ifosfamide and doxorubicin/epirubicin.

HPLC is capable of resolving these issues. In the first case, HPLC separates the excipient from the active dosage component. Second, it differentiates molecules that are chemically and spectrally similar, because of different retention times on a chromatography column.

HPLC Analysis

The objective of this study was to optimize a method for fast analysis (< 3 minutes) by HPLC to differentiate two chemotherapies. Cyclophosphamide and ifosfamide, and doxorubicin and epirubicin, were chosen as test mixtures, since they are difficult to resolve spectrally. Cyclophosphamide and ifosfamide are nitrogen mustard alkylating agents. Doxorubicin and epirubicin are anthracycline antibiotics closely related to daunomycin, a natural product originally isolated from streptomyce peucetius.

Although these molecules are never mixed, they were chosen as pairs with limited separation to demonstrate the feasibility of the proposed HPLC method. The ultimate purpose was to develop a routine, analytical process for the verification of anticancer chemotherapies.

The studied molecules possess spectral features in the UV and visible domain that are quasi-identical (Figures 1 to 6).

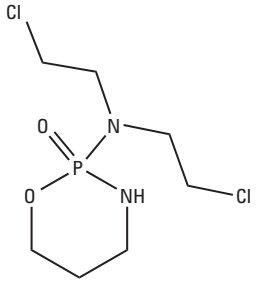

Figure 1. Cyclophosphamide.

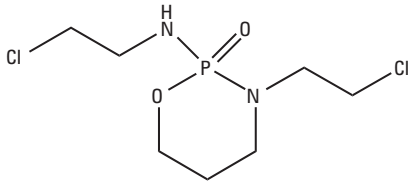

Figure 2. Ifosfamide.

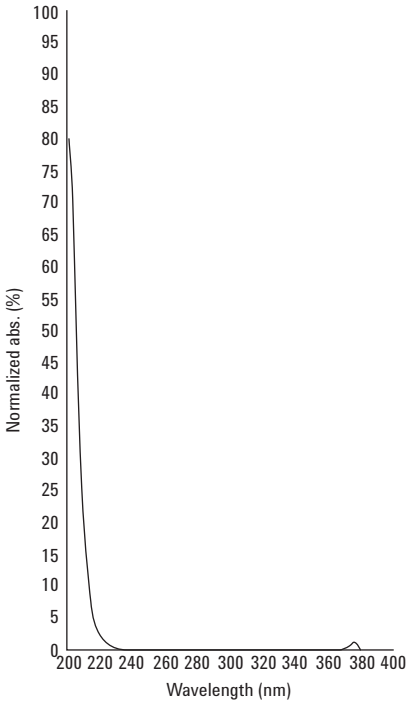

Figure 3. UV spectrum of cyclophosphamide and ifosfamide.

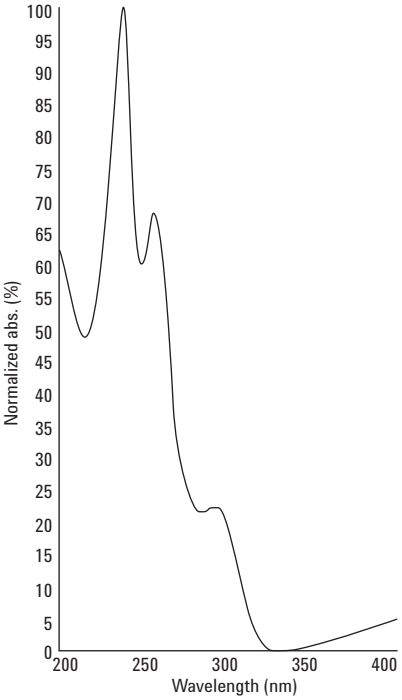

Figure 6. UV spectrum of doxorubicin and epirubicin.

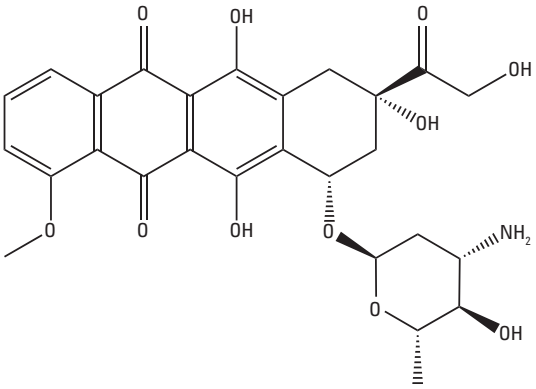

Figure 4. Doxorubicin.

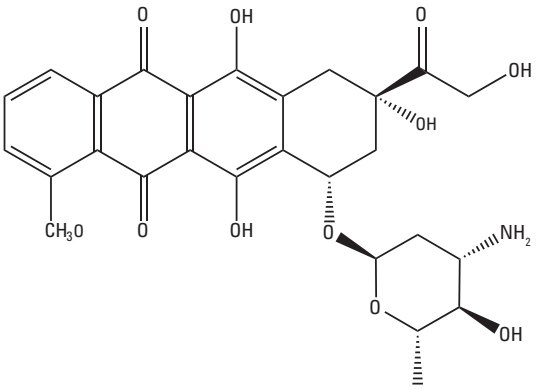

Figure 5. Epirubicin.

Materials and Methods

The rationale for the choice of the materials for liquid chromatography and the qualitative and quantitative compositions of the mobile phases are explained in the discussion section.

Calibration standards were made from cyclophosphamide (Endoxan 1,000 mg, Baxter), ifosfamide (Holoxan 1,000 mg, Baxter), doxorubicin (Doxorubicin Teva 50 mg, Teva), and epirubicin (Farmorubicine 50 mg, Pfizer).

Mobile phases were acetonitrile, osmotic water, sodium hydrogen phosphate, and hydrochloric acid 1 N. Dilution solvents were glucose 5% (m/v) (G5%) for the doxorubicin and epirubicin, and sodium chloride 0.9% (m/v) for the cyclophosphamide and ifosfamide.

Conditions

Cyclophosphamide/ifosfamide

Column	Agilent Polaris C18, 4 × 30 mm, 3 µm
Mobile phase	H_2O:ACN 75:25 v/v
Flow rate	1.0 mL/min
Pressure	45 bar
Injection volume	10 µL

Doxorubicin/epirubicin

Column	Agilent Pursuit XRs Ultra Diphenyl, 4 × 50 mm, 2.8 µm
Mobile phase	Sodium phosphate buffer 0.1 M, pH 7.5:ACN 75:25 v/v
Flow rate	0.8 mL/min
Pressure	250 bar
Injection volume	50 µL

Standard Solutions

Cyclophosphamide	Calibration solutions (1, 3, 5, 7, 9 mg/mL) and quality controls (2, 4, 8 mg/mL) prepared from Endoxan, 20 mg/mL
Ifosfamide	Calibration solutions (1, 3, 6, 9, 12 mg/mL) and quality controls (2, 5, 10 mg/mL) prepared from Holoxan, 40 mg/mL
Doxorubicin	Calibration solutions (0.1, 0.5, 1.0, 1.5, 2.0 mg/mL) and quality controls (0.25, 0.75, 1.75 mg/mL) prepared from Doxorubicin Teva, 2 mg/mL
Epirubicin	Calibration solutions (0.1, 0.5, 1.0, 1.5, 2.0 mg/mL) and quality controls (0.25, 0.75, 1.75 mg/mL) prepared from Farmorubicine, 2 mg/mL

Validation Procedure

Every method was assessed for linearity, repeatability, reproducibility, and accuracy. Limit of detection (LOD) and limit of quantification (LOQ) were calculated. To accomplish this, we measured one range per day over six days at five concentration levels, each one repeated six times. To determine the accuracy, six repetitions were performed at every level of verification.

Results and Discussion

For cyclophosphamide and ifosfamide, UV spectra were obtained between 200 and 400 nm and quantification occurred at 205 nm. The analysis time was 1.8 minutes. Results for cyclophosphamide and ifosfamide are shown in Tables 1 to 5 and Figures 7 to 9.

For doxorubicin and epirubicin, UV spectra were also obtained between 200 and 400 nm. Quantification took place at 235 nm with an analysis time of 2.5 minutes. Results for doxorubicin and epirubicin are shown in Tables 6 to 9 and Figures 10 to 12.

Table 1. Range, LOD, and LOQ of Cyclophosphamide and Ifosfamide

	Range (mg/mL)	Limit of detection (mg/mL)	Limit of quantification (mg/mL)
Cyclophosphamide	1 - 9	0.110	0.329
Ifosfamide	1 - 12	0.009	0.269

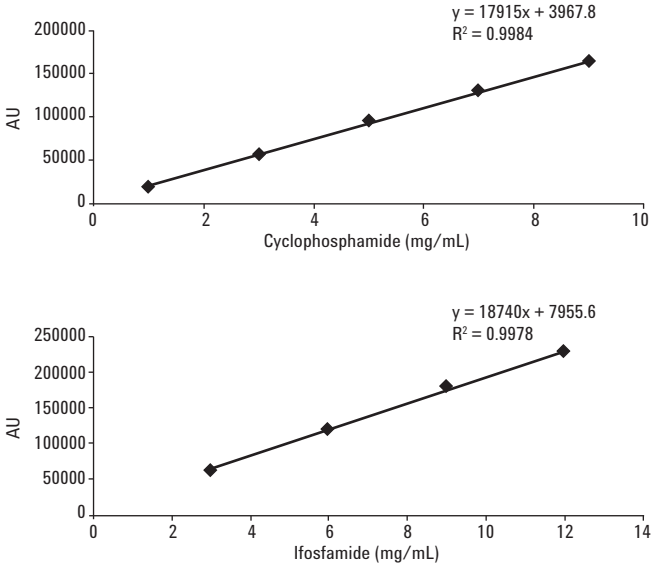

Figure 7. Calibration curves for cyclophosphamide and ifosfamide.

Table 2. Repeatability and Reproducibility of Cyclophosphamide

Concentration cyclophosphamide (mg/mL)	1	3	5	7	9
CV repeatability	2.5	1.6	0.9	0.7	0.5
CV reproducibility	4.5	6.3	2.8	2.8	3.0

Table 3. Repeatability and Reproducibility of Ifosfamide

Concentration ifosfamide (mg/mL)	1	3	6	9	12
CV repeatability	1.6	0.3	0.3	0.6	1.6
CV reproducibility	2.6	1.7	1.7	1.4	1.6

Table 4. Accuracy of Cyclophosphamide

Control (mg/mL)	2	4	8
Cyclophosphamide (%)	98.3	100.3	100.5

Table 5. Accuracy of Ifosfamide

Control (mg/mL)	2	5	10
Ifosfamide (%)	96.7	103.6	100.8

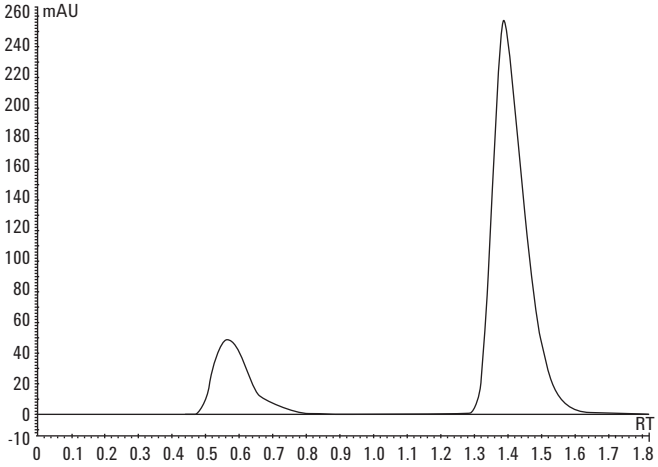

Figure 8. Chromatogram of cyclophosphamide at two different wavelengths (RT = 1.37 ± 0.05 min).

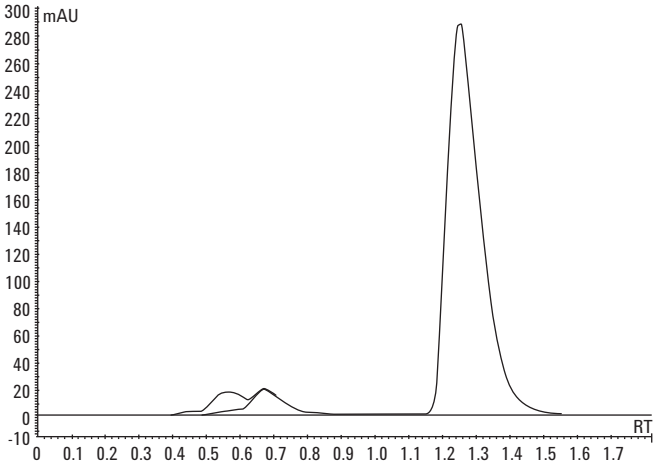

Figure 9. Chromatogram of ifosfamide at two different wavelengths (RT = 1.25 ± 0.05 min).

Table 6. Range, LOD, and LOQ of Doxorubicin and Epirubicin

	Range (mg/mL)	Limit of detection (mg/mL)	Limit of quantification (mg/mL)
Doxorubicin	0.1 - 2	0.009	0.028
Epirubicin	0.1 - 1	0.011	0.034

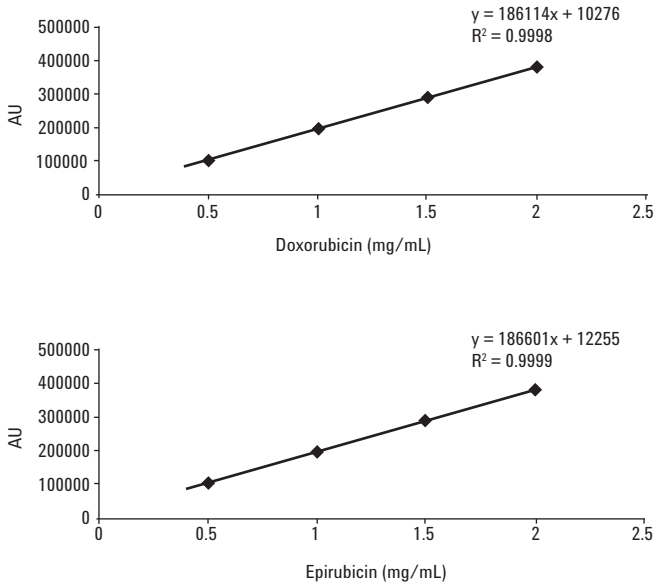

Figure 10. Calibration curves for doxorubicin and epirubicin.

Table 7. Repeatability and Reproducibility of Doxorubicin

Concentration doxorubicin (mg/mL)	0.1	0.5	1.0	1.5	2.0
CV repeatability	2.4	0.4	0.7	0.4	0.6
CV reproducibility	3.6	1.5	0.9	1.0	1.2

Table 8. Repeatability and Reproducibility of Epirubicin

Concentration epirubicin (mg/mL)	0.1	0.5	1.0	1.5	2.0
CV repeatability	2.5	0.9	0.7	0.8	1.4
CV reproducibility	5.0	1.9	2.1	1.2	1.5

Table 9. Accuracy of Doxorubicin and Epirubicin

Control (mg/mL)	0.25	0.75	1.75
Doxorubicin (%)	96.7	103.6	100.8
Epirubicin (%)	99.4	103.6	101.5

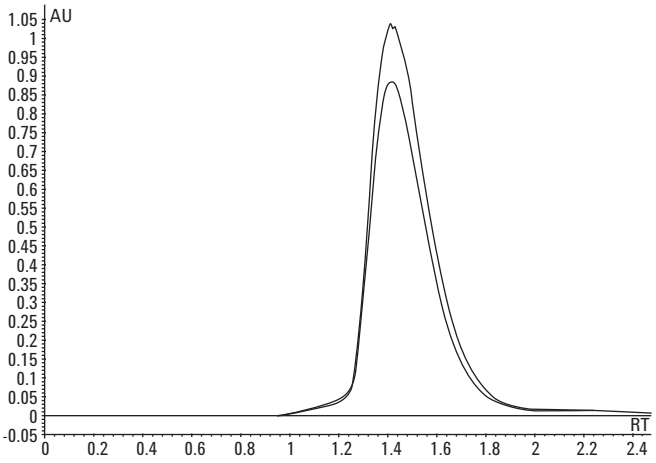

Figure 11. Chromatogram of doxorubicin at two different wavelengths (RT = 1.4 ± 0.1 min).

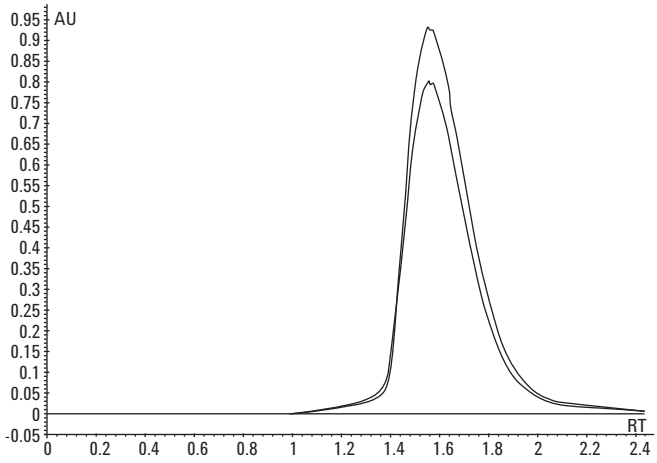

Figure 12. Chromatogram of epirubicin at two different wavelengths (RT = 1.7 ± 0.15 min).

Assessing dosage by HPLC, with the aim of verifying the contents of chemotherapeutic bags, rests on two main principles: speed and simplicity of use. To achieve speed, it is necessary to use short columns (in general, 3 or 5 cm) with small particle diameters (2.8 or 3 µm). Short columns deliver short analysis times, and small particles are more efficient than larger particles.

Analysis time to verify cyclophosphamide and ifosfamide was fast, given their relative different chemical structures and distribution coefficients. The difference was situated in the position of a chlorinated carbon chain (CH_2-CH_2-C_1), which allowed the use of a reversed-phase column (C18). The mobile phase was a mix of water and acetonitrile that permitted differentiation of the two components in two minutes.

Verifying doxorubicin and epirubicin appeared to be more difficult because the difference between the two molecules was very small. In fact, these molecules are epimers on the OH-function of the daunosamine cycle, which determined the choice of column. During preliminary tests, the use of a column with a C18 polar reversed-phase and sodium dodecyl sulfate (SDS) to obtain ion exchange was considered. We were confronted with the solubility of SDS at different pH values and differences in retention. Therefore, a diphenyl-based column was selected with π interactions, given the richness of double bonds present.

For the mobile phase, we first used a mix of water/acetonitrile that did not reveal differences in retention times. It was therefore decided to modify the ionization of the two molecules given their different pKa values, even if these were small (pKa = 7.7 for epirubicin and pKa = 8.2 for doxorubicin), using phosphate buffer. This produced a significant difference in retention times between these two molecules.

There was a significant problem of cross contamination. Anthracyclines are colored red in solution and are adsorbed on the wall of the chemotherapy bags and in the chromatography system. This could influence the analysis of certain molecules that show major absorption in UV (such as 5-fluorouracil and gemcitabine). There was a decrease of the signal and thus an underestimation of the concentrations of the verified bags after injections of anthracyclines. To avoid this contamination, a rinsing program was developed with a flow of 10 mL/min of 100% acetonitrile as mobile phase directly into the detector for 1 minute, followed by water at 1 mL/min for 30 seconds.

The difference in the retention times was observed in every validation, on all the 196 injections performed for each molecule. The performance of the confidence interval (0.05 min for cyclophosphamide/ifosfamide and 0.08 min for doxorubicin/epirubicin) was an indicator of true reliability. The lowest and highest retention times were observed, and the mean of these was used to set up an average retention time for the different molecules. The different retention times for each group of molecules were determined with a confidence interval that did not overlap, therefore allowing differentiation without ambiguity. This was the essential point of the validation since it was the only discrimination parameter for similar molecules. It was for this reason the observation of the retention times was performed on the 196 injections necessary for the validation.

Nevertheless, this differentiation of the retention times necessitates perfect equilibration of the column, to ensure reproducibility. It is also important to note that for each molecule, the different parameters of validation are compatible with a routine system, since the different variation coefficients do not exceed 5% (repeatability, reproducibility). Another consideration is that these methods are precise (accuracy between 96% and 104%).

The absence of incorrect spectral recognition of different molecules or groups of molecules was verified in order to avoid problems of identification. A recognition minimum of 95% of the library spectrum was established in order to validate the identification.

Conclusion

The use of HPLC permitted the differentiation of drug molecules chemically, with the purpose of developing an analytical verification of chemotherapeutic bags containing active components. Techniques of low dosing that allow, in the context of analytical verification, confident differentiation of doxorubicin and epirubicin had not been previously described. The results presented here suggest a way forward for the identification of these two anthracyclines.

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