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Fluorescence detection combined with either HPLC or HPTLC for pharmaceutical quality control in a hospital chemotherapy production unit: Application to camptothecin derivatives

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Abstract

In order to achieve the analytical assessment of the manufactured batches of chemotherapy preparations, post-production quality control has been developed. The common use of camptothecin derivatives (i.e. irinotecan (CPT-11) and topotecan (TPT)) as part of protocols in Institut Gustave Roussy (IGR) has led to develop an efficient analytical method that could assess an increasing number of samples with high throughput, good specificity and practicality. Due to the difference of concentration between batches containing irinotecan or topotecan, HPLC and HPTLC both combined with fluorescence detection were investigated. Those two techniques made identity, purity and quantitation assays possible. The chromatographic conditions that were chosen allowed identification of each drug through their rate of flow (R_f), 0.10 and 0.35, or their retention time (t_R), 2 and 7 min for topotecan and irinotecan, respectively. A calibration curve was plotted for each molecule and validated by three quality controls (high, medium and low). Coefficients of variation of repeatability (CV_r) and intermediate precision (CV_i) were determined for both methods. Considering their values and the concentration ranges (from 100 to 500 mg/L for HPTLC and from 0.1 to 1 mg/L for HPLC), it was decided to perform analysis using HPTLC for irinotecan preparations and HPLC for topotecan preparations. These inferences seemed appropriate regarding the number of preparations to be assayed.
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Keywords: Irinotecan; Topotecan; HPTLC; HPLC; Quality control; Fluorescence

1. Introduction

Irinotecan (CPT-11) and topotecan (TPT) are both analogs of camptothecin (CPT) which is a drug substance extracted from the Chinese plant *Camptotheca acuminata*. Camptothecin derivatives are known to have an antitumor activity but also to inhibit HIV-1 replication in human peripheral blood lymphocytes [1,2]. The antitumor activity of such drugs is due to inhibition of topoisomerase I, thus preventing DNA replication. Camptothecin derivatives are mostly used as part of chemotherapy treatments of colorectal, ovarian and small-cell lung cancers, and multiple clinical trials are carried out in order to evaluate their usefulness in other cancers [3].

The Institut Gustave Roussy (IGR) is the leading European cancer research and treatment institution. Many different drugs are used as part of chemotherapy protocols in Institut Gustave Roussy, resulting in a high number of drug preparations that need to be assayed by the Department of Clinical Pharmacy (DCP) [4–6]. Some preparations of widely used drugs (e.g. 5-FU or *cis*-Platinum) are produced in large quantities every day. For such drugs the use of a chromatography system under a technician's supervision is justified, considering the number of samples to be assayed. Other drugs, of occasional use (amongst which are CPT-11 and TPT), give rise to fewer preparations, and therefore, fewer samples to be assayed by the DCP. In order to gain time but also to free materials and technicians, we have chosen to develop a method that would make it possible to assay both CPT-11 and TPT preparations at the same time. A validated method was already used for the assay of CPT-11 preparations by high performance

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thin-layer chromatography (HPTLC) [4] and, even though the detection range of such a method was suitable mostly for high doses, it was tried to adapt it to TPT preparations. In the original method, detection was performed using a fluorescence quenching technique and, in order to improve sensibility, we decided to use direct fluorescence combined with the application of a paraffin film on the plates for our simultaneous method. At the same time we wanted to try and set a method that used high performance liquid chromatography (HPLC), coupled with a fluorimetric detector. Such HPLC techniques have been described in many papers from prior literature, but most of them are intended to assay camptothecin derivatives in blood or plasma [7–9]. This work deals with the comparison of two chromatographic methods according to their precision but also to their suitability for a quality control purpose.

2. Materials and methods

2.1. Materials and chemicals

2.1.1. Chemicals and solvents

Irinotecan (Campto®, Aventis, Paris, France) and topotecan hydrochloride (GSK, Stevenage, United Kingdom), were used to prepare standards. Potassium dihydrogen phosphate and formic acid (Carlo Erba, Rodano, Italy) and orthophosphoric acid (Merck, Darmstadt, Germany) were analysis grade. Paraffin oil was purchased from Gifrer-Barbezat (Decines, France). Organic solvents; methanol, heptane, methylene chloride and acetonitrile were HPLC grade (Carlo Erba, Rodano, Italy).

2.1.2. HPLC system

Analyses were performed using D-7000 HSM® software (Merck-Hitachi, Darmstadt, Germany) configured with a HPLC chromatographic system consisting in a quaternary pump L-7100 (Merck-Hitachi, Darmstadt, Germany), a MIDAS® autosampler equipped with a column oven (Spark, AJ Emmen, Netherlands). Analytes were separated on a Macherey–Nagel Nucleodur® gravity C_{18} 5 μm 100 Å column, 150 mm × 4 mm i.d. (Macherey–Nagel, Hoerdt, France). Fluorimetric detection was performed on a FP-1520® spectrofluorimetric detector (Jasco, Tokyo, Japan).

2.1.3. HPTLC CAMAG® analytical station

HPTLC CAMAG® (CAMAG, Muttenz, Switzerland) is composed of four separated modules: (1) an HPTLC Vario® chamber for optimization of mobile phases; (2) two TLC sampler III® automated sampler applicators; (3) five solid Teflon (PTFE) migration chambers; and (4) a TLC scanner 3® densitometer controlled by the CATS 4® software (4.05 version). The stationary phases (Nano-SIL®-20 UV_{254}), manufactured by Macherey–Nagel, (Hoerdt, France) were made of uniform 0.2 mm thin silica layers that were placed on a glass surface of 20 cm × 10 cm. The granulometry phase (2–10 μm) is guaranteed by the manufacturer and its homogeneity is one of the key factors contributing to quality separation.

2.2. Methods

2.2.1. Qualitative and quantitative HPLC fluorimetric assay

2.2.1.1. Samples, standards and quality control preparations (QCs). Commercially available CPT-11 solution (20 mg/mL) was dissolved in a methanol/water (50:50, v/v) mixture to obtain a 2 mg/mL working solution. TPT working solution (2 mg/mL) was prepared using crystalline powder dissolved in methanol and stored at −20 °C. Standard concentrations were set at 0.1, 0.2, 0.4, 0.8, 1.0 mg/L by dilution in a methanol/water (50:50, v/v) mixture. Three quality control preparations (low QC_L, medium QC_M and high QC_H) set at 0.25, 0.6 and 0.9 mg/L were prepared from another commercial solution, according to the same procedure. The standards, QCs and samples (i.e. therapeutic solution diluted in a methanol/water (50:50, v/v) mixture) were transferred to glass snap-ring clipped vials, which were arranged on the autosampler's rack.

2.2.1.2. Chromatographic conditions. The pH of the aqueous buffer was set to 3.5 to prevent the opening of the lactone cycle, which would result in an equilibrium state between the original molecules (i.e. CPT-11 and TPT) and their corresponding carboxylates (see Fig. 1). Retention times of the lactone forms and the associated carboxylate forms are not the same considering that the lactone is more hydrophobic, and as a result, two peaks per molecule instead of only one would have been detected [7,10]. The mobile phase was acetonitrile and potassium dihydrogen phosphate 50 mM in water (pH 3.5). An elution gradient was used to separate the analytes and equilibrate the column (17% of acetonitrile during the three first minutes, 30% from the fourth to the eighth and back to 17% from the ninth to the thirteenth) was applied in order to decrease retention times (especially that of CPT-11). The analytical column was thermostated at 50 °C. The injected volume was 100 μL and the elution flow was set at 1 mL/min. The excitation wavelength was set at 355 nm and the emission wavelength at 515 nm.

2.2.2. Qualitative and quantitative HPTLC fluorodensitometric assay

2.2.2.1. Samples, standards and quality control preparations. The working solutions prepared for HPLC assay were also used for the HPTLC assay. Standard concentrations were set at 100, 200, 300, 400, 500 mg/L by dilution in a methanol/water (50:50, v/v) mixture. Three quality controls (low QC_L, medium QC_M and high QC_H) set at 150, 250 and 450 mg/L were prepared from another commercial solution, according to the same procedure. The standards, QCs and samples (i.e. therapeutic solution diluted in a methanol/water (50:50, v/v) mixture) were transposed in glass snap-ring clipped vials, which were arranged on the autosampler's rack.

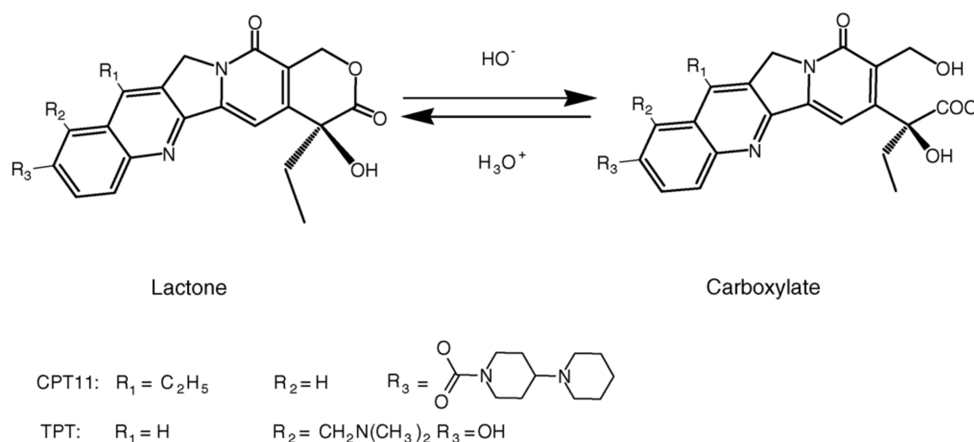

Fig. 1. Structures and pH influence.

2.2.2.2. Chromatographic conditions. HPTLC CAMAG System can be used for quantitation purposes because of its accuracy (up to a nanoliter) and the reproducibility of the autosampler. 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 were sprayed onto the plate to form 3 mm bands, 5.5 mm apart. In a single run, it is possible to assay five standards, three QCs and 24 samples placed within the QCs.

A methylene chloride/methanol/formic acid/water mixture (82:24:2:1, v/v/v/v) was developed for CPT-11 and TPT chromatography using a 5 cm horizontal sandwich migration method. After chromatography the plates were dried with a hair dryer. Signal enhancement was performed by automated soaking of the chromatography plate in paraffin-heptane solution (15%, v/v). The chromatography plate was dried a second time with a hair dryer and heated at a temperature of 70 °C for 5 min.

The plate was analysed on a TLC scanner 3® densitometer controlled by the CATS 4® software. The densitometric analysis was performed in a fluorescence-reflection mode with the setting of the excitation wavelength at 366 nm and the detection above 400 nm with the use of an optical filter. The surface area of both CPT-11 and TPT were automatically measured for each sample. CPT-11 or TPT amounts were calculated for each standard, each QC and each sample. The calibration curve was set taking these amounts into account. QCs and samples were calculated according to the response amount and the calibration curve equation. Each compound is defined according to its rate of flow (R_f), which is the ratio between substance migration and solvent migration distances. With this parameter, CPT-11 or TPT can be qualitatively assayed in pharmaceutical preparations.

2.2.3. Validation of the methods

2.2.3.1. Selectivity. CPT-11 and TPT are administrated to patients in dextrose 5% solutions contained in infusion bags. Influence of dextrose on analysis was investigated on therapeutic solutions.

2.2.3.2. Calibration. The calibration functions, i.e. relationship between compound peak areas and its applied amount, were determined for both drugs by linear regression over a defined range from 0.1 to 1 mg/L for HPLC and by Michaelis–Menten regression over a defined range from 100 to 500 mg/L for HPTLC. For routine use, each calibration curve was validated using three QCs. Samples were assayed within the QCs. Six calibration curves were done to ensure that the regression model was the most accurate for quantitative purposes. The limit of detection (LOD) and the limit of quantification (LOQ) of the technique for each active substance was obtained by use of the mean value of the slope (b') and the standard deviation of the intercept (S.D. a) from six calibration curves as defined by the ICH Topic Q2A and Q2B [11,12].

2.2.3.3. 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 each drug as described previously. These low, medium and high QCs were analysed individually six times. Means, bias and coefficient of variation (CV) were calculated.

2.2.3.4. 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 [5,6].

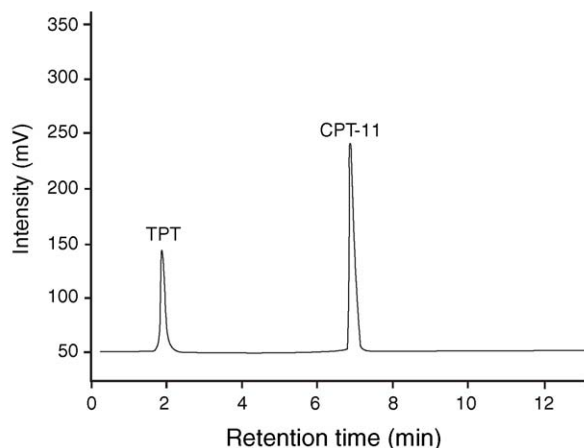

Fig. 2. HPLC chromatogram of QC_M at 0.6 mg/L.

3. Results

3.1. High performance liquid chromatography

Considering the similar structures of the two drugs as shown on Fig. 1, the same excitation and detection wavelengths apply to both TPT and CPT-11. According to the molecules' fluorescence spectra, a few wavelength couples where tested and it was decided to set the excitation wavelength at 355 nm and the detection wavelength at 515 nm.

In order to reduce the analysis time, a gradient mode elution was developed. A chromatogram with the elution program using different proportions of acetonitrile in an aqueous KH_2PO_4 buffer is reported on Fig. 2. This separation method gave retention times of 2 min for TPT and 7 min for CPT-11. Thus, the two compounds were well separated with baseline return and symmetrical peaks were obtained (Fig. 2). For the infusion bag assay, we verified that there was no analytical interference with dextrose 5% as shown on Fig. 3.

The calibration function was determined by linear regression from 0.1 to 1 mg/L for both drugs, with r^2 over 0.995 (see Table 1). Six series of measurements were performed for each drug allowing determination of a mean equation of the model and the range of measurement as shown on Table 1. Bias of back-calculated concentrations of the standards was

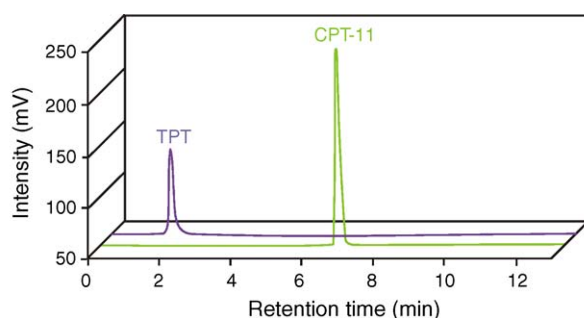

Fig. 3. HPLC chromatograms of infusion bag samples (0.6 mg/L) in dextrose 5%.

Table 1
Results of the calibration study

		Range (mg/L)	LOD (mg/L)	LOQ (mg/L)	r^2 (mean ± S.D.)
HPLC	CPT11	0.1–1	0.05	0.15	0.997 ± 0.001
	TPT	0.1–1	0.05	0.15	0.995 ± 0.002
HPTLC	CPT11	100–500	20	62	0.993 ± 0.002
	TPT	100–500	27	80	0.991 ± 0.003

The values were calculated on six different measurements.

Table 2
Results of the accuracy study

		QC_L bias (%)	QC_M bias (%)	QC_H bias (%)
HPLC	CPT11	2.04	−0.07	2.51
	TPT	0.04	2.02	0.45
HPTLC	CPT11	0.88	1.87	2.89
	TPT	4.57	4.63	4.88

The values were calculated on six different measurements.

found below 4.6% and below 5.0% for CPT-11 and TPT, respectively. The limit of detection (LOD = 3.3 × S.D. a/b') was calculated at 0.05 mg/L for both compounds while the limit of quantitation (LOQ = 10 × S.D. a/b') was calculated at 0.15 mg/L. These results demonstrate the linearity of the method in the assessed range, for both compounds.

The method proved to be accurate for the determination of both compounds, according to the mean values and the CV values (i.e. R.S.D.) calculated from six analyses of each control level (QC). No bias was found to be over 2.51% for the HPLC method with both molecules (Table 2). Concerning the precision, the CV values for repeatability (CV_r) and intermediate precision (CV_i) for each active substance are summarized in Table 3. The observed CV_r and CV_i values were all found to be below 5.08%.

3.2. High performance thin-layer chromatography

For the HPTLC system, the mercury lamp of the scanner was set to 360 nm (excitation wavelength), and a filter was applied in order to detect all signals of a wavelength higher than 400 nm (excitation wavelength). After chromatography, silica plates were dipped into a solution of paraffin–heptane (15:85, v/v) and then dried. The enhancement of fluorescence, which has been studied in previous works, can be explained by the fact that paraffin causes excitation energy to be less

Table 3
Results of the repeatability (CV_r) and intermediate precision (CV_i) study

		CV_r (%)			CV_i (%)		
		QC_L	QC_M	QC_H	QC_L	QC_M	QC_H
HPLC	CPT11	5.04	4.28	3.82	3.53	2.00	4.23
	TPT	4.49	4.97	5.08	2.68	3.15	2.47
HPTLC	CPT11	2.23	1.38	2.50	3.90	5.41	1.95
	TPT	4.14	4.65	3.04	4.54	7.71	5.12

The values were calculated on six different measurements.

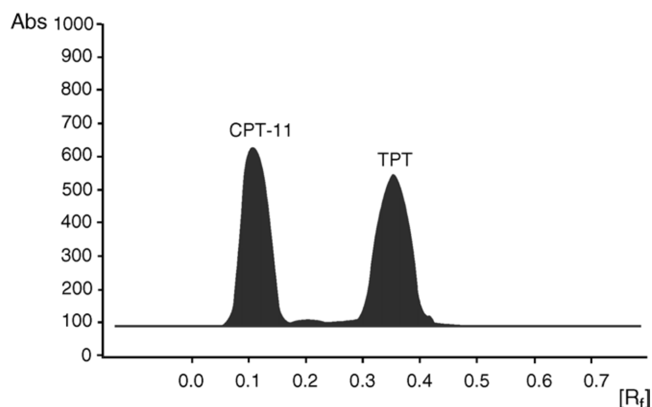

Fig. 4. HPTLC chromatogram of QC_M at 250 mg/L.

dissipated during vibration relaxation processes [13]. This last step enhanced the fluorescence signal to about five-fold in our conditions. A solvent system composed of methylene chloride/methanol/acetic acid/water (82:24:2:1, v/v/v/v) was chosen for the elution of the compounds. This mobile phase allowed a good separation of TPT and CPT-11 as shown on Fig. 4.

In order to confirm that infusion bags have been prepared with the right drug, one needs to be able to identify the compounds. This qualitative assay of infusion bags was made possible for CPT-11 and TPT, by specific rates of flow (0.1 and 0.35, respectively, as shown on Fig. 5).

The calibration function was determined by Michaelis–Menten regression (which is preferred to linear regression because of detector saturation issues) from 100 to 500 mg/L for both drugs with r^2 over 0.991 (see Table 1). Six series of measurements were performed for each drug allowing the determination of a mean equation of the curve and the range of measurement as shown on Table 1. Bias of back-calculated concentrations of the standards was found below 6.1% and below 5.1% for CPT-11 and TPT, respectively (except for standard 1; 11.8 and 13.3%, respectively). The LOD was calculated at 20 and 27 mg/L for CPT-11 and TPT, respectively while the LOQ was calculated at 62 and 80 mg/L for CPT-11 and TPT, respectively. These results show an excellent correlation to the model.

For the HPTLC method, all bias was found to be below 4.88% for TPT and below 3% for CPT-11, confirming the accuracy of the method. Regarding precision, CV_r values and CV_i values were found to be below 5.41% except CV_r found at 7.71% for TPT.

4. Discussion

In order to improve the HPTLC method that was already in use in our laboratory, the detection technique was changed from fluorescence quenching (using the silica plate's fluorescent agent) to fluorescence (that is selective and more sensitive). Furthermore, a fluorescent enhancer as a paraffin film was applied on the plates to improve sensibility.

A major drawback of the method was the non-linear regression model used for calibration, which is known not to give an equal accuracy throughout the concentration range, since the slope is not constant. In our example, the problem was pretty much just theoretical considering the fact that the model was very close to linearity.

The concentration range that was used for validation of the method was limited by the sensibility of the technique and such an array did not allow any dilution of TPT samples (CPT-11 preparations are much more concentrated than TPT ones). This could have been a major issue considering the reconstitution of TPT preparations in dextrose 5% which can interfere if detection is performed by fluorescence

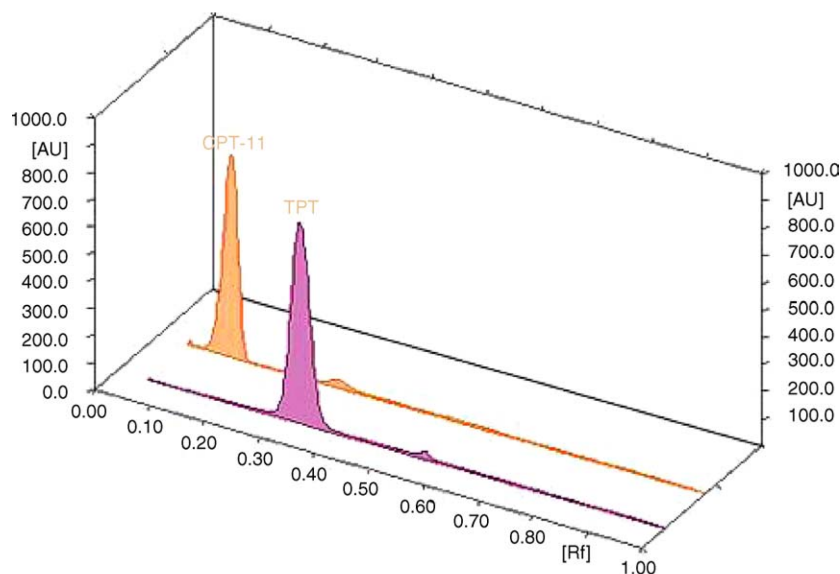

Fig. 5. HPTLC chromatograms of infusion bag samples (400 mg/L) in dextrose 5%.

quenching (not selective). The selective fluorescence detection of camptothecin derivatives solved the problem.

The overall time for a single full run of 24 samples would be around an hour, making HPTLC the most convenient of the two methods when results are needed rapidly, but one needs to keep in mind that a technician is needed (dipping, scanning, data analysis are not performed automatically).

With the HPLC system concentrations to be assayed are much lower (approximately 500-fold) than the ones needed for HPTLC, and a dilution step is needed when infusion bags are dealt with rather than blood samples for example. The time of analysis is rather long, taking more than 12 min per sample, but the process does not need any supervision and can run by itself from the moment it is launched and until all data are analyzed. It can therefore, be useful when only a few samples need to be analyzed, considering that the total duration of the analysis is closely related to the number of samples (which is not the case with HPTLC).

5. Conclusion

With this work, we have demonstrated that both HPLC and HPTLC are suitable for the analysis of TPT and CPT-11. As a general conclusion, taking all the elements mentioned above into consideration, it was decided that CPT-11 analysis would be performed using HPTLC and TPT analysis using HPLC. The fact that our lab deals with infusion bags that contain TPT or CPT-11 (and not both of the drugs), and that there are more samples containing CPT-11 than samples containing TPT made the difference, considering that HPLC is well adapted to the analysis of only a few samples whereas HPTLC is adapted for the analysis of a larger number of samples. Nevertheless, one can use either HPLC or HPTLC for the simultaneous determination of CPT-11 and TPT in infusion bags prepared for clinical chemotherapy.

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