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Computer-assisted UHPLC–MS method development and optimization for the determination of 24 antineoplastic drugs used in hospital pharmacy

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ABSTRACT

This study reports the use of retention modeling software for the successful method development of 24 injectable antineoplastic agents. Firstly, a generic screening of several stationary and mobile phases (using various organic modifiers and pH) was achieved. Then, an optimization procedure of mobile phase temperature, gradient profile and mobile phase binary composition was conducted through only 28 real experiments using retention modeling software for data treatment. Finally, the optimized separation was achieved with a mobile phase consisting in 10 mM acetic acid at pH 5.1 (A) and acetonitrile (B). A Waters CORTECS® T3 column (100 × 2.1 mm, 1.6 μm) operated at 25 °C with a gradient time of 17.5 min (0–51%B) at a flow rate of 0.4 mL/min was used. The prediction offered by the retention model was found to be highly reliable, with an average error lower than 1%. A robustness testing step was also assessed from a virtual experimental design. Success rate and regression coefficient were evaluated without the need to perform any real experiment. The developed LC–MS method was successfully applied to the analysis of pharmaceutical formulations and wiping samples from working environment.

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1. Introduction

In recent years, an increase of the chemotherapies compounded in hospital pharmacies, in conjunction with the growing number of patients treated for a cancer, has been noticeably observed. Active ingredients of these pharmaceutical formulations, called antineoplastic drugs, are highly toxic compounds characterized by a high reactivity and a non-selective pharmacological action. Most of antineoplastic agents have mutagenic, carcinogenic and/or teratogenic effects. As a result, chemotherapies are considered as high-risk pharmaceutical formulations not only for the patients, but also for healthcare professionals and their environment. Development of analytical methods to identify and quantify antineoplastic drugs in various matrices is of utmost importance to ensure an appropriate therapeutic treatment for the patient (i.e. quality control of chemotherapy, stability studies and therapeutic drug monitoring) and to protect human being and environment (i.e. toxicology studies, environmental contamination monitoring) [1]. However, the development of generic methods for the analysis of antineoplastic drugs constitutes a real challenge because of their high toxicity and the great diversity of physico-chemical properties of these compounds (pK_a, lipophilicity, solubility, stability ...). Multiple techniques have been employed for the analysis of antineoplastic drugs. However, liquid chromatography coupled to mass spectrometry (LC–MS) remains one of the most widely used techniques for the analysis of antineoplastic drugs [1,2]. The high selectivity offered by the wide choice of mobile and stationary phases combined with the high sensitivity of MS detection constitute the major advantages of LC–MS. Other separation techniques such as CE-UV also demonstrated a real potential for the analysis of antineoplastic drugs [3]. However, its inherent low sensitivity and the need for compounds with chromophore moiety constitute the main limitations.

Generic analytical methods, allowing the simultaneous determination of several target compounds, are particularly interesting in terms of time saving, costs and handling. However, most of the published LC–MS methods were intended for the analysis of one or possibly up to 2 or 3 different antineoplastic drugs. To our knowl-

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Table 1
Molar masses, detection mode, detected *m/z* ratio, pKa, Log P, log D at pH 3, 5 and 9 of the 24 antineoplastic agents. (Calculated with Chemicalize software).

Peak ID	Name	Molar mass (g/mol)	ESI mode	m/z (cone voltage)	Pka (acidic)	Pka (basic)	LogP	LogD (pH 3)	LogD (pH 5)	LogD (pH 9)
1	5-Fluorourcil	130.1	–	129.0 (34 V)	7.18	–	−0.66	−0.656	−0.659	−2.241
2	Cytarabine	243.2	+	244.1 (42 V)	12.55	–	−2.80	−2.798	−2.798	−2.798
3	Fludarabine	365.2	–	364.1 (26 V)	1.35	0.63	−1.97	−3.281	−3.977	−6.194
4	Gemcitabine	263.2	+	264.1 (52 V)	11.52	–	−1.47	−1.467	−1.467	−1.469
5	Dacarbazine	182.2	+	183.1 (26 V)	6.64	1.72	−0.43	−0.446	−0.435	−1.401
6	Methotrexate	454.4	+	459.2 (24 V)	3.25	2.8	−0.24	−0.237	−2.692	−7.073
7	Pemetrexed	427.4	+	455.2 (40 V)	3.34	2.43	1.49	1.311	−1.086	−5.551
8	Busulfan	246.3	+	264.1 (22 V)	–	–	−0.76	−0.760	−0.760	−0.760
9	Raltitrexed	458.5	+	498.2 (26 V)	3.72	1.24	1.97	1.893	0.033	−5.049
10	Etopophos	668.5	+	669.1 (54 V)	1.46	–	0.51	−0.988	−1.857	−4.031
11	Topotecan	421.5	+	422.2 (40 V)	8.00	9.75	−0.33	−2.665	−2.542	−0.327
12	Ifosfamide	261.1	+	261.1 (40 V)	14.64	0.12	0.10	0.096	0.096	0.096
13	Cyclophosphamide	261.1	+	261.1 (40 V)	13.43	0.08	0.10	0.097	0.097	0.097
14	Irinotecan	423.5	+	428.2 (38 V)	11.69	9.47	2.78	−1.549	−0.707	2.177
15	Doxorubicin	543.5	+	544.2 (28 V)	8.00	9.93	0.53	−1.528	−1.448	0.527
16	Etoposide	588.6	+	606.2 (32 V)	9.33	–	1.16	1.160	1.160	0.995
17	Epirubicin	543.5	+	587.3 (78 V)	8.00	9.93	0.53	−1.528	−1.448	0.527
18	Daunorubicin	527.5	+	544.2 (26 V)	8.00	9.93	1.34	−0.711	−0.630	1.344
19	Idarubicin	497.5	+	528.2 (20 V)	8.02	9.94	1.51	−0.553	−0.476	1.509
20	Vincristin	825.0	+	825.4 (80 V)	10.85	8.66	3.13	−3.862	−3.092	2.943
21	Vinblastine	811.0	+	811.4 (72 V)	10.87	8.86	4.18	−2.812	−2.275	3.907
22	Vinorelbine	779.0	+	779.4 (62 V)	10.87	8.66	4.65	−2.331	−1.347	4.469
23	Docetaxel	807.9	+	846.2 (80 V)	11.90	–	2.92	2.917	2.917	2.916
24	Paclitaxel	853.9	+	892.2 (80 V)	11.77	−1.18	3.54	3.539	3.539	3.538

edge, only few generic analytical methods were reported for large sets antineoplastic drugs assays [4–6]. It can be noted that the development of these methods was characterised by a rapid optimisation of chromatographic conditions based on the use of a fast gradient of two predefined mobile phases on only one column with a focus on a short analysis time; which is done, in most cases, to the detriment of the complete separation of drugs. Even if this lack of selectivity can be mitigated by MS detection, the developed methods may suffer from unwanted matrix effects between compounds and should provide unreliable quantitation of compounds.

The great versatility of LC–MS could also become a limitation during method development because of the very large number of possible operating conditions that need to be tested (i.e. type of stationary phase, temperature, gradient steepness, pH, ionic strength, organic modifier nature). Traditionally, strategies for the development of specific and robust UHPLC methods have most relied on a trial and error procedure. Development time for a single target molecule can be fast but finding optimal conditions for the analysis of multiple compounds can be tedious and time-consuming even for the most experienced chromatographer. In this context, retention modeling software considerably reduce development time by simulating chromatograms from a small set of experiments and also reduce the manipulation of toxic substances, such as antineoplastic drugs.

The aim of this work was to develop a generic UHPLC-MS method for the analysis of 24 antineoplastic drugs used in hospital pharmacy compounding unit, with the help of a chromatographic modeling software. Only optimization of chromatographic conditions were reported in this article. This method could be applied in routine laboratories to determine antineoplastic drugs in various matrices, including traces in the environment for example. To the best of our knowledge, it is the very first generic UHPLC-MS method for such antineoplastic compounds supported by computer assisted method development.

2. Experimental

2.1. Chemicals and sample

All solvents were MS grade and all chemicals were obtained in the highest analytical quality available. Acetonitrile (ACN), formic acid, acetic acid and ammonium hydroxide were purchased from Merck (Darmstadt, Germany). Methanol (MeOH) and Dimethyl Sulfoxide (DMSO) were purchased for Sigma-Aldrich (Buchs, Switzerland). Ultrapure Type 1 water was obtained from a Milli-Q purification system from Millipore (Bedford, MA, USA).

5-Fluorouracil, Methotrexate and Dacarbazine were purchased from Tokyo Chemical Industry (Zwijndrecht, Belgium). Gemcitabine was obtained from Acros Organic (Geel, Belgium). Busulfan was obtained from Sigma-Aldrich (Buchs, Switzerland). Cytarabine, Epirubicin and Topotecan were obtained from Torronto Research Chemicals (North York, ON, Canada). Raltitrexed, Pemetrexed, Docetaxel, Paclitaxel, Vincristine, Vinblastine, Vinorelbine, Doxorubicin, Daunorubicin, Idarubicin, Etoposide, Etoposide Phosphate, Irinotecan and Fludarabine phosphate were obtained from Pharmaserv (Stansstad, Switzerland). Cyclophosphamide and Ifosfamide were obtained from Baxter AG (Opfikon, Switzerland) as Endoxan® and Holoxan® lyophilisate for injection, respectively. Physico-chemical properties of the compounds were summarized in Table 1.

Stock solutions of standards were prepared by dilution of standard compounds in DMSO at 1 mg/mL and were kept at −80 °C.

2.2. Safety consideration on antineoplastic agents handling

Because antineoplastic agents are highly toxic compounds, their handling required strict safety precaution to limit analyst and environment exposure. All powders were weighted and solubilized under horizontal laminar airflow safety cabinet equipped with HEPA H14 filters. Most of the dilutions were performed with an automated liquid handing workstation (Tecan Freedom EVO®, Männedorf, Switzerland). All instruments and materials in contact with toxic compounds were treated as hazardous waste. Personal protective equipment (gloves, gown, mask . . .) were chosen following literature recommendations.

2.3. Equipment and software

All analyses were performed using an ultra-high-pressure liquid chromatograph system coupled to a mass spectrometer consisting in a thermostated auto-sampler (Acquity H-Class FTN, Waters), a quaternary-flow solvent-delivery system (Acquity H-Class QSM,

Waters), a high vacuum pump (Sogevac SV40BI, Leybold), and a triple-quadrupole mass spectrometer detector (TqD, Waters). Data acquisition and instrument control were performed by MassLynx 4.1 software (Waters). Retention and resolution modeling was performed with Drylab® 4 software (Molnar-Institute, Berlin, Germany).

Cortecs UPLC C18 1.6 μm, 2.1 mm x 100 mm, Cortecs UPLC C18 + 1.6 μm, 2.1 mm x 100 mm, Cortecs UPLC T3 1.6 μm, 2.1 mm x 100 mm, Acquity UPLC CSH Fluoro-Phenyl 1.7 μm, 2.1 mm x 100 mm, Acquity UPLC HSS PFP 1.8 μm, 2.1 mm x 100 mm, Cortecs UPLC Shield RP18, 1.6 μm, 2.1 × 100 mm, Cortecs UPLC Phenyl 1.6 μm, 2.1 × 100 mm were all purchased from Waters (Milford, MA, USA).

2.4. Mobile phase composition and sample preparation

As mobile phase buffers (mobile phase A), 10 mM ammonium formate (pH = 3.0), and 10 mM ammonium acetate (pH = 5.0) were freshly prepared. ACN and MeOH were chosen as organic modifiers (mobile phase B) for the screening procedure. Later on, during the optimization procedure, three pH values were set for the 10 mM ammonium acetate, namely pH_1 = 4.5, pH_2 = 5.0 and pH_3 = 5.5.

Stock solutions of antineoplastic drugs were thaw at room temperature for 30 min and vortexed before dilution at 100 ng/mL in deionized water.

2.5. Mass spectrometry parameters

The mass spectrometer was operated in both positive (ESI+) and negative (ESI-) ion electrospray ionization mode. Electrospray settings were as follows: capillary voltage, 3 kV (1 kV for ESI-); source and desolvatation temperature were 150 °C and 400 °C, respectively. Desolvatation and cone gas flow were set to 800 and 30 L/h, respectively. Detected *m/z* and cone voltages for each compound were summarized in Table 1.

2.6. Preliminary experiments

A preliminary screening was performed to select the most promising column chemistry, organic modifier and pH range for the analysis. The 24 studied antineoplastic agents were analyzed on 7 stationary phases possessing different selectivities and hydrophobicities at two pH values (3 and 5) with either acetonitrile or methanol in gradient mode, using MS detection. Seven different UHPLC columns with identical dimensions (100 × 2.1 mm) were used. In term of column chemistries, five core-shell materials with C18, C18+ (with positively charged silica particles), Shield-C18 (with carbamate as polar embedded groups), endcapped C18 and Phenyl were selected. Charged surface hybrid (CSH) and high strength silica (HSS) stationary phases both bonded with pentafluorophenyl (PFP) moieties were also selected to tune selectivity. The overall peak shapes, retention of highly polar compounds (i.e. 5-fluorouracil, cytarabine) and the separation of isomers (doxorubicin/epirubicin and cyclophosphamide/ifosfamide) were considered in this initial step to find out the most suitable conditions. In order to correctly assign peaks and avoid mismatching of isobaric compounds, the 24 antineoplastic drugs were injected from two pools of 12 compounds. A generic gradient from 2 to 98% B in 12 min at 0.5 mL/min flow rate was systematically applied. In all cases, column temperature was set to 35 °C.

2.7. Systematic method development (3D retention model)

After the initial screening procedure, the gradient time (tG), mobile phase pH and temperature (T) were further optimized using the most promising combination of stationary phase, buffer and organic modifier. For this purpose, a 3D retention model was built up based on twelve additional experiments (tG × T × pH). The following levels of the three variables were studied: $pH_1 \sim 4.5$, $pH_2 \sim 5.0$ and $pH_3 \sim 5.5$, T_1 = 25 °C and T_2 = 50 °C and tG_1 = 10 min and tG_2 = 30 min. At this stage, the gradient was run from 0 to 75%B at flow rate of F = 0.5 mL/min. All these conditions were then combined in a full factorial experimental design (3 pH × 2 T × 2 tG = 12 runs).

2.8. Method transfer (refinement for routine use) and robustness testing

After defining the optimal conditions, the method was routinely applied. A continuous increase of pressure was observed with the number of injections. Therefore, in order not to work close to the maximal pressure limit of the column - and thus expectedly to improve column lifetime – the flow rate was decreased from 0.5 mL/min down to 0.4 mL/min. The previously built retention model was used to perform a geometrical method transfer. The selected final conditions were F = 0.4 mL/min, with a gradient of 0–51%B in 17.5 min at T = 25 °C and pH = 5.1. All the method transfer procedure was performed *in silico*, and the simulated results were then experimentally verified.

As a last step of method development and refinement, a simulated robustness testing was performed. Besides the three model variables used for the optimization (i.e. tG, T, pH), the flow rate, as well as initial and final compositions of the mobile phase represent the investigated factors in the built-up robustness model.

The effect of nine factors was calculated at three levels (3^6 full factorial design corresponding to 729 experiments) and their impact on critical resolution was calculated. The modeled deviations from the nominal values were the following: the gradient time was set to tG = 17.3, 17.5 and 17.7 min, temperature was set to 24, 25 and 26 °C, mobile phase pH was set to 5.0, 5.1 and 5.2, flow rate was set to 0.39, 0.40, 0.41 mL/min. The mobile phase compositions were set at 1% B levels. Finally, frequency distribution plots and the impact of the factors were calculated.

3. Results and discussion

An analytical process involves three main steps: (1) sample preparation, (2) separation and detection and (3) data handling. Nowadays, the sample preparation step plays a secondary role and merely cleans the sample in order not to contaminate the analytical system. Indeed, the method selectivity is mainly based on the separation and the detection step when LC–MS technique is used. When analysing a complex mixture, efforts should focus on the chromatographic separation of isobaric compounds. However, the development of a generic method for the simultaneous determination of more than 20 compounds (including three pairs of isobaric compounds) is not that simple. Especially since the compounds to analyze have a wide range of physico-chemical properties. To reduce the number of experiments and therefore the manipulation of toxic compounds, the use of chromatographic modeling appeared as a very attractive approach. Such a software was used in our case to develop a LC–MS method for the simultaneous analysis of 24 antineoplastic drugs that can be applied in many areas such as quality control of chemotherapies, stability tests, biological samples analysis (TDM, toxicological analysis) and environmental studies.

3.1. Analytical target profile

Every analytical method development starts with the definition of Analytical Target Profile (ATP), which specifies the objectives of the analytical method to be developed. ATP can vary depending

on multiple criteria such as critical resolution (lowest resolution between two peaks), number of separated compounds, robustness of the method and total analysis time. The ideal method that satisfies all the criteria is often unreachable with complex mixtures. In this study, attention was mainly paid on the separation of isobaric compounds, the absence of a total co-elution between compounds to limit interferences at ionisation ($R_S > 0.8$), sufficient retention of the most polar compounds and the total analysis time (lower than 30 min).

3.2. Stationary phase selection (preliminary measurements)

At the first step of the method development, three parameters were evaluated, namely the column chemistry, mobile phase pH and organic modifier. This scouting procedure is similar to the approach applied in former studies [7,8]. The entire screening consisted in 28 experimental conditions, which could be completed in 3 working days.

Mobile phase pH had a strong impact on overall peak shape since numerous antineoplastic drugs were ionizable (see Table 1). At pH 3, poor peak shape was observed with all tested columns for polar compounds such as cytarabine and dacarbazine (Fig. 1). A similar behavior was observed for the compounds belonging to the vinca alkaloids (i.e. vincristine, vinblastine and vinorelbine). For these compounds, less acidic pH (pH 5) significantly improved peak shapes and increased retention time. Chromatograms obtained for vincristine on the seven stationary phases were reported in Fig. 2. Basic pH values were not tested due to poor stability of most of the selected stationary phases reported by the manufacturer at pH values higher than 8. Therefore, mobile phase pH of 5 seemed to be promising for this antineoplastic drug mixture.

No important selectivity or sensitivity difference was observed when using MeOH rather than ACN as organic modifier. Therefore, MeOH was not selected as organic modifier, due to its higher viscosity and therefore the high pressure generated in gradient mode leading to reduced column lifetime, and ACN was preferred as organic modifier.

On the seven tested stationary phases, HSS-PFP offered the highest retention of 5-fluorouracil (Fig. 3) and cytarabine (Fig. 1), due to the π - π interactions with aromatic moieties, but this stationary phase was not compatible with highly lipophilic compounds, such as vinca-alkaloids which were eluted as broad peaks (Fig. 2). CSH column offered a sufficiently high retention of 5-fluorouracil (k = 0.8) and an acceptable peak shape for other compounds at pH 5, but this stationary phase showed a decrease in retention after about 200 injections (data not shown). Even if an overall acceptable peak shape for most of the compounds was obtained with RP-18 Shield, Cortecs® C18 and Cortecs® Phenyl columns, they were not considered for further experiments because of the low retention of the most hydrophilic compounds. All tested columns - except CSH-PFP - were able to separate isobaric drugs (doxorubicin/epirubicin and cyclophosphamide/ifosfamide) at pH 5 (Fig. 4).

With Cortecs® UPLC T3 column, good peak shape was observed for most of the compounds and acceptable retention was obtained for the polar drugs. Another important advantage of this column was its compatibility with 100% aqueous mobile phases, which helped to achieve appropriate retention for the most polar compounds. Under these conditions, the Cortecs® UPLC T3 column was selected for further experiments.

3.3. 3-D experimental design and retention model

Gradient steepness, temperature and mobile phase pH were selected as model variables to create a cube (3D) resolution map, which shows the critical resolution of the peaks to be separated against the three factors [9,10]. Indeed, based on some preliminary

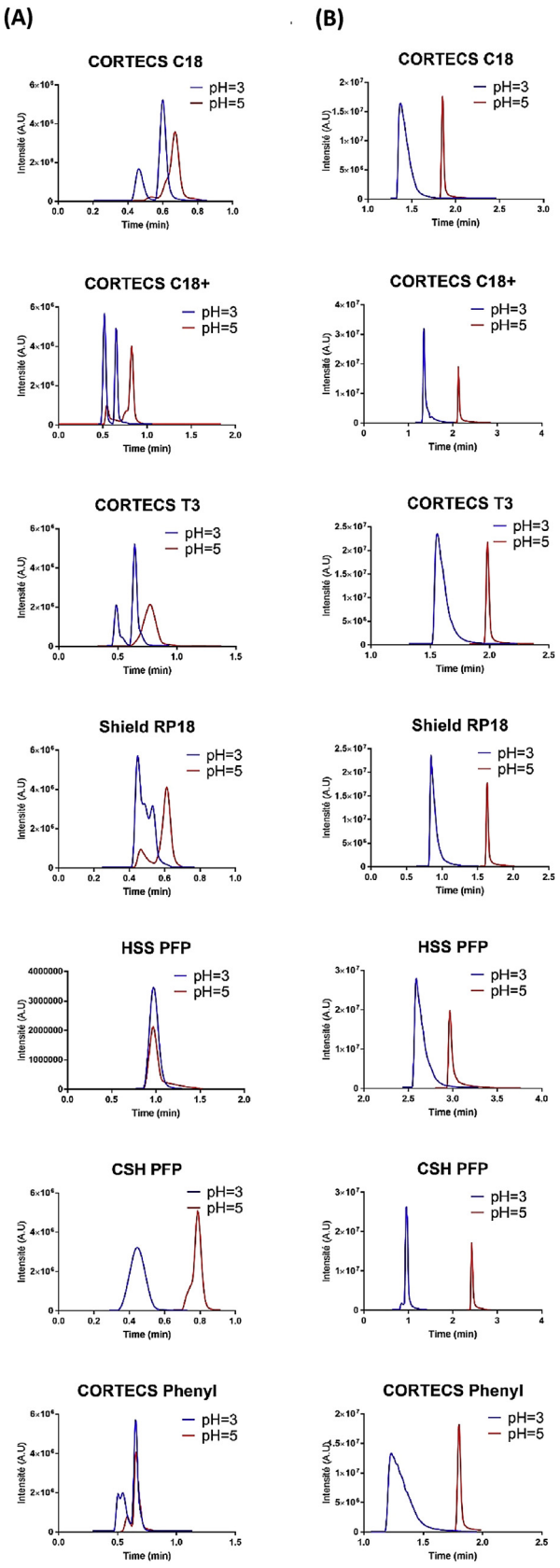


Fig. 1. Chromatograms of cytarabine (A) and dacarbazine (B) on seven stationary phases at pH = 3 and pH = 5.

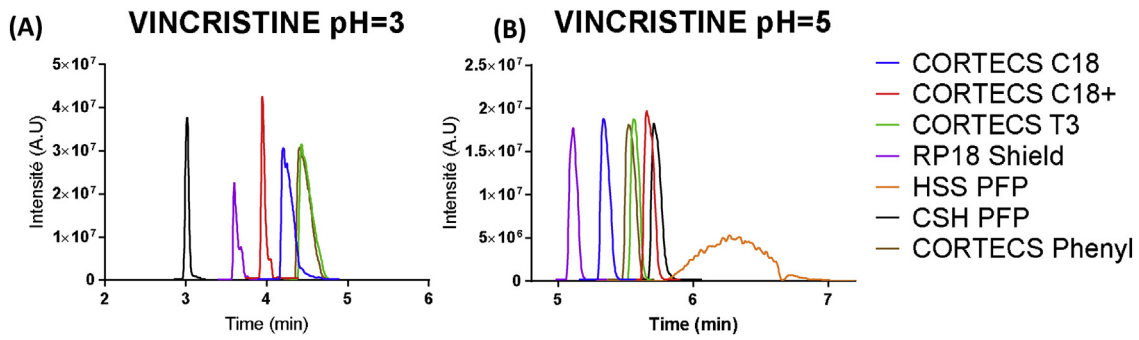


Fig. 2. Chromatograms obtained for vincristine on the seven stationary phases at pH = 3 (A) and pH = 5 (B).

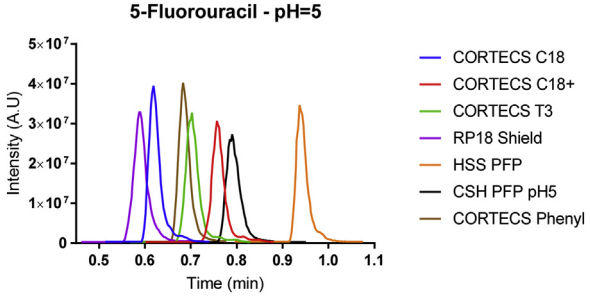


Fig. 3. Chromatograms of 5-fluorouracil on seven stationary phases at pH = 5.

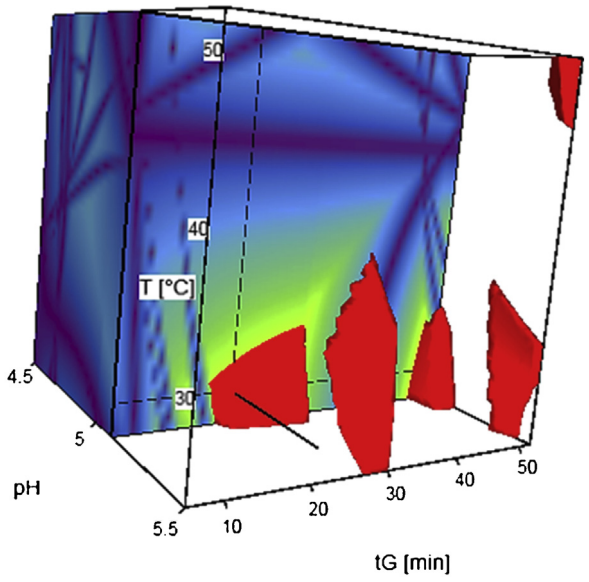


Fig. 5. 3-D resolution map for the mixture of 24 compounds in the experimental domain. Red color indicates the space where critical resolution Rs > 0.8 and dark blue color indicates co-elutions. (Column: Cortecs T3, tG = 20 min, T = 25 °C, pH = 5.1, gradient: 0–75% B, F = 0.5 mL/min) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

experiments, it appears that these three variables have the most significant effect on the selectivity and resolution for this type of analytes.

Peak tracking was supported by MS detector. The created resolution map is shown in Fig. 5. It suggested that appropriate resolution and robustness could be attained at a working point corresponding to tG = 20 min, T = 25 °C and pH ~ 5.1. Then, by applying a linear gradient from 0 to 75%B, $Rs_{,min}$ = 0.89 could be achieved between the most critical peak pair, namely methotrexate and pemetrexed (peaks 6 and 7 on Fig. 6 A). Due to the high number of peaks to be separated, it was not possible to perform baseline separation for all peak pairs within an acceptable analysis time (less than 30 min). Since MS detection was used, a resolution > 0.8 appeared more than sufficient to identify closely eluting peaks. However, the mixture contained some isobaric compounds or compounds detected at same *m/z* ratio due to molecular adducts (i.e. busulfan/gemcitabine, ifosfamide/cyclophosphamide, and doxorubicin/epirubicin) that need to be baseline resolved since they cannot be distinguished with MS in case of co-elution. Therefore, the selectivity and resolution between isobaric compounds should be sufficient. By using retention modeling software, it is possible to select and study some particular peaks of interest and not the whole mixture. Fig. 6B shows the predicted chromatogram of the mixture containing possible isobaric compounds. Because of their large difference in chemical structure, busulfan and gemcitabine had very different chromatographic behaviour, leading to a very high selectivity and resolution. The situation was slightly different with the two other peak pairs: ifosfamide/cyclophosphamide (structural isomers) and doxorubicin/epirubicin (diastereoisomers). Under the tested conditions, baseline separations were obtained for these two pairs, with Rs values of 2.63 and 2.74 for ifosfamide/cyclophosphamide and

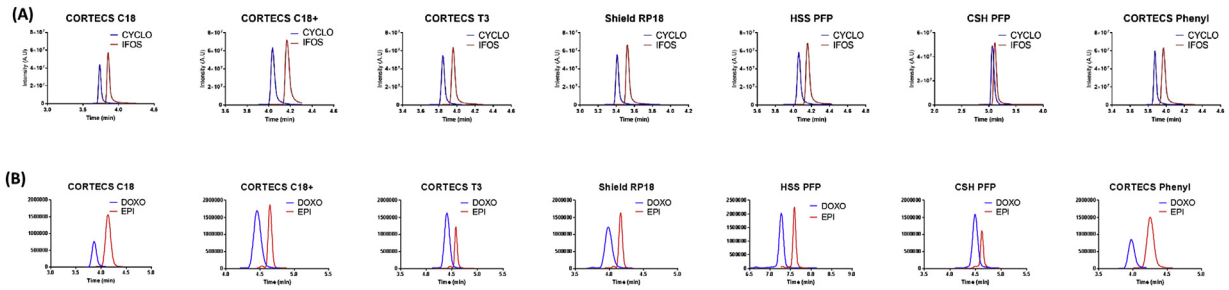


Fig. 4. Chromatograms of cyclophosphamide (CYCLO) and ifosfamide (IFOS) (A) and chromatograms of doxorubicin (DOXO) and epirubicin (EPI) (B) on the 7 tested stationary phases.

Table 2
Predicted and experimental retention time of the 24 compounds (Column : CORTECS T3, tG = 20 min, T = 25 °C, pH = 5.1, gradient: 0–75%B, F = 0.5 mL/min).

Peak ID	Name	Predicted tR [min]	Experimental tR (min) Mean ± RSD, n = 3	Error (%)
1	5-Fluorourcil	0.92	0.92 ± 0.01	0.4
2	Cytarabine	1.39	1.39 ± 0.03	0.2
3	Fludarabine	1.96	1.98 ± 0.02	1.0
4	Gemcitabine	2.15	2.14 ± 0.01	−0.5
5	Dacarbazine	3.18	3.19 ± 0.02	0.3
6	Methotrexate	4.17	4.20 ± 0.02	0.6
7	Pemetrexed	4.26	4.28 ± 0.02	0.5
8	Busulfan	5.25	5.24 ± 0.01	−0.1
9	Raltitrexed	5.46	5.43 ± 0.01	−0.5
10	Etopophos	5.76	5.80 ± 0.01	0.7
11	Topotecan	5.89	6.03 ± 0.02	2.4
12	Ifosfamide	6.82	6.81 ± 0.01	−0.1
13	Cyclophosphamide	7.05	7.05 ± 0.01	0.0
14	Irinotecan	8.33	8.40 ± 0.01	0.8
15	Doxorubicin	8.66	8.70 ± 0.01	0.5
16	Etoposide	8.83	8.82 ± 0.01	−0.2
17	Epirubicin	9.03	9.08 ± 0.05	0.5
18	Daunorubicin	9.92	10.01 ± 0.01	0.8
19	Idarubicin	10.48	10.56 ± 0.01	0.7
20	Vincristin	11.29	11.32 ± 0.03	0.3
21	Vinblastine	12.17	12.4 ± 0.01	1.9
22	Vinorelbine	12.90	13.13 ± 0.06	1.8
23	Docetaxel	14.02	14.01 ± 0.01	−0.1
24	Paclitaxel	14.36	14.35 ± 0.01	0.0

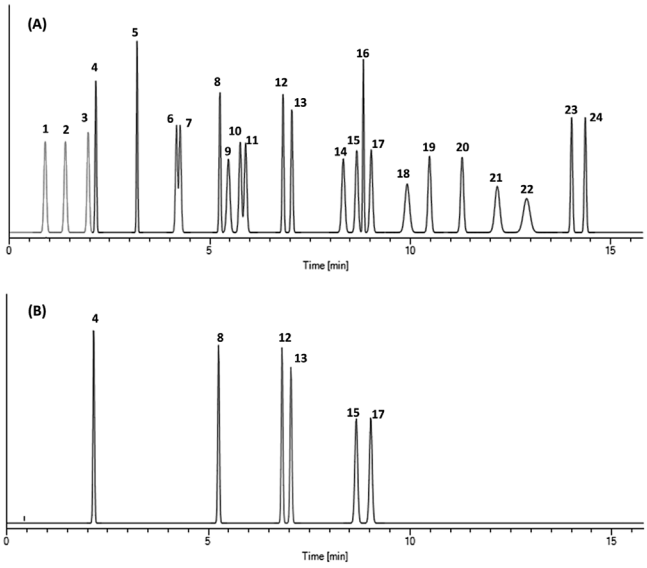


Fig. 6. Predicted chromatograms of the 24 compounds (A) and of the isobaric compounds (B) under the optimized conditions (Column: Cortecs T3, tG = 20 min, T = 25 °C, pH = 5.1, gradient: 0–75% B, F = 0.5 mL/min).

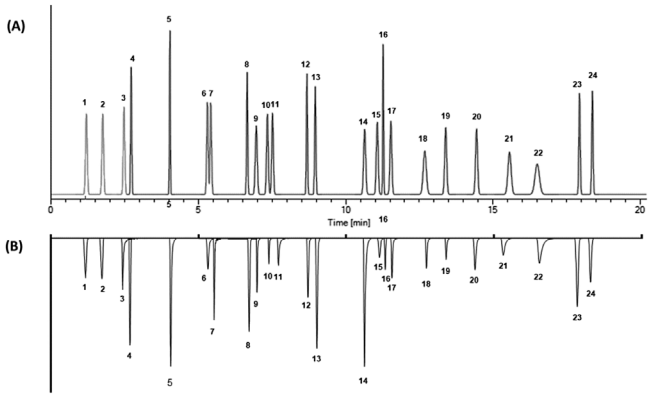


Fig. 7. Predicted (A) and experimental (B) chromatograms of the 24 compounds at transferred conditions. (Column: Cortecs T3, tG = 17.5 min, T = 25 °C, pH = 5.1, gradient: 0–51% B, F = 0.4 mL/min).

doxorubicin/epirubicin, respectively. Therefore, the selected conditions were appropriate for the analysis of all 24 antineoplastic drugs.

Since no peaks eluted after 15 min, the gradient was stopped at the corresponding mobile phase composition (~51%B) and the analysis time was reduced accordingly.

Retention time prediction was experimentally verified at the working point. Excellent agreement was found between measured and predicted values. The average error, expressed in retention time prediction was 0.5%. The deviation values were between +2.4% and -0.5%. (Table 2) It worth mentioning that in some cases, when more complicated matrix effect is expected, then a more robust working point should be selected (i.e. more centered within the design space) despite the fact it may require longer analysis time [11,12]

3.4. Method transfer and simulated robustness testing

As previously mentioned, whilst using the method in routine, systematic pressure increase was observed after several injections. Therefore, the flow rate was decreased down to 0.4 mL/min to increase column lifetime. For this purpose, a simulated geometrical method transfer was performed [13]. Fig. 7 shows the predicted and experimental chromatograms at 0.4 mL/min flow rate. Working at lower flow rate resulted in higher critical resolution (Rs = 1.19 between peaks 6/7 and Rs = 3.6 between peaks 12/13). No significant gain on sensitivity was observed at reduced flow rate. The virtually transferred method was experimentally verified. Again, very good agreement was found between predicted and measured retention times (average error was 0.4% with minimal value -0.2% and maximal value +1.5%).

The last step of the method development and refinement was the estimation of the robustness. Fig. 8 A and B show the frequency distribution plots of virtual robustness test for the 24 compounds and the selected isobaric compounds, respectively. The frequency of a given value was plotted against the resolution. As shown for the 24 antineoplastic drugs, the resolution varied between 0.58 and 1.5, while it ranged between 3.46 and 3.89 for the isobaric compounds.

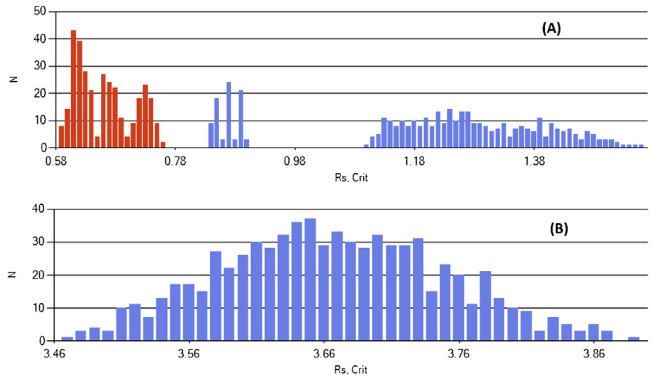


Fig. 8. Frequency distribution plots of simulated robustness tests considering all 24 compounds (A) and only isobaric compounds (B) at transferred conditions. (Column: Cortecs T3, tG = 17.5 min, T = 25 °C, pH = 5.1, gradient: 0–51% B, F = 0.4 mL/min).

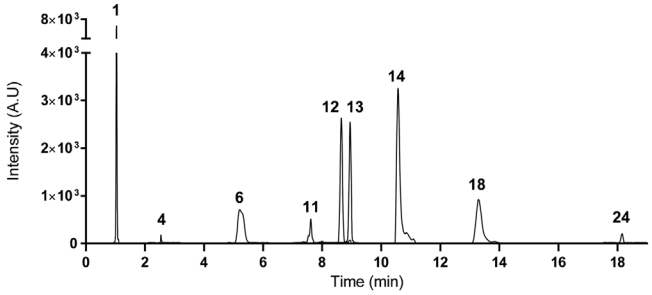


Fig. 9. Typical chromatogram obtained after wipe sampling procedure in chemotherapy compounding unit (work bench of an isolator).

When considering Rs > 0.8 criterion, the success rates were 55 and 100%, for the 24 antineoplastic drugs and the isobaric compounds only, respectively. Since MS detection was used, and isobaric compounds were baseline separated; this 55% probability to perform Rs > 0.8 was more than sufficient. The method can be considered as a robust one. When calculating the impact of the method variables, the mobile phase pH was found to be the most influential variable. Beside this, the initial %B of the gradient program was also found to be significant.

3.5. Application of the final method to real samples

In order to demonstrate the applicability of the developed LC–MS method to real samples, the analyses of the 24 antineoplastic drugs was achieved in pharmaceutical formulations for quality control and in wiping samples for environmental monitoring.

For quality control, 3 pharmaceutical formulations were analysed by diluting the samples to a target value of approximately 150 ng/mL compatible with a LC–MS analysis. In all cases, an unambiguous identification of the antineoplastic drugs contained in pharmaceutical formulations was achieved (data not shown).

The method was also successfully applied to 3 environmental samples. Surface contamination by antineoplastic drugs was detected in wiping samples from chemotherapies production area in hospital. In all tested samples, at least one antineoplastic drugs was detected. A typical chromatogram obtained for the LC–MS analysis of a wiping sample from the work bench of an isolator used for the compounding of chemotherapies was reported in Fig. 9.

4. Conclusions

The use of predictive modeling software allowed the successful development of a generic LC–MS method for the simultaneous analysis of 24 antineoplastic drugs. Only a few initial experiments were required for optimization, which is both time-saving and safer for analyst, as limited manipulation of toxic compounds was required. The high selectivity of the developed method paves the way for a wide field of applications such as the pharmaceutical formulation analysis (quality control or stability studies), environmental monitoring and biological samples analysis (TDM, toxicological studies). Indeed, the applicability of the method was demonstrated for the analysis of chemotherapies and wiping samples. Next experiments to be conducted concern the evaluation of quantitative performance according to the routine application and the nature of sample matrix through a validation process of the overall analytical procedure including sample preparation, separation and detection.

The perfect adequacy between the predicted and the experimental data have emphasized the real potential of the predictive modeling software for the fast and robust development of chromatographic separation. Even if the work of the analyst appears reduced in terms of manipulation, his role remained important in the definition of the analytical objective of the method in a quality by design framework.

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