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Liquid Chromatographic Method for Simultaneous Determination of Five Antineoplastic Drugs

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***SUMMARY*. Therapeutic importance and benefices caused by antineoplastic drugs are unquestionable however unfortunately well-known are their side effects. So, the extensive use and the exposure to multiple agents may be at risk to health care workers involved in the preparation and administration of these drugs. It is therefore important to have accurate methods for simultaneous analysis for evaluation of the occupational exposure. In this study, we have developed a method for simultaneous determination of 5-fluorouracil (5-FU), methotrexate (MTX), doxorubicin (DOX), cyclophosphamide (CP) and ifosfamide (IF). The assay was performed by HPLC-UV, detection in 195 nm, with a C18 column (250 x 4 mm, 5 μm) with a similar guard- column. Mobile phase was constituted by water pH 4: acetonitrile: methanol (70:17:13, v/v/v) with a flow of 0.4 mL min^{-1} up to 13 min and after this, 1 mL min^{-1}. For cleaning of surfaces, we used a solution of acetonitrile: methanol (50:50, v/v). The method presented a linear calibration in a range from 0.25 to 20 μg mL^{-1}, for 5-FU and MTX and from 0.5 to 20 μg mL^{-1} for IF, DOX and CP, with correlation coefficients (r^2) upper to 0.997. The repeatability, expressed in terms of percent relative standard deviation, was ≤ 10% and recovery was > 70%, in surfaces contaminated with the analytes. The results obtained suggest that the method developed can be applicable for simultaneous determination of the five drugs studied and can be considered useful in exposure assessment.**

INTRODUCTION

Chemotherapy is the only systemic treatment modality for cancer. However, cytotoxic drugs are not selective for cancer cells, but also effect the growth and reproduction of healthy cells [1]. It has been widely documented in the last 20 years that nurses and pharmacy personnel working in hospitals are exposed to antineoplastic agents and the relevant exposure pathways are through the skin and by inhalation [2]. During the preparation of cytotoxic infusions, a variety of drug manipulations are performed, resulting in the generation of aerosols and droplets, which are known to contaminate the areas in which they disperse into, including isolators and surrounding surfaces [3-11]. Gloves utilized by health care workers, in the chemotherapy handling sites, can also increase the risk of exposure in other areas of a hospital [12]. Touzin *et al.* [13] recently published a paper that evaluated contamination on the external surfaces of cyclophosphamide vials, during storage in pharmacy departments, and demonstrated the drug presence.

According to the International Agency for Research on Cancer (IARC), at least nine alkylating cytostatic drugs are classified as carcinogenic to humans (Group 1). In addition, several cytostatic drugs are classified, by the IARC, in Groups 2A and 2B (probably and possibly carcinogenic to humans, respectively) [14].

During the 1980's, a series of guidelines and recommendations from professional organizations and government agencies were developed and promoted, recommending policies and procedures for the safe handling of antineoplastic agents [15].

A more recent report has been issued by Na-

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tional Institute for Occupational Safety and Health (NIOSH) [16] which released a comprehensive analysis and description of specific recommendations entitled "Preventing Occupational Exposures to Antineoplastic and other Hazardous Drugs in Healthcare Settings". The alert recommends ways to reduce occupational risks in healthcare settings by controlling job-related exposure [15].

Based on current scientific knowledge, it is impossible to set a level of exposure that can be considered to be safe. For this reason, exposure to cytostatic agents has to be kept at the lowest possible level. Nevertheless, even when protective measures are taken and safety guidelines are adhered to, contamination occurs. Biological and environmental monitoring are therefore essential to identify the main exposure routes and to quantify potential health risks. However, risk assessment calls for accurate standardized sampling techniques and analytical methods. Wipe sampling is very useful to evaluate the presence of residual contaminants in the workrooms and moreover the effectiveness of personal protective equipment and decontamination techniques [14].

High performance liquid chromatography with ultra-violet detection (HPLC-UV) is most often referred to in current literature on analytical methods for determination of antineoplastic agents. This technique appears to be most feasible for attaining the maximum sensitivity (lower limit of detection) when used for detection of multiple antineoplastics in both air and surface samples [14,17].

In this study, the aim was to develop a HPLC method able to detect the presence of five structurally different drugs, extensively used in the clinical practice, on surfaces, in a single analysis. This capability can provide information on exposure to personnel from these drugs. The drugs evaluated were methotrexate (MTX), 5-fluorouracil (5-FU), cyclophosphamide (CP), doxorubicin (DOX) and ifosfamide (IF). The approach used to decide which agents to include in this analytical method was use frequency in cancer hospitals and potential human health hazard.

MATERIALS AND METHODS

Materials

Cyclophosphamide, methotrexate and 5-fluorouracil were purchased from Sigma, Aldrich chemical company, doxorrubicin (Adriblastina®) was donated from a Cancer Hospital and ifosfamide (Holoxan®) was donated from a Laboratory of Industrial Hygiene and Toxicology. Acetonitrile and methanol (HPLC grade) were obtained from Mallinckrodt®.

HPLC conditions

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 ambient temperature (21 °C). The detection was, firstly, performed with a Shimadzu SPD-M10Avp (Kyoto, Japan) diode array detector (DAD) and after this, the analysis was performed in a Shimadzu SPD-10Avp (Kyoto, Japan) UV detector. Chromatographic separation was achieved using a SupelcosilTM LC-18 (250 x 4.6 mm, 5 mm) column protected by a similar guard-column (4 x 4.6 mm). The mobile phase consisting of a mixture of water adjusted to pH 4: acetonitrile: methanol (70:17:13, v/v/v), was delivered at a flow rate of 0.4 mL min^{-1} by 13 min after this, the flow was increased to 1.0 mL min^{-1}. Data acquisition and treatment was performed by a Class-VP software (Shimadzu).

Standard and stock solutions

Stock standard solutions were prepared by dissolution of each drug in methanol to obtain a concentration of 1 mg mL^{-1}. These solutions were stored at –20 °C between experiments. The working solutions were prepared each day by making a 10-fold dilution of the stock solution in methanol.

Confidence parameters

Validation of this study was in compliance with IUPAC guidelines [18]. The following parameters were assayed: robustness, linearity, lower limit of detection (LOD) and quantification (LOQ), precision and stability.

Robustness was performed in middle level (5 µg mL^{-1}) and was explored using mobile phase flow rate and column temperature. 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 0.25 a 20 µg mL^{-1}, in six replicates for each level. A calibration curve was generated for each analytical run, in duplicate, and it consisted of a blank and six non-zero samples covering the expected range, including LOQ.

LOD was calculated as 3 SD (standard deviation) of six independent complete determinations of analyte concentration in a typical matrix

blank, with no censoring of zero or negative results and LOQ obtained by the successive dilutions for determined the lowest concentration with accuracy and precision, as 10% RSD (relative standard deviation), and with a signal-to-noise ratio of 10:1.

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 was assessed by coefficient of variation (CV %), which was calculated as 100 x SD/mean measured concentration.

Test surface coating intentionally contaminated with analytes

Intentional contamination of surfaces was performed in order to evaluate the method. The test was made, according to Roberts *et al.* [1], by a transverse sectioning through a barrel of a 10 mL syringe at 5 cm intervals. The resulting rings were then cut in half, giving rectangular surfaces of 3.5 x 3 cm. Polypropylene, an inert surface, was used to eliminate any contribution from the surface on the tests carried out. The surfaces (n = 6) were coated by placing between 20 µL of the drug solutions (100 µg mL^{-1}) on the concave side and blank consisted in non-coated surface. All surfaces were allowed to dry until no solution remained and desorption of dried drug was made with 2 mL of acetonitrile: methanol (50:50, v/v) into a centrifuge tube. The tubes were centrifuged for 5 min at 1500 g. The supernatant was transferred to an auto-sampler vial for assay by HPLC. Recovery was determined comparing the peak areas obtained against a standard (taken as 100%) which had not been subjected to these conditions.

RESULTS AND DISCUSSION

Despite the use of protective measures, it is still necessary to check if there is exposure to antineoplastic drugs. Groups exposed to antineoplastic include patients, individuals working in the pharmaceutical industry, workers who prepare and administer the drugs, cleaning personnel, and family members of patients and researchers.

In occupational health, several techniques are available to monitor exposure, dose or effect. In several studies, wipe samples were taken and analysed from different surfaces (safety cabinets and floors, in production, preparation and administration rooms) and objects (tables and vials). In addition, gloves and sleeve protectors, for personal protection, were frequently contaminated and can increase the risk of exposure in other areas of a hospital [12,19]. Sessink *et al.* [20] detected CP in the urine of pharmacy technicians and nurses didn't directly involve in the preparation and administration of this drug.

The drugs can be easily detected on environmental and biological samples, according to the following priorities: measurements on surface samples, on biological samples and on environmental samples [21].

Since workers are exposed to a wide variety of antineoplastic drugs, it is necessary to identify certain substances that can be used as indicators or to develop methods able to detect multiple agents. Currently, acceptable analytical methods do exist for several antineoplastics, but usually only for an individual agent or for small groups of chemically similar agents.

In this study, it was to develop a method able to detect the presence of five structurally different drugs (Fig. 1), extensively used in the clinical practice, on surfaces, in a single analysis. It provides the capability to conduct a more comprehensive evaluation of antineoplastic drugs exposure.

In Figure 1 is shown the UV spectra of the drugs in a DA detector, after this it was possible verify that 195 nm is a satisfactory wavelength to detect all compounds studied, simultaneously, in agreement to other study [17].

Satisfactory chromatographic separation (Fig. 2) of 5-FU, MTX, IF, DOX and CP was isocratically obtained using a reverse phase column and mobile phase constituted by water adjusted to pH 4: acetonitrile: methanol (70:17:13, v/v/v). It isn't possible to obtain a separation between 5-FU and MTX with a flow rate of 1.0 mL min^{-1}, during the chromatographic run. So, the mobile phase was delivered at a flow rate of 0.4 mL min^{-1} by 13 min after this, the flow was increased to 1.0 mL min^{-1}. With these conditions, it was possible detected all five analytes in a run time of 30 min, which can be applied in routine. Analysis of mobile phase, analytes free, did not show any interference in the retention time of the compounds studied. Methanol has a UV cut off at 205 nm and this was a factor to limiting the solvent level in the mobile phase to less than 15%, so the sensitivity of the detector wasn't affected.

Before performing validation experiments, system suitability was evaluated. These tests are used to verify if the resolution and repeatability of the system are adequate for the analysis and

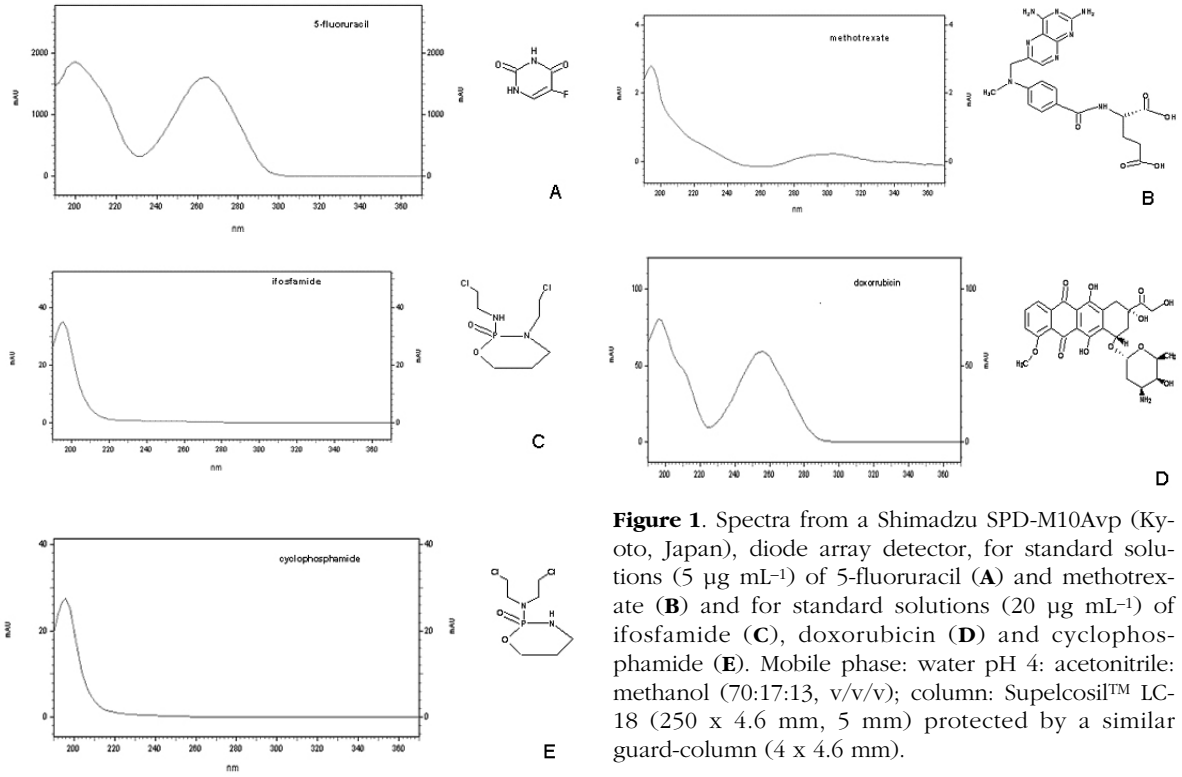

Figure 1. Spectra from a Shimadzu SPD-M10Avp (Kyoto, Japan), diode array detector, for standard solutions (5 µg mL^{-1}) of 5-fluoruracil (**A**) and methotrexate (**B**) and for standard solutions (20 µg mL^{-1}) of ifosfamide (**C**), doxorubicin (**D**) and cyclophosphamide (**E**). Mobile phase: water pH 4: acetonitrile: methanol (70:17:13, v/v/v); column: Supelcosil™ LC-18 (250 x 4.6 mm, 5 mm) protected by a similar guard-column (4 x 4.6 mm).

they are utilized to checking of system performance [22]. Parameters such as plate count, tailing factors and resolution were determined and compared against the specifications, as demonstrated in Table 1. It is possible observed that the system was suitable since the results of the test were considered satisfactory, according to Shabir [22] that reported an acceptable range of plate count > 2000, resolution > 2.0 and tailing factor between 0.5 and 2.0.

Linearity was demonstrated over the concentration range of 0.25-20 µg mL^{-1} for 5-FU and MTX and of 0.5-20 µg mL^{-1} for IF, DOX and CP. These results can be observed in Table 2 and were acceptable, since the correlation coefficients (r^2) were ≥ 0.997.

Robustness was demonstrated using ten per-

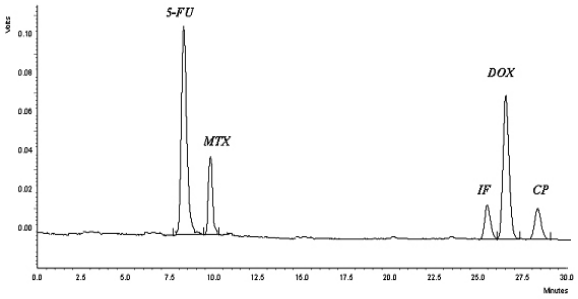

Figure 2. Typical HPLC chromatograms, in optimal conditions evaluated: 5 µg mL^{-1} of 5-fluoruracil (5-FU), methotrexate (MTX) and 10 µg mL^{-1} of ifosfamide (IF), doxorubicin (DOX) and cyclophosphamide (CP). Mobile phase: water pH 4: acetonitrile: methanol (70:17:13, v/v/v); column: Supelcosil™ LC-18 (250 x 4.6 mm, 5 mm) protected by a similar guard-column (4 x 4.6 mm).

Analyte	Retention Time	Plate count (N)	Resolution** (Rs)	Tailing Factor (T)	Capacity factor (k)
5-FU	7.8	3456	-	1.4	6.8
MTX	9.4	3388	2.6	2.1	8.4
IF	26.1	27719	-	1.1	25.1
DOX	27.3	28566	2.0	1.1	26.9
CP	28.9	26807	2.7	1.2	27.9

Table 1. System suitability parameters* for HPLC-UV method evaluated for simultaneous determination of 5-fluoruracil (5-FU), methotrexate (MTX), ifosfamide (IF), doxorubicin (DOX) and cyclophosphamide (CP). *Reference values: N ≥ 2000; Rs ≥ 2; 0,5 ≤ T ≤ 2; k > 2. ** Resolution was calculated between: MTX and 5-FU; DOX and IF; CP and DOX.

Parameters	5-FU	MTX	IF	DOX	CP
Linearity (r^2)	0.9999	0.9996	0.9986	0.9997	0.9974
Range (μg mL^{-1})	0.25- 20	0.25-20	0.5-20	0.5-20	0.5-20
Slope (standard error)	597902 (24932.4)	417030 (12070.3)	12890 (455.3)	35185 (2235.3)	5805.9 (340.4)
Intercept (standard error)	-6698.4 (2566.9)	-39640 (1242.7)	- 4295.5 (4687.9)	- 48324 (2511.6)	- 14542 (9362.2)
LOD (μg mL^{-1})	0.1	0.1	0.3	0.3	0.3
LOQ (μg mL^{-1})	0.25	0.25	0.5	0.5	0.5

Table 2. Linearity, detection and quantification limits for simultaneous determination of 5-fluorouracil (5-FU), methotrexate (MTX), ifosfamide (IF), doxorubicin (DOX) and cyclophosphamide (CP) by HPLC-UV.

Concentration (μg mL^{-1})	Precision (%CV)			Concentration (μg mL^{-1})	Precision (%CV)	
	Analyte				Analyte	
	IF	DOX	CP		5-FU	MTX
1	9.6	6.1	5.5	0.5	7.9	7.6
5	4.6	4.4	7.1	2.5	2.1	4.3
20	5.7	2.0	4.3	10	3.1	3.2

Table 3. Precision (repeatability) for simultaneous determination of 5-fluorouracil (5-FU), methotrexate (MTX), ifosfamide (IF), doxorubicin (DOX) and cyclophosphamide (CP) by HPLC-UV.

cent deviation in flow rate of mobile phase and column temperature and these variations hadn't influenced the results. They were compared with those obtained by the proposed method and the relative standard deviation was ≤ 5.0 %.

The lower limit of detection was 0.1 μg mL^{-1}, for 5-FU and MTX and 0.3 μg mL^{-1}, for IF and CP. Two criteria were used for LOQ, accuracy and precision and signal-to-noise ratio, and the results were closed. LOQ was 0.25 μg mL^{-1} for 5-FU and MTX and 0.5 μg mL^{-1} for IF and CP (50 μL was injected onto the column). These were considered satisfactory, mainly for 5-FU and MTX, drugs considered indicators of the occupational exposure, since are frequently used in clinical practice.

Roberts *et al.* [1] investigated the removal and deactivation of antineoplastic contamination from surfaces of a pharmaceutical isolator workstation. The three marker were used: 5-fluorouracil, cyclophosphamide and doxorrubicin. For the analysis, three differents HPLC methods (100 μL was injected onto the column) were validated and used to quantify the amount of the parent drug, remaining after the study phases. Detection and quantification limits were, respectively, for 5-FU, 0.2 and 0.5 μg mL^{-1}; for CP, 2.5 and 10 μg mL^{-1}; for DOX, 0.25 and 1 μg mL^{-1}.

The limits of detection for 5-FU and MTX were, respectively, for boxes and drugs vials/ampoules, 0.3 and 3 μg, obtained by Sessink *et al.* [9], when HPLC methods were used. The difference between the analysis was the mobile phase, that was constituted by a sodium acetate buffer for 5-FU, however for elution of MTX was necessary to use a blend of sodium acetate buffer and methanol.

The results obtained from repeatability can be considered satisfactory from the three levels evaluated in this method (Table 3) however, low levels presented relative standard deviations around 8 % for 5-FU and MTX and 10% for IF.

Recovery from drug-coated surfaces was > 70%. The method is reproducible with a coefficient of variation of <5% for intra-assay precision, as showed in Table 4, in surfaces contaminated with the drugs, in six replicates.

The question of whether exposure can be diminished by a reduction in handling is difficult to answer. Normally, it is reasonable to assume a positive correlation between use and exposure and currently, no recommended exposure limits (RELs), permissible exposure limits (PELs), or threshold limit values (TLVs®) have been established for antineoplastics drugs [16,20]. A balance must be achieved to continue the use of these beneficial drugs in patients, while assuring the health of personnel administering them.

Drug	Recovery	Precision (%CV)
5- Fluorouracil	103	4
Methotrexate	81	5
Ifosfamide	106	4
Doxorubicin	99	4
Cyclophosphamide	74	1

Table 4. Recovery by HPLC-UV method for simultaneous determination of 5-fluorouracil, methotrexate, ifosfamide, doxorubicin and cyclophosphamide in surfaces spiked, in six replicates, with 2 μg mL^{-1}.

CONCLUSIONS

For simultaneous analysis of five drugs: 5-fluorouracil, methotrexate, ifosfamide, doxorubicin and cyclophosphamide, a simple, reproducible and robust HPLC-UV method was developed and validated. This method is reliable, precise and linear in the range evaluated and provides the ability to detect the presence of five different agents, simultaneously. Monitoring the occupational exposure to antineoplastic is important to control and to protect the health of workers involved in the preparation and administration of these drugs.

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