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Journal of Chromatography B, 726 (1999) 95–103

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatography of methotrexate for environmental monitoring of surface contamination in hospital departments and assessment of occupational exposure

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Received 26 June 1998; received in revised form 3 November 1998; accepted 3 December 1998

Abstract

In the frame of applicative research in occupational hygiene of hospital workplaces, we investigate hospital indoor contamination as a consequence of the use of antineoplastic drugs (ANDs), with the purpose of assessing exposure of medical and nursing personnel to potentially harmful doses of ANDs, and ultimately of yielding advice on safe operating procedures for manipulation of ANDs in hospitals and in house-care of cancer patients. Among the large number of currently employed ANDs, methotrexate (MTX) has been selected as a tracer of surface contamination, on the basis of its wide use in therapy, its ease of measurement and of its chemical properties relevant to persistence and transport in the indoor environment. MTX is a polyelectrolyte, with a high water, but lower organic solvent solubility, a negligible vapour pressure and a high chemical robustness to environmental stress, thus allowing to measure surface-to-surface carryover (e.g. from spillage or glove fingerprint) and indoor contamination due to aerosol transport (e.g. from syringe manipulation procedures). Monitoring of MTX in environmental samples such as swab washings of surfaces and objects requires an analytical method with characteristics of sensitivity, reproducibility, precision, analytical speed, ease of automation and robustness. We have therefore developed an analytical procedure which employs simple short-column RP-HPLC with UV detection, automated sample injection and a close analogue internal standard for improved precision and solid-phase extraction (SPE) for sample concentration. Our method has proven suitable for detecting traces of MTX on a wide variety of surfaces and objects, with a limit of quantification in the range of 50 $\mu g/dm^3$ for direct injection of unconcentrated washings, corresponding to the possible detection of surface contamination as low as 1 $\mu g/m^3$ and a limit of detection in the range of 10 ng/m^2 for samples as large as 100 dm^3 concentrated by SPE. We present preliminary results from a recent hospital case-study, assessing the contamination level of furniture and equipment in drug preparation areas. Spillage fractions as high as 5% of the employed mass (70–260 mg/day) are measured on the polythene-backed paper disposable hood cover sheet; traces of MTX in the microgram range can also be measured on floor surfaces, furniture and handles, even at a distance from the preparation hoods.

Keywords: Methotrexate

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00561-1

1. Introduction

Concern regarding exposure of hospital staff to antineoplastic drugs (ANDs) is an emerging facet of occupational medicine in hospital premises, triggered by the epidemiological suspicion of an increased infertility, abortion and number of tumor cases in hospital nursing staff preparing cytostatic drugs and administering antitumoral therapy to patients [1–7].

To correctly assess the occupational health risks associated with manipulation of ANDs and to rationally advice intervention priorities for exposure abatement, reliable quantitative information on the exposure levels to individual compounds is needed, thus prompting the set-up of adequate monitoring procedures [8–10]. However, due to the large number of drugs currently employed in antineoplastic therapy and to the considerable differences in their properties and in the manipulated amounts, a first-choice approach to environmental monitoring involves the use of suitable tracer compounds, rather than detailed multiresidue analysis. Among the large number of currently employed ANDs, methotrexate (MTX) is a promising tracer of the contamination levels of workspaces due both to surface-to-surface carryover (e.g. from spillage or glove fingerprint) and to aerosol transport (e.g. from syringe manipulation procedures). In fact, MTX is a polyelectrolyte, with a high water, but low organic solvent solubility, a negligible vapour pressure and a high chemical robustness to environmental stress. It is widely employed not only in oncologic chemotherapy, but also as a general immunosuppressant in the therapy of autoimmune diseases, such as rheumatoid arthritis, and is a toxicologically relevant compound, for which teratogenicity has been demonstrated.

Several high-performance liquid chromatographic (HPLC) methods have been published for the determination of methothrexate (and of some metabolites and structurally related compounds) in biological samples (e.g. [11–22]), mostly for pharmacokinetic purposes, but in a few studies also for biological and environmental monitoring of occupational exposure [11,16]. This paper deals with the set-up of a HPLC method for measurement of contamination from MTX in hospital drug preparation areas and describes some relevant applications in the exposure monitoring in oncologic hospital departments.

2. Experimental

2.1. General

2.1.1. Chemicals

All solvents and reagents were analytical or the highest grade available. Standard methothrexate (MTX; L-4-amino-N^{10}-methylpteroyl-glutamic acid [133073-73-1]) was obtained as a 5 mg lyophilized pharmaceutical preparation of the sodium salt (Ledertrexate, Lederle) obtained from a hospital pharmacy. Aminopterin (AMP; 4-amino-pteroyl-glutamic acid [54-62-6]) was purchased from Aldrich (The Netherlands). All analytical standards and solutions were kept frozen in the dark in the smallest aliquots suitable for daily use. SPE disposable cartridges (Bond-Elut Jr®, C_{18}, 1000 mg) were purchased from Varian (USA).

2.1.2. Equipment

HPLC analyses were performed on a modular computer-controlled liquid chromatography system composed of an AS1000 auto sampler fitted with a fixed-volume 20 μl sample loop and employing 2-ml glass vials, a P1000 isocratic pump, a CrocoCil thermostat oven with temperature controller and a UV1000 UV–vis detector, all interfaced to the proprietary PC-1000 ver. 3.0 data system (Thermo Separation, USA).

2.1.3. Standard solutions

The stock solution of MTX was prepared by dissolving the 5 mg pharmaceutical preparation in 5 ml of deionized water for in vivo use; that of the selected chromatographic internal standard (I.S.), AMP, was prepared by dissolving a weighed amount of the pure compound (≈5 mg) in the minimum volume of 1 m*M* NaOH and diluting the resulting solution to 50 ml. Appropriate amounts of the stock solutions were further diluted to the required analytical concentrations with deionized water for in vivo use. Calibration solutions containing 1 μl/ml of AMP as the intenal standard (I.S.) and five linearly

decreasing concentrations of MTX (1, 0.5, 0.25, 0.125, 0.062 μg/ml) were freshly prepared and analyzed with each sample series.

2.2. Liquid chromatography

2.2.1. Column

All separations were accomplished on a column system composed of a 0.5 μm frit filter, a 10×4.6 mm I.D. Supelguard column placed before a 33-mm long Supelcosil LC-18 analytical column, both packed with spherical 3 μm RP-18 bonded silica particles (Supelco, USA).

2.2.2. Mobile phase

A 5 *M* solution of ammonium acetate, filtered through a 0.20-μm filter under water-jet vacuum and stored at 4°C was employed as stock, freshly diluted to the required concentration and mixed with the organic solvent to prepare all mobile phases. The final mobile phase was prepared by adding 4 ml of acetonitrile to 96 ml of 10 m*M* ammonium acetate (NH_4OAc) aqueous buffer, acidified with AcOH to pH 6. Flow-rate was set to 2 ml/min and the column was thermostated to 40°C. The compounds were detected by absorbance monitoring at 310 mm.

2.2.3. k' values

k' values of analytes were calculated as: $k'_{CPD} = (t_{CPD} - t_{VOID})/t_{VOID}$. The void elution time was derived, for each autosampler injection of the standard solutions, from the earlier spike peak due to switch of the automated Rheodyne valve from the LOAD to the INJECT position.

2.2.4. Analytical calculations

MTX quantification in samples was accomplished with the I.S. technique (peak area ratio vs. concentration plot) by comparison with a standard curve obtained from calibrators in water solution. Complete statistical calculations of the regression curve including 95% confidence limits for concentration calculations were performed off-line, on a standard computer spreadsheet. The lower limit for detection was calculated as the concentration corresponding to a (MTX/AMP) peak ratio given by the intercept plus three times its upper confidence limit, calculated on the lower curve of the confidence limit; percent accuracy was calculated as the relative difference between calculated and nominal concentration of the calibrators and percent precision as the standard error of determination of the calibrators [23].

2.3. Environmental sampling and analysis

The collection of MTX contamination on laboratory furniture and surfaces (e.g., board and inside surfaces of the preparation hood, nearby floor, window panes, cupboards) and on objects (e.g., drawer handles, laboratory equipment, telephones) was accomplished by thoroughly wiping the surfaces with cotton swabs dipped in 0.1 *M* ammonium acetate solution. A typical volume of washing solution of 20 ml can be employed to sample from as a wide surface as ≈ 0.5 m^2. The liquid was removed from the swollen cotton swabs by thoroughly pressing them in a 100-ml plastic syringe and measuring the volume in a graduated glass cylinder. Removable objects, such as used gloves and the disposable cover cloth of the hood board (where employed) were taken to the laboratory, dipped (as a whole or as appropriate representative sub-samples of larger items) into appropriate volumes of washing solution and analyzed.

All washings were at first analyzed as such, after adding 1 μg of I.S. to a 1-ml sample; negative or dubious samples were re-analyzed, concentrating an appropriate larger aliquots (10–100 ml) of the sample by solid-phase extraction (see below) and adding the same amount of internal standard before injection. A calibration curve in the 62.5–1000 μg/ml range was analyzed along with each sample series, consisting of 6–13 samples for each investigated department, depending on its characteristics.

Results referring to contamination of flat surfaces (such as hood surfaces and board, floor, walls) were expressed as mass of MTX (i.e. the measured analytical concentration in the chromatographic sample multiplied by the appropriate volume fraction of the washing or extraction sample) per unit surface (measured with an approximation of ±1 cm). With a view to comparing the contamination level between flat surfaces and objects, such as handles, telephones, gloves, or between different objects, contamination

was calculated also for irregular-shaped objects, by estimating surface from their approximate geometric size: for instance, the surface of a telephone was calculated as being ≈ 0.015 m^2.

2.3.1. Enrichment of environmental samples

The MTX contained in environmental extracts at a higher dilution than that allowing detection by direct injection of washings was concentrated by SPE on a RP-18 disposable cartridge. Briefly, cartridges were previously activated by washing with 3 ml of methanol, then 3 ml of water and thoroughly dried by gentle pressurization. The sample solution was previously filtered through filter paper if grossly turbid and a volume of 20–100 ml was passed through the cartridge by vacuum suction. After thorough cartridge drying with 5 ml of air, the retained organics were eluted with 2×l-ml portions of a freshly prepared methanol solution containing 1% v/v aqueous ammonia. The filters were pooled, dried under vacuum, reconstituted with 1 ml of aqueous I.S. solution (1 μg/ml) and transferred to the autosampler vial.

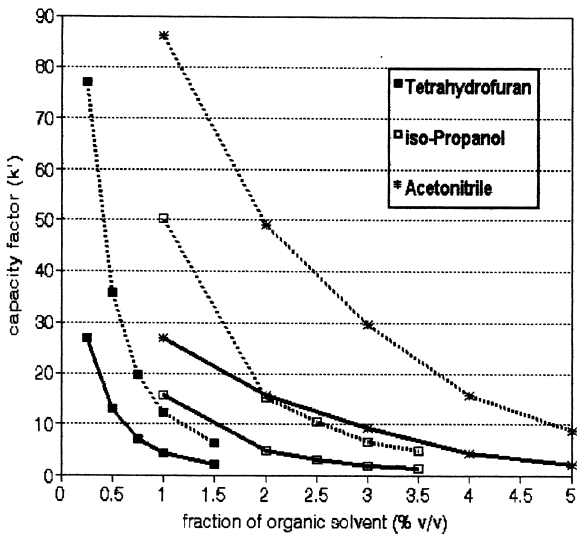


Fig. 1. Relationship between proportion of organic solvent (tetrahydrofuran or isopropanol or acetonitrile) in the aqueous mobile phase (100 m*M* ammonium acetate, pH 6.85) and the capacity factor (k') of MTX (dotted lines) and of the internal standard, AMP (solid lines). Separations were carried at room temperature on the standard (10+33)×4.1 mm column at a flow-rate of 2 ml/min; detection by UV was at 310 nm.

3. Results

3.1. Analytical method

3.1.1. Chromatographic separation

The influence of buffer strength, pH value, nature and proportion of organic solvent (either tetrahydrofuran, THF; isopropanol, i-PrOH or acetonitrile, MeCN) in the eluent was carefully studied in order to optimizing the analytical conditions. It was decided to avoid use of ion-pairing reagents and of non-volatile buffers (such as phosphate) in the chromatographic eluent, in order to allow the simplest interfacing to off-line mass spectrometric detection for confirmative analyses.

Fig. 1 shows the relationship between the proportion of organic modifiers in the mobile phase and the capacity factor (k') of MTX (dotted lines) and of the I.S., AMP (solid lines). In all cases, retention decreased with the increasing proportion of organic solvent: in this regard, THF yielded the strongest analyte elution among the three organic solvents considered, followed by i-PrOH and MeCN. Since MeCN yielded the best compromise between separation of the early-eluting AMP peak from the chromatographic front and delay in MTX elution, it was selected as the organic component of the chromatographic eluent.

The effect of pH on retention of AMP and MTX was studied at pH 5 and 7, since, at a lower value, UV absorbance of the highly specific band around 300 nm employed for selective detection and quantification decreases (possibly due to protonation of the pterine ring; spectrophotometric study data not shown). Under several chromatographic conditions, elution of both AMP and MTX was delayed at the more acidic pH (e.g. at pH 5: $k'_{AMP} = 9.6$; $k'_{MTX} = 31.2$; at pH 7: $k'_{AMP} = 4.7$; $k'_{MTX} = 19.9$); however, the two peaks eluted closer to each other (e.g., $k'_{AMP}/k'_{MTX} = 3.2$ at pH 5 vs. 4.3 at pH 7) thus improving peak shape (and therefore detectability) of the later-eluting MTX. This behaviour is similar to that reported by Hirai et al. for MTX and for its 7-OH metabolite [22]. The effect of buffer ion strength on retention was also tested with mobile phases composed of 10, 50 or 100 m*M* ammonium

acetate buffer at pH 5 and containing 5% MeCN, which yielded no difference in retention times; it was therefore decided to employ the lowest ionic strength. Heating the column to 40°C yielded an improvement over room temperature (20–22°C) in that k' values for both compounds were lower by ≈20%, thus shortening the chromatographic run without loss of resolution and also lowering column backpressure.

Fig. 2 reports typical chromatographic separations of MTX and the selected I.S., AMP. The chromatogram refers to direct analysis of a washing from a contaminated surface. The early eluting I.S. is fairly well separated from the interfering peaks at the front, even with a short chromatographic run of 8 min, thus allowing the analysis of as many as seven samples per hour with the use of an autosampler. Under standard operating conditions, a volume of 200 ml of the mobile phase could be continuously recycled for the analysis of real-life samples, with no apparent effect on chromatographic resolution, column backpressure or analytical sensitivity, for as long as 1 week.

3.1.2. Linearity, sensitivity and reproducibility

The analytical validation of the method was performed concurrently to sample analysis. The intra-assay accuracy and precision was evaluated by preparing and analyzing four series of calibrators in the range 62.5–1000 ng/ml; inter-assay accuracy and precision was evaluated from the seven individual calibration curves, each associated with many study samples, prepared and analyzed within a 6-month period. One typical linear calibration curve with complete statistical parameters of the assay is reported in Fig. 3.

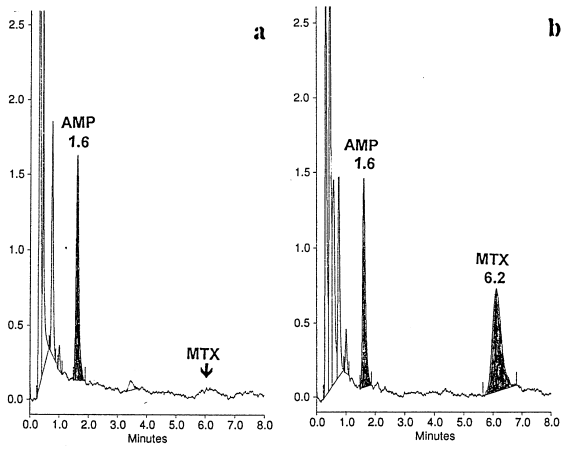


Fig. 2. Typical chromatographic separation of MTX and the internal standard, AMP. (a) Blank sample by direct injection (b) washing sample from a contaminated surface (direct injection; MTX concentration of ≈200 ng/ml).

3.1.3. Sample stability

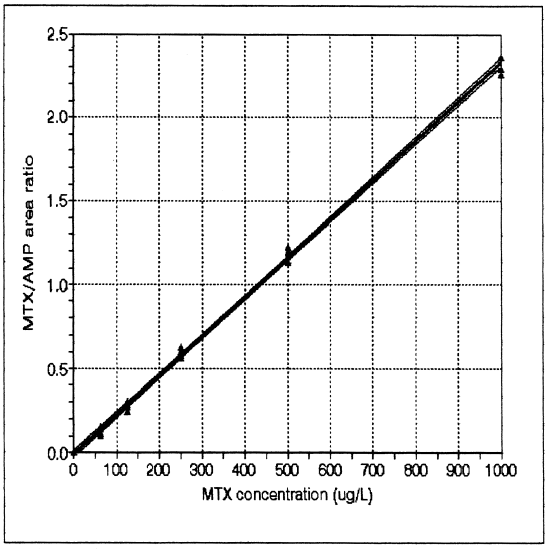
Samples were usually analyzed immediately after return of the industrial hygiene team to the laboratory (same day) and the global analytical procedure, including SPE sample enrichment was usually completed within 48 h; therefore an explicit study of sample stability was not carried out. Aliquots of few interesting samples were stored at −20°C in the disposable autosampler vials for as much as 6 months and those reanalyzed did not show any deterioration, as far as their chromatographic profile is concerned.

3.2. Analysis of environmental samples

The chromatograms reported in Fig. 2 show typical results obtained in monitoring surface contamination of preparation rooms in hospital departments. Sample a was obtained from rinsing a surface in the drug preparation area of a department where MTX was not in use. Sample b was obtained from rinsing the front surface and the handles of a small chest of drawers beneath the preparation hood (approx. surface 0.35 m^2) with cotton swabs wet with ammonium acetate (total volume of the washing 20 ml). The analytical concentration of MTX in the sample was ≈0.2 μg/ml, corresponding to ≈11 μg/m^2 on the surface.

3.2.1. Sample concentration by solid-phase extraction

A single, abundant sample taken from a monitoring study was analyzed to test the efficacy of sample concentration by SPE with reference to the sample volume accepted from the cartridges and to recovery of the analyte. Direct analysis of a 1-ml sample of a 20-ml washing of laboratory furniture (the inside surface of the hood top) gave a concentration of ≈99 ng/ml; after SPE concentration of



linear regression of calibration curve [a]	
range of calibration (ng/mL)	62.5-1000
intercept	-0.011
error-of intercept	±0.013
slope	$2.34*10^{-3}$
error-of-slope	$\pm 0.02*10^{-3}$
regression coefficient r^2	0.9991
number of curves	4
limit of detection (ng/mL; S/N > 3)	47

target concentration (μg/L)	intra-assay (n=4) [b]				inter-assay (n=7) [c]			
	measured	± st.dev	accuracy (%)	precision (%)	measured	± st.dev	accuracy (%)	precision (%)
1000	1015	57	+1.5	5.6	1012	20	+1.2	2.0
500	510	17	+2.0	3.3	471	41	-5.9	8.2
250	255	11	+2.2	4.3	254	21	+1.6	8.6
125	120	11	-3.9	9.1	133	19	+6.7	15.5
62.5	57	10	-8.1	17.4	67	20	+7.3	32.3

Notes: [a] the calibration curve shown in Figure is elaborated from four independent preparations of the calibrators, prepared and analyzed in the same day; [b] same data of the curve shown in Figure; [c] data from the individual calibration curves prepared and analyzed with the study samples.

Fig. 3. Plot of calibration curve and evaluation of the statistical parameters for intra- and inter-assay validation of MTX quantification vs. AMP internal standard.

the remaining sample, the concentration of the injected sample amounted to ≈1760 ng/ml. The percent recovery of MTX in the 20-ml sample (containing a total MTX mass of ≈1.98 μg) thus exceeds 93% (data not shown).

The chromatogram of Fig. 4a represents direct injection of a washing sample taken from a laboratory shelf (≈0.25 m^2): MTX concentration is below the chromatographic limit of detection. Fig. 4b shows the chromatogram obtained by concentrating on a C_{18} SPE cartridge all of the remaining sample (≈19 ml). The observed peak corresponds to a mass of MTX in the entire swab sample of 280 ng, i.e., to a surface contamination ≈1 $\mu g/m^2$, a value considered to correspond to very low or 'background' contamination.

3.3. Results from field measurements

The described method is currently employed to measure the MTX contamination levels in hospital drug preparation areas. A preliminary survey campaign was performed within a large general hospital in Milan with as many as six departments or departmental services dispensing antineoplastic chemotherapy (with several ANDs; MTX was employed in only four of them) to in-hospital or outpatients. Departments varied widely in lay-out

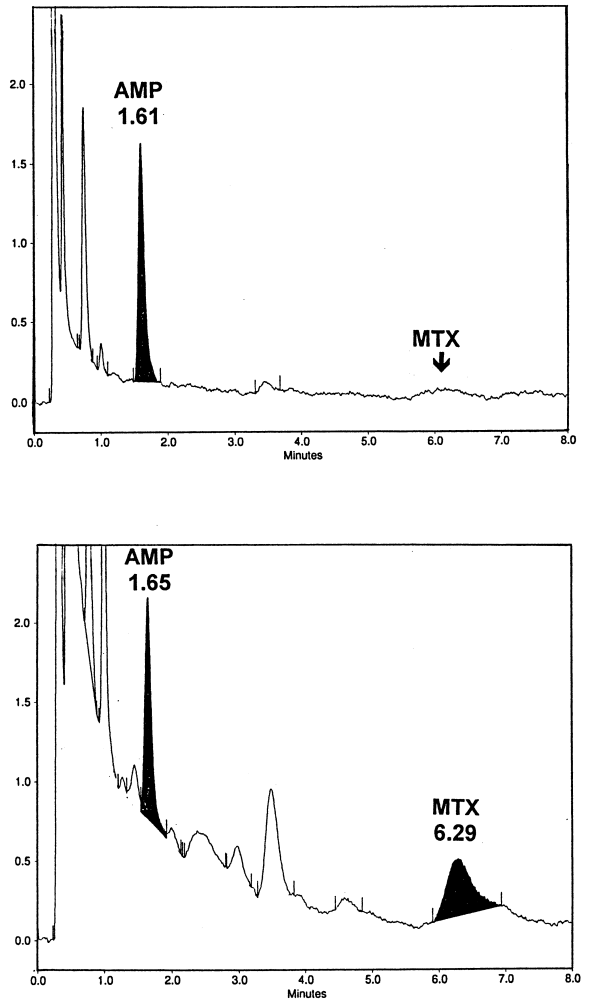


Fig. 4. Detection of a weak MTX contamination in a drug preparation area. (a) Direct injection of a sample of the washing (b) sample after concentration on a C_{18} SPE cartridge of ≈20 ml of the washing (MTX content of ≈280 ng).

and operational characteristics, ranging from day-hospital and inpatient premises to restricted-access isolation rooms for immunosuppressed patients awaiting bone-marrow transplantation (BMT). To proof the applicability of the method, the results of some measurements are summarized in Table 1. A total of 34 samples were collected from four preparation rooms, all equipped with a preparation hood (two chemical, with a charcoal filter; two laminar flow; all hoods recycling ambient air). In all four rooms, washing samples were taken from the hood inside walls, the hood board (and the disposable blanket cover, when used), the floor beneath and around the hood (1 m depth on the front and sides); random samples were taken from room walls, window panes and horizontal and vertical furniture surfaces (shelves, cupboard and drawer chests). Only nine samples could be directly measured, while 25 needed concentration; 20 samples (60% of the total number) yielded contamination figures above the analytical limit of quantification of 15 ng/ml. Surface contamination levels from MTX ranged from 0.5 to 60 $\mu g/m^2$, the higher values being measured in the preparation room of the day-hospital oncologic department, the lower in the BMT department. A single highest values of 645 $\mu g/m^2$ was measured on the disposable paper blanket covering one hood board.

4. Discussion

The analytical conditions selected for measurement of MTX surface contamination in hospital premises are a compromise between several conflicting needs: a high sample throughput is necessary to expeditously manage the large number of samples obtained from on-site inspections; specificity and sensitivity should be adequate to trace surface contamination in the $\mu g/m^2$ range and, finally, switching between different analytical methods should be as fast as possible.

Most of the several HPLC methods recently published for the determination of methothrexate (and of some metabolites and structurally related compounds) in biological samples deal with pharmacokinetics and therapy monitoring in patients, while one study reports method set-up and application to biological (but not environmental) monitoring of exposure in oncologic nurses [10,16] and one deals with measurement of hospital workers exposure to aerosols of several antineoplastic agents including MTX, but without parallel biological monitoring [8,11]. All methods employ separation on reversed-phase C_{18} columns with mixtures of organic solvents with aqueous buffers at several values of pH ranging from almost neutral down to 2.7. Detection by UV monitoring at the 300–320 nm

Table 1
Synopsis of contamination from MTX measured in four departmental areas for preparation of cytostatic drug within a large general hospital

Sampling position within preparation area		No. of samples	No. of negative samples	Min–max ($\mu g/m^2$)
Hoods	Inside	3	1	3; 25
(1 chemical	Board	2	0	1; 7
3 laminar flow)	Blanket	2	0	62; 645
Surfaces within 1 m	Chest of drawers	4	2	0.5; 11
of hood	Table board	1	1	n.d.
	Shelves	5	4	1
	Floor	4	1	1–53
Other surfaces	Telephone	1	0	11
and objects	Handles	2	1	3
	Window panes	4	4	N.d.
	Others	6	0	24–200
	Total	34	14	0.5–645

absorption band of the pterine ring allows specific measurement of MTX concentrations in the low ng/ml range [11,12,14–16,18,20]. A further increase in sensitivity as high as 20-fold has been obtained by post-column oxidation and fluorescence detection [13,17,19,21]; however the method appears rather difficult to carry out under routine conditions. Only four papers employ an internal standard to improve assay precision: three of them use the only commercially available analogue of MTX, AMP [12,14,15], while the other employs a structurally unrelated compound, 8-chloroteophylline [20]. Under several chromatographic conditions tested, AMP elutes much earlier than MTX ($k'_{MTX}/k'_{AMP} > 3$), thus limiting the possibility to optimize the peak shape of the analyte without resorting either to gradient elution [14], or to the use of ion-pairing reagents [20].

In particular, the method set-up and applied by Mader et al. to biological monitoring of hospital nurses [16] is able to attain a high sensitivity (as low as 4 ng/ml in urine or 40–200 pg on column) with simple UV detection, but needs substantial sample preparation and a lengthy and complicated column-switching liquid chromatographic separation procedure requiring more than 45 mm between successive sample injections; therefore it is hardly adequate for high sample throughput in general-purpose industrial hygiene laboratories which need short analysis times and fast switching between a number of different monitoring methods.

Our assay is characterized by a simple and fast chromatographic run on a short column, which allows the analysis of a large number of samples within a work-day; the use of isocratic conditions and of a mobile phase with a percent-low organic content allows recycling, inexpensive and unrestricted disposal of exhausted material and minimal exposure of laboratory personnel to potentially noxious chemicals. The limit of detection of the method approaches 50 ng/ml (corresponding to $\approx$1 ng on-column): as shown by the data of Fig. 3, precision within 10% can be obtained for quantification of samples at a concentration of $\approx$250 ng/ml, which corresponds to detection of 5 μg of MTX in a typical 20-ml washing. Considering that a such volume of washing can be employed to sample from a surface as wide as 0.50 m^2, contamination of surfaces can be reliably determined in the range of 10 $\mu g/m^2$, and detection is feasible at <1 $\mu g/m^2$. The analytical method is therefore suitable for routine detection of surface contamination from methotrexate in hospital drug preparation areas. Moreover, one advantage of environmental with respect to biological samples is that they are dilute, rather than highly contaminated: washings can therefore be easily concentrated by SPE. Concentration of surface washings by SPE on C_{18} silica cartridges of volumes as high as 100 ml

without breakthrough of solutes allows a sensitivity increase of 10- to 50-fold, thus lowering the limit of quantification to $<1\ \mu g/m^2$, a sensitivity adequate to detect even MTX contamination due to surface-to-surface carryover (e.g. from glove fingerprint) on such objects as drawer handles, telephones and gloves.

5. Conclusions

Adequate quantitative methods for measuring the environmental concentrations or levels of antineoplastic drugs are a prerequisite to the assessment of health risk associated to the occupational exposure to these compounds and to the rational planning of interventions aimed at exposure reduction and improvement of the safety of hospital personnel. The described analytical method is now routinely employed in our laboratory to monitor the contamination level of surfaces in the preparation rooms, even far from the work bench. Reliable quantitative information on the dissemination pathways of tracer antineoplastics is of utmost importance to establish correct operating procedures for drug preparation, disposal of residues and decontamination of surfaces.

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