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# Determination of 5-fluorouracil in environmental samples by solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

Giuseppina Micoli, Roberta Turci, Marta Arpellini, Claudio Minoia*

*Laboratory of Environmental Hygiene and Industrial Toxicology, Salvatore Maugeri Foundation, via Alzaia 29, Pavia, Italy*

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**Abstract**

5-Fluorouracil (5-FU) is one of the most widely used antineoplastic drugs. It can be therefore considered to be a model compound for the identification of exposure routes during preparation and administration of cytostatic agents, especially for nucleoside analogue drugs. In this study, an HPLC–UV method was validated for determination of 5-FU in wipe samples by direct analysis of the aqueous solutions and in air samples by using solid-phase extraction (SPE). When samples were pre-treated on styrene–divinylbenzene resin SPE columns, a 20-fold preconcentration of the analyte was achieved. As regards air samples, correlation coefficients were always higher than 0.998 and the limit of detection was assessed at 15 ng on filter. In order to verify the reliability of these procedures, 5-chlorouracil was used as internal standard. The procedure presented here has been applied to the environmental monitoring of occupational exposed subjects. The amount of 5-FU ranged from 0.043 to 0.23 $\mu g/m^3$ in air samples and from 0.2 to 470.1 $\mu g/dm^2$ in wipe samples. 5-FU was also detected on the internal side of the gloves (0.07 to 3.77 $\mu g$/pair of gloves). 

*Keywords:* 5-Fluorouracil

## 1. Introduction

5-Fluorouracil (5-FU) is an antimetabolite antineoplastic agent widely used for the treatment of several types of malignancies, such as colorectal cancer [1]. The International Agency for Research on Cancer (IARC) has included 5-FU into group 3 (inadequate evidence of carcinogenicity in humans) [2–4]. Nevertheless, 5-FU is a mutagen and a teratogen, as well as most of the antineoplastic drugs commonly used in chemotherapy [5,6].

Hospital personnel handling these agents may be exposed by three routes: inhalation of aerosolised drug, transdermal absorption and accidental ingestion. In particular, to assess exposure levels and hence identify the main exposure route, it is necessary to measure the amounts of the drugs in environmental matrices, such as filters, pads, gloves and wipe samples [7–10]. As regards the analytical technique, it should be cost-saving and easily available in most laboratories. Thus, high efficiency liquid chromatography coupled with ultraviolet detection (HPLC–UV) can be considered to be the most suitable technique for the determination of 5-FU in environmental samples. In this case, the sample

*Corresponding author. Fax: +39-382-578-764.
*E-mail address:* igamb@fsm.it (C. Minoia).

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treatment just involves dissolving the analyte in water, on condition that pH is properly adjusted. On the other hand, since even very low exposure levels may result in adverse effects, high sensitivity is required. In particular, determination of airborne 5-FU levels calls for a very low quantitation limit. In order to obtain a 20-fold preconcentration of the analyte (and consequently a lower LLQ), a solid-phase extraction (SPE) procedure was developed.

In this study, two different procedures for determination of 5-FU in environmental matrices (direct analysis and SPE), are presented. The HPLC–UV method was validated by using 5-chlorouracil as internal standard, and good precision and accuracy were obtained, so that this method can be successfully applied to the evaluation of occupational exposure to 5-FU.

## 2. Experimental

### 2.1. General

#### 2.1.1. Chemicals

5-fluorouracil and 5-chlorouracil were supplied by Sigma–Aldrich, Inc. (St. Louis, MO, USA). Stock solutions were prepared by dissolving the compounds in water. The standard solutions were freshly prepared every day, stored in the dark and refrigerated. Distilled and deionised water was produced from a Milli-Q Plus water purification system.

HiperSolv for HPLC™ ammonium acetate (purity>98%), acetic acid glacial, methanol (purity>99.8%), ethyl acetate (purity 99.8%) and diethyl ether (purity 98%) were obtained from BDH Laboratory Supplies, Poole, UK.

Isolute® $C_{18}$, $C_8$, $C_N$, ENV+, SAX (IST House, Duffryn Industrial Estate, UK) and Waters Oasis™ HLB (Waters, Milford, MA, USA) extraction cartridges were used. LC-Diol and ENVI ™Chrom P SPE tubes were obtained from Supelco (Bellefonte, PA, USA).

#### 2.1.2. Equipment

A Hewlett-Packard 1040 M Series II diode array detector was interfaced to an HPLC system, incorporating an HP HPLC 1090 ternary pump. A Chemstation HPLC-HP was also used to integrate peak areas.

### 2.2. Liquid chromatography

All separations were accomplished on a LiChrospher 100 RP18, 250×4 mm, 5 μm (LiChroCART® 250-4, Merck) and a guard column $C_{18}$ (4×4 mm). The selected wavelength was 265 nm. The mobile phase consisted of a methanol–0.02 *M* ammonium acetate buffer pH 4.7 (2:98, v/v). The flow-rate was 0.8 ml/min under isocratic conditions, with an injection volume of 100 μl.

### 2.3. Extraction procedure

Each filter or pad was pre-treated with 10 or 50 ml of pH 8 acetate buffer, respectively. After shaking, an appropriate aliquot of the samples was extracted by using SPE devices. A Varian VAC ELUT SPS 24 solid-phase extraction vacuum manifold was used to simultaneously process up to 24 samples. Isolute® ENV+ (packing 200 mg/6 ml column reservoir) SPE columns were first conditioned with 6 ml of methanol and 6 ml of 0.02 *M* ammonium acetate buffer (pH 5). In order to ensure that the SPE packing did not dry before sample addition, about 1 mm of the buffer solution was allowed to remain above the top tube frit. Then, a 10-ml aliquot of each pre-treatment solution was transferred to the tube and the sample was allowed to pass through the extraction device. To maximise recoveries of the analyte, the interference elution solvent was maintained at the same pH as that of the sample solution. Hence, the tubes were washed with 2 ml of pH 5 acetate buffer. After the column washing step, the ENV+ sorbent bed was dried thoroughly by applying a mild vacuum to the manifold.

Finally, 5-FU was dropwise eluted with three 1-ml aliquots of methanol–ethyl acetate (1:1, v/v). The eluate was evaporated to dryness under a nitrogen stream and the residue was reconstituted with 500 μl of mobile phase and then injected onto the HPLC column.

### 2.4. Validation study

During environmental monitoring of occupational exposed subjects, contamination of the working areas was measured by using wipe samples (WS). Four cotton gauzes were soaked in acetate buffer (pH 4.7) and objects and surfaces were swept clean [11].

Calibration samples were prepared by spiking each blank wipe sample with 500 μl of appropriate solutions so as to obtain 5-FU amounts of 1, 2, 4, 8, 16, 32 μg on the matrix. Each sample is then put into a glass bottle with 50 ml of an aqueous solution adjusted to pH 8, vortexed and kept in the dark overnight. Since expected drug levels on wipe samples are rather high, preconcentration is not necessary and an aliquot of the sample solution can be directly injected onto the HPLC column.

In order to test the intra- and inter-day precision and accuracy of the method, wipe samples were spiked in quadruplicate at three levels, low (3 μg), middle (12 μg) and high (24 μg), on three subsequent days.

With a view to evaluating the exposure level depending on potential inhalation, total airborne particulate matter was collected on borosilicate microfiber filters (Ø 20 mm) by using active samplers at a flow-rate of 2 l/min [11].

Calibration samples were prepared by spiking each blank filter with 100 μl of appropriate solutions so as to obtain 5-FU amounts of 15, 30, 60, 120, 240 and 480 ng on the matrix. Each sample was then pretreated with 10 ml of an aqueous solution adjusted to pH 8, vortexed and shaken for 1 h. Since expected drug levels on filters are low, a further treatment is required so that the sample solution can be concentrated enough for detection. To this end, the calibration air samples (AS) were purified as reported in the Section 2.3.

In order to test intra- and inter-day precision and accuracy of the method, air samples were spiked in quadruplicate at three levels, low (45 ng), middle (180 ng) and high (360 ng), on three subsequent days.

All the samples were also spiked with 5-chlorouracil (32 μg for wipe samples and 480 ng for air samples), which was used as internal standard.

Peak areas were measured by a Chemstation HPLC-HP and the ratios of the peak area of 5-FU to that of 5-CU were plotted against theoretical 5-FU amounts. The resulting slopes and intercepts of the standard calibration curves were used to back-calculate the values for quality control samples. Accuracy is defined as the percent difference between the measured mean concentrations and the corresponding nominal ones. The lower limit of quantitation (LLQ) is the lowest concentration which can be determined with an inter-day relative error and precision less than or equal to 20%. The limit of detection (LOD) is defined as the concentration yielding a signal intensity three times the background value.

## 3. Results and discussion

### *3.1. Extraction procedure*

5-FU solubility in aqueous solutions increases with increasing pH of the solution [12]. When analysing WS, high contamination levels are expected, so preconcentration procedures can be avoided. In order to quantify the recovery of the analyte from the matrix, WS were spiked with 5-FU so as to obtain a final concentration of 0.05 and 0.4 μg/ml, for low and high levels respectively. Then, each WS was put into a glass bottle with 50 ml of water and an aliquot of this solution was injected onto the chromatographic system. This experiment was carried out in duplicate for each concentration and at 5 different pH (range 4–8). The highest recovery was obtained at pH 8.

When considering other matrices, for example filters or pads, expected 5-FU levels are much lower, so that preconcentration of the sample is necessary to obtain a lower limit of quantification. At first, liquid–liquid extraction was taken into consideration. Nevertheless, the recovery was less than 20% for all the mixtures and solvents considered, and a solid-phase extraction (SPE) procedure was therefore developed.

In order to choose the most suitable cartridge for SPE, seven different tubes were conditioned with 3 ml of methanol and 3 ml of pH 5 buffer. A 1-μg/ml 5-FU solution was adjusted to pH 5 and 3-ml aliquots were then added to the cartridges. From the analysis of the effluent, it resulted that ENVI-Chrom P can retain the highest percentage of the analyte. Table 1 shows the retention efficiencies for each SPE column.

To select the best elution solvent or mixture, corresponding to the highest recovery, an experiment in triplicate was carried out. ENVI-Chrom P tubes (250 mg/6 ml) were conditioned with 3 ml of MeOH and 3 ml of pH 5 buffer. After adding 3-ml aliquots of a 1-μg/ml 5-FU solution (pH 5), ethyl acetate, mixture A (MeOH–diethyl ether, 1:1, v/v)

Table 1
SPE columns and corresponding retention efficiencies

| SPE column | 5-FU retained by the sorbent (%) |
|---|---|
| Isolute® $C_{18}$ | 0.2 |
| Isolute® $C_8$ | 32.5 |
| Isolute® $C_N$ | 1.3 |
| Isolute® SAX | 33.1 |
| LC-Diol | 41.0 |
| ENVI-Chrom P | 87.1 |
| OASIS™ | 0.3 |

and mixture B (MeOH–ethyl acetate, 1:1, v/v), were used to elute the analyte. After elution with mixture B, a mean recovery of 85% was obtained.

As regards the sample pH, no significant differences were observed for pH values less than 7, but a considerable fall in 5-FU recovery was noticed when increasing pH of the loaded solution. However, the intermediate value of 5 has been chosen, so as to standardise procedures.

In order to enhance sensitivity, a larger sample volume was added to SPE tubes. Nevertheless, when increasing the sample volume applied to ENVI-Chrom P, a lower 5-FU recovery was observed. Therefore, some extra experiments were carried out by using Isolute® ENV+ SPE columns (200 mg/6 ml), which are very similar to ENVI-Chrom P, but are characterised by a higher surface area (about 1100 $m^2/g$). Two 5-FU solutions (0.2 and 1.0 μg/ml) were adjusted to pH 5 and different volumes were loaded in duplicate on both ENVI-Chrom P and ENV+ SPE devices. The analyte was eluted with 3 ml of mixture B, evaporated to dryness and re-dissolved in 500 μl of mobile phase. When ENVI-Chrom P was used, recovery depended on the applied sample volume. Otherwise, when using ENV+, recovery was independent of both 5-FU concentration and applied volume, as you can see in Fig. 1a and b. If the applied volume was 10 ml, the mean recovery was 94.1% if using ENV+, compared to 33.4%, if using ENVI-Chrom P. On the basis of these results, ENV+ has been chosen as the most suitable SPE column for 5-FU clean up.

### *3.2. Chromatographic separation*

A typical chromatogram of a wipe sample spiked with 12 μg of 5-FU and 32 μg of 5-CU (I.S.) is shown in Fig. 2. Similarly, Fig. 3 shows the chromatogram of a filter spiked with 180 ng of 5-FU and 480 ng of 5-CU (I.S). The chromatographic run lasted 15 min and the retention times were about 4.85 min and 7.95 min for 5-FU and 5-CU respectively. Diode Array Detection (DAD) provides for high peak purity. Moreover, no endogenous peaks were detected during the chromatographic analysis.

### *3.3. Validation study*

Linearity of detection response was assessed for both aqueous standards (WS) and extracted standards (AS) over the range 1 to 32 μg and 15 to 480 ng, respectively. The regression curves, obtained by plotting peak area ratio against theoretical 5-FU amounts had correlation coefficients always higher than 0.998 and intercepts were not significantly different from zero (Table 2).

Precision, between days and within day, was calculated as the observed coefficient of variation (C.V.%) at each level. The intra-day C.V.% ranged from 0 to 8.5%, while day-to-day precision was always less than 13.1%. The method is also accurate with a relative error$<$109% and 110% for inter-day and intra-day experiments, respectively (Table 3).

As regards wipe samples, the limit of detection (LOD) was 1 μg and the lower limit of quantitation was assessed at 3 μg. At this level, day-to-day C.V.% was less than 20% and inter-day accuracy was about 100% (Table 4).

Solid phase extraction allows a 20-fold preconcentration of the analyte, and consequently improves sensitivity. As regards air samples, LLQ was 30 ng and the precision of the determination was less than 16%, with an adequate assay accuracy (98.0%). The limit of detection was assessed at 15 ng on filter.

This method is therefore simple and sensitive enough to be applied to the environmental monitoring of hospital personnel occupationally exposed to 5-FU.

### *3.4. Environmental samples*

Sixteen subjects occupationally exposed to antineoplastic agents participated in this study. Sampling occurred in four units of an Italian hospital on two

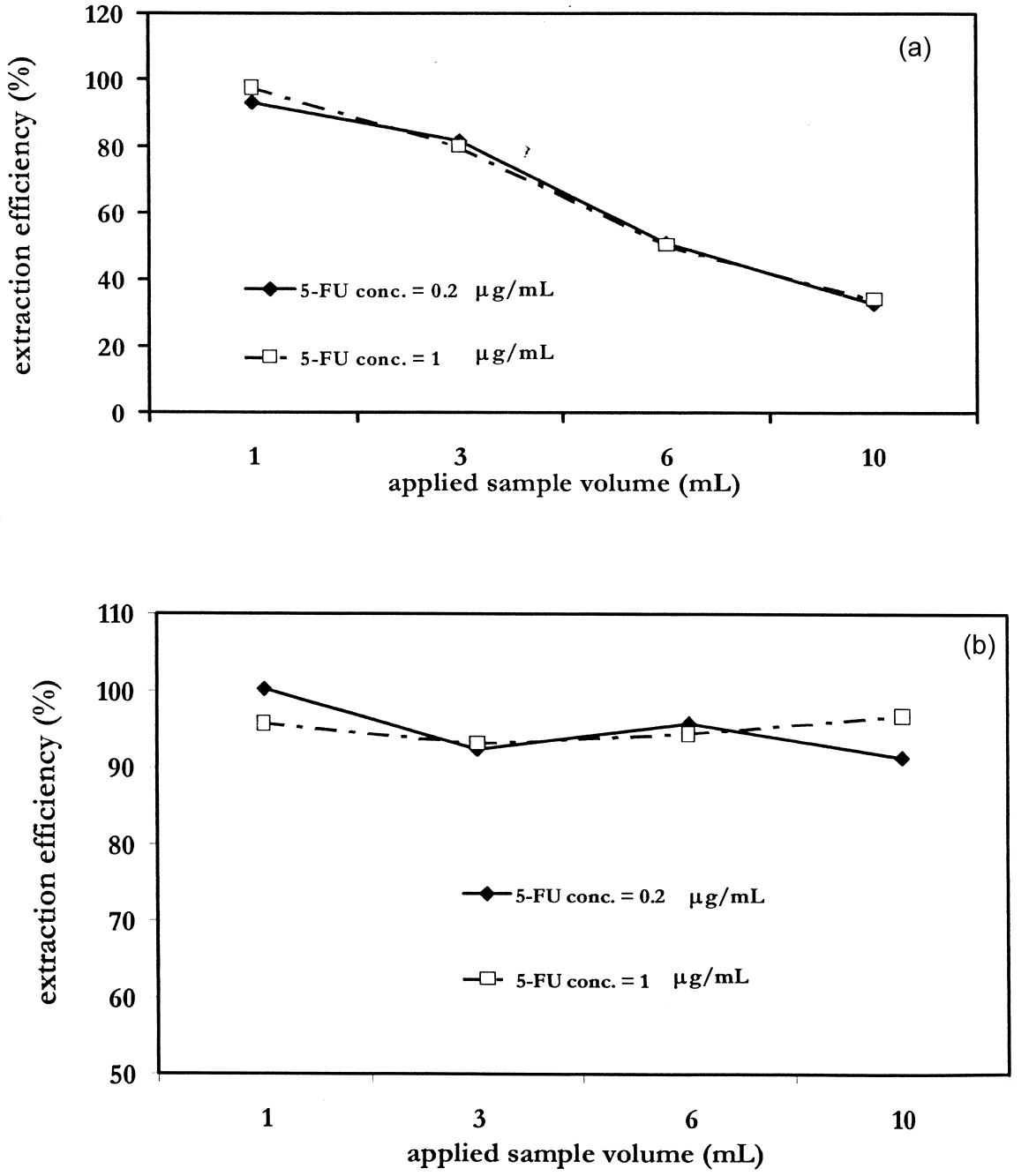


Fig. 1. (a) Extraction efficiency depending on applied sample volume when using ENVI-Chrom P (pH of the sample=5; elution volume=3 ml). (b) Extraction efficiency depending on applied sample volume when using ENV+ (pH of the sample=5; elution volume=3 ml).

consecutive days. On the first day, the personnel were monitored during preparation only, whereas on the following day, monitoring was also carried out during administration.

*3.4.1. Air samples*

As regards stationary samples, 5-FU was detected in one (out of 12) sample taken from the centre of the preparation area (0.043 $\mu g/m^3$). Three out of 27

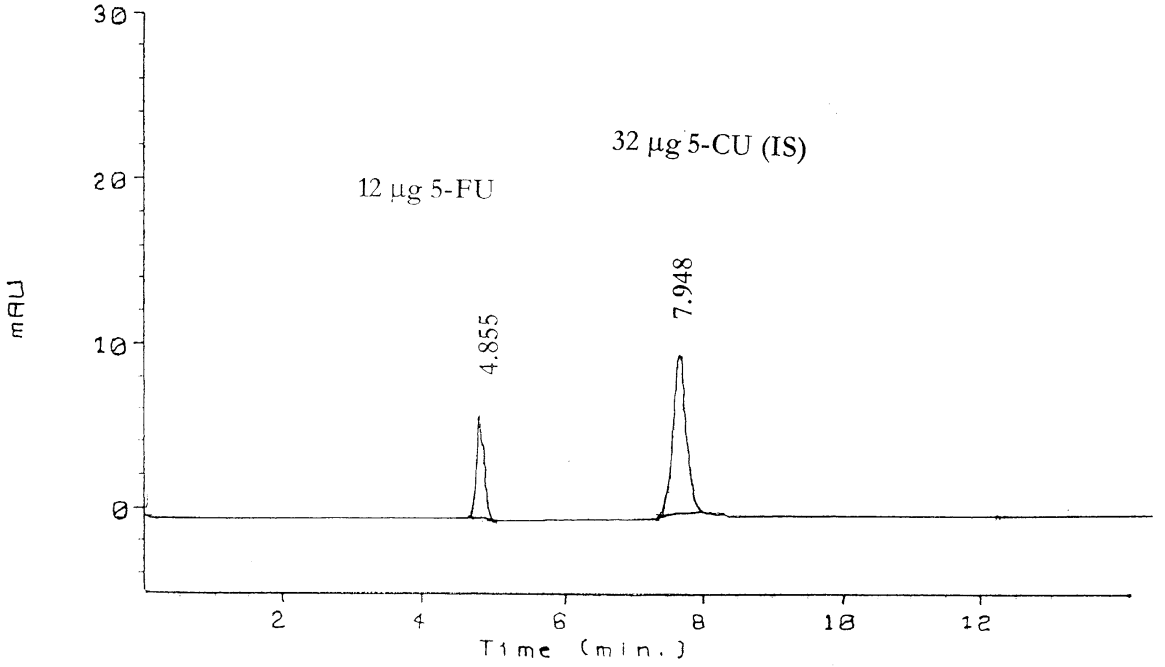


Fig. 2. Representative chromatogram obtained from the analysis of a spiked wipe sample (12 μg 5-FU and 32 μg 5-CU).

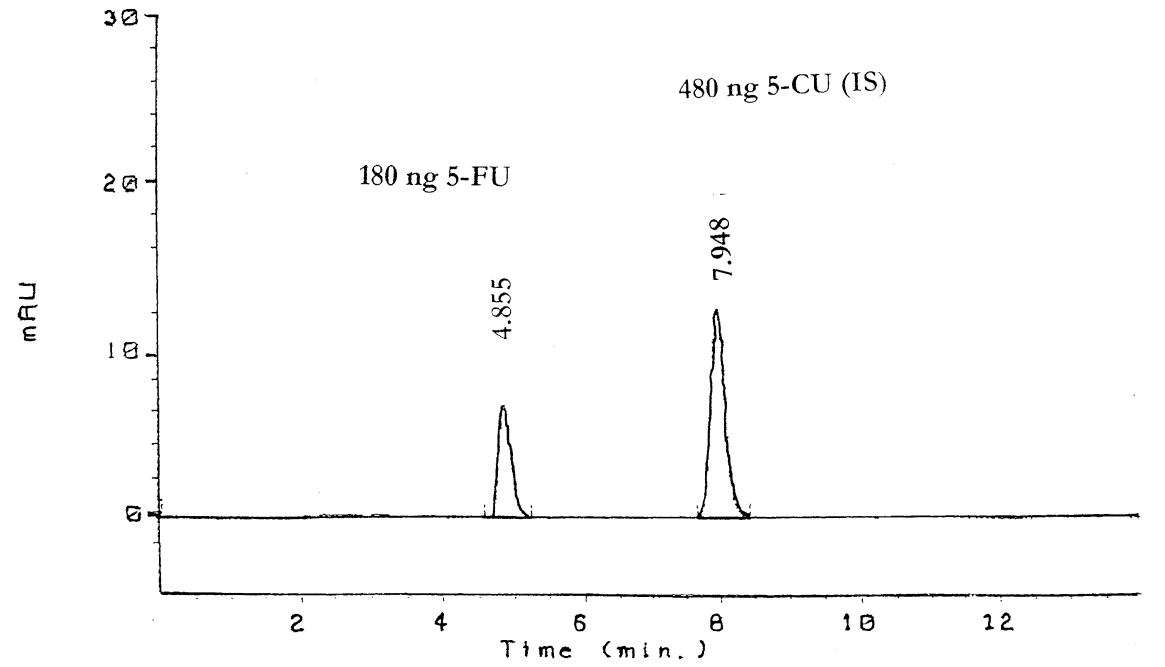


Fig. 3. Representative chromatogram obtained from the analysis of a spiked filter (180 ng 5-FU and 480 ng 5-CU).

Table 2
Calibration parameters for quantitation of 5-FU on air and wipe samples

| | Wipe samples | Air samples |
|---|---|---|
| Range of calibration | 1–32 μg | 15–480 ng |
| Intercept mean | 0.043 | 0.025 |
| Intercept S.D. | 0.018 | 0.021 |
| Slope mean | 0.379 | 3.00E-03 |
| Slope S.D. | 0.288 | 1.73E-04 |
| Regression coefficient (mean) | 0.9989 | 0.9991 |
| No. of curves | 3 | 3 |
| Limit of detection | 1 mg | 30 ng |

personal samples taken during drug preparation were positive for 5-FU (0.05–0.23 $\mu g/m^3$).

### *3.4.2. Wipe samples*

On the first day, 5-FU was detected in 30 out of 61 samples taken from different locations in four units. The highest amounts were determined inside the hood (up to 71.5 $\mu g/dm^2$). It is also noticeable that significant amounts of 5-FU were also detected on the floor in front of the hood (1.2 $\mu g/dm^2$), on the phone (0.5 $\mu g/dm^2$), on the fridge handle (0.5 $\mu g/dm^2$) and at the top of the hood near the HEPA filter (8.0 $\mu g/dm^2$). On the following day, higher 5-FU levels were detected, ranging from 0.2 to 470.1 $\mu g/dm^2$, depending on the sampling location. For example, 211 $\mu g/dm^2$ 5-FU at the top of the hood and 146.1 $\mu g/dm^2$ on the floor in front of the hood were detected in unit 4.

### *3.4.3. Gloves*

The procedure presented here is also suitable for the determination of 5-FU on the internal side of the gloves. The contamination levels ranged from 0.07 to 3.77 μg/pair of gloves used during preparation and from 0.12 to 3.29 μg/pair of gloves used during administration.

## 4. Conclusions

A sensitive and reliable HPLC–UV procedure, incorporating solid-phase extraction (SPE), has been

Table 3
Intra-day and inter-day precision and accuracy of the method for determination of 5-FU in wipe samples

| | 5-FU theoretical amount (μg) | *n* | 5-FU experimental amount (μg) (mean±S.D.) | C.V. (%) | Accuracy (%) |
|---|---|---|---|---|---|
| Intra-day precision | 3 | 4 | 2.8±0.2 | 7.0 | 93.7 |
| | 12 | 4 | 12.2±1.0 | 8.5 | 101.3 |
| | 24 | 4 | 25.5±0.8 | 3.0 | 106.1 |
| Inter-day precision | 3 | 3 | 2.7±0.4 | 13.1 | 89.1 |
| | 12 | 3 | 12.7±1.1 | 8.4 | 106.0 |
| | 24 | 3 | 25.3±1.5 | 5.9 | 105.5 |

Table 4
Intra-day and inter-day precision and accuracy of the method for determination of 5-FU in air samples

| | 5-FU theoretical amount (ng) | *n* | 5-FU experimental amount (ng) (mean±S.D.) | C.V. (%) | Accuracy (%) |
|---|---|---|---|---|---|
| Intra-day precision | 45 | 4 | 49.0±2.2 | 4.5 | 108.8 |
| | 180 | 4 | 180.3±3.4 | 1.9 | 100.2 |
| | 360 | 4 | 356.2±0.0 | 0.0 | 99.0 |
| Inter-day precision | 45 | 3 | 50.0±4.9 | 10 | 110.0 |
| | 180 | 3 | 180.0±6.0 | 3.3 | 99.7 |
| | 360 | 3 | 348.7±15.1 | 4.3 | 96.9 |

developed and validated in order to detect even very low 5-FU levels in environmental matrices. Moreover, SPE enables a 20-fold concentration of the analyte with a limit of quantitation of 150 ng on wipe sample and 30 ng on filter. These results suggest that this method can also be suitable for the analysis of pads and gloves, and thus for determination of the main exposure routes during preparation and administration of 5-FU.

This method is a part of a national project granted by the Italian Ministry of Health, aiming at development of new analytical procedures whose main objective is risk assessment in antineoplastic drugs units. After cyclophosphamide, ifosfamide, platinum compounds, taxol and methotrexate [11,13–16], 5-FU has been taken into consideration. Unfortunately, mass spectrometry cannot be used for 5-FU detection, unless this drug is derivatised. On the other hand, derivatisation is a time-consuming procedure and is not suitable for routine analyses. However, SPE enables to enhance sensitivity and the diode array detection assures peak purity.

In summary, the presented method can be a feasible tool for the detection of 5-FU in environmental samples and thus for assessment of the effective degree of protection offered by personal protective devices and biological safety cabinets.

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