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Journal of Chromatography A, 726 (1996) 179–184

JOURNAL OF CHROMATOGRAPHY A

Trace analysis of the antineoplastics ifosfamide and cyclophosphamide in sewage water by two-step solid-phase extraction and gas chromatography–mass spectrometry

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Received 21 July 1995; revised 4 September 1995; accepted 20 September 1995

Abstract

A sensitive, specific and highly reproducible method for the analysis of the two antineoplastics, ifosfamide and cyclophosphamide, in sewage water at the ppt-level is presented. The method includes a two-step solid-phase extraction (SPE) on C_{18} and SiOH material and GC–MS analysis using single ion monitoring (SIM). The method was applied to degradational studies of the two drugs in laboratory-scale sewage treatment plants (LSSTPs) and to quantification in hospital sewage water samples. It was shown that the drugs were not degraded in LSSTPs and that they turned up in hospital sewage water.

Keywords: Solid-phase extraction; Waste water; Ifosfamide; Cyclophosphamide

1. Introduction

The two oxazaphosphorines, ifosfamide (IF) and cyclophosphamide (CP), belong to the most frequently used antineoplastic agents in cancer therapy. The agents are administered in dosages ranging from 1.0 g/m^2 to 5.0 g/m^2 body surface for IF [1] and from 0.7 g/m^2 to 2.8 g/m^2 body surface for CP [2] per day. Total dosage over the whole time of therapy may reach 60 g/m^2 body surface [3], approximately equivalent to 150 mg/kg body weight. Due to the mutagenic [4,5] and carcinogenic [6] potential, concern has arisen as to whether the drugs might present a health risk for the personnel handling and administering the drugs [7–9]. Aside from the release of the drugs during handling a second route of entry presenting a potential environmental risk is excretion of IF and CP via urine or feces of chemotherapeutically treated cancer patients. Approximately 14–53% of the administered drug dosage is excreted unmetabolised into urine [1]. Urine peak concentrations of 50.6 mg/l were reported 2 h after administration of IF [10]. The drug concentrations in sewage water vary with the number of treated patients and the water consumption in a hospital. A rough estimate calculated from the annual drug and water consumption and an average excretion rate of 20% for IF and CP predicts concentrations in the range of 1 μg/l to 10 μg/l for medium to large sized hospitals treating patients using chemotherapy.

To our knowledge nothing is known about degradation, or persistence, of these drugs in the environ-

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0021-9673/96/$15.00
SSDI 0021-9673(95)01063-7

ment. Tracking the fate of the drugs in environmental compartments with conventional pharmaceutical analyses is neither sufficiently sensitive nor sufficiently specific enough. HPLC-methods, as proposed by Burton and James [11], are not suitable for waste water samples due to low sensitivity and possible interference by sewage water components in the low UV-range (200 nm). Reported GC methods coupled with an electron-capture detector (ECD), or MS, resulted in higher sensitivity and specificity [7,10]. The conventional method for extracting the drugs from plasma or urine samples is liquid–liquid extraction [11–13]. Furthermore, solid-phase extraction methods using polymeric, silica or cyclohexyl bonded-silica solid phases were proposed by several authors [14–16]. Derivatisation is recommended by most of the authors [14,12]. The most frequently used reactant is trifluororacetic anhydride [16].

This study describes a method for the trace-level analysis of IF and CP in sewage water at the ppt-level with high reproducibility. The developed method was applied to LSSTPs in which degradation of the drugs was investigated and to real sewage water samples from a hospital effluent.

2. Experimental

2.1. Antineoplastic agents

The drugs IF, CP and trofosfamide (TF) were of highest available purity (>99%) donated by ASTA Medica AG, Frankfurt, Germany.

2.2. Extraction and clean-up

A 500-ml LSSTP effluent or sewage water sample was filtered consecutively through filter paper circles (Schwarzband 589 and Grünband 589/6, diam. 55 mm, Schleicher and Schuell, Dassel, Germany) and glass fiber filters (0.45 μm pore size, diam. 55 mm, Macherey and Nagel, Düren, Germany). The filtered sample was applied to a C_{18} SPE cartridge (Chromabond, 500 mg, Macherey and Nagel) preconditioned with 3 ml hexane (Pestanal, Riedel-de-Haën, Seelze, Germany), 3 ml methanol (Pestanal, Riedel-de-Haën) and 10 ml deionised water. The cartridge was then washed with 10 ml of deionised water. The flow-rate for all steps was set at 3 ml/min. To displace residual water, the cartridge was evacuated with a vacuum pump, dried further under a stream of nitrogen (purity 99.996%) and finally washed with 500 μl hexane. The drugs were eluted with 2 ml of methanol–acetone (95:5, v/v). To concentrate the sample the eluate was dried with nitrogen and the residue dissolved in 200 μl ethyl acetate (Pestanal, Riedel-de-Haën). For further clean-up this extract was applied to a SiOH SPE cartridge (Chromabond, 100 mg, Macherey and Nagel) preconditioned with 1 ml hexane. Ethyl acetate was sucked through and the sample was eluted consecutively with 1 ml of acetone–hexane (9:1, v/v) and 1 ml acetone. The eluate was dried with a stream of nitrogen. For samples other than sewage water, the residue was dissolved in 200 μl ethyl acetate and analyzed. Sewage water samples were further cleaned by washing the residue twice with 100 μl hexane, which was discarded. The remaining residue, that was insoluble in hexane was then redissolved twice in 100 μl di-2-propyl ether (puriss., Fluka, Buchs, Switzerland) and transferred to a new vial. This solvent change allowed for further discrimination of interfering sewage water components. The ether fraction was then evaporated to dryness and redissolved in 100 μl ethyl acetate.

All glassware and other equipment, with the exception of new autosampler vials, were rinsed with acetone prior to use.

2.3. Derivatisation with trifluoroacetic anhydride

To 100 μl of the final ethyl acetate fraction, 100 μl trifluoroacetic anhydride (TFA) (99%, Sigma, Deisenhofen, Germany) were added and the mixture was derivatised for 20 min at 80°C in a 4-ml Teflon-lined screw-cap vial. After completion of the reaction the remaining liquid was evaporated with nitrogen and the residue was redissolved in 100 μl toluene (Nanograde, Promochem, Wesel, Germany).

2.4. Chromatography

The gas chromatograph used was a Fisons GC 8065 equipped with a Fisons A 200 S autosampler and a mass spectrometer MD 800 (Fisons Instruments, Mainz-Kastel, Germany). Data acquisition

and evaluation was accomplished with Fisons PC-based Lab-Base.

For separation of both derivatised and underivatised samples a capillary column (Permabond SE-52-DF, 0.25 μm film thickness, 25 m × 0.25 mm I.D.; Macherey and Nagel) connected with a deactivated fused-silica retention gap (350 mm × 0.5 mm I.D., type 160-2537; J&W; Fisons Scientific, Mainz-Kastel, Germany) was used with automated on-column injection. Injection volumes ranged from 0.5 μl to 2 μl and were corrected for quantification with an internal standard [1,2,3,5-tetrachloro-benzene (TCB; AccuStandard, New Haven, CT, USA) for underivatised samples and trofosfamide (TF) for derivatised samples].

Carrier gas was helium (purity 99.996%) at an inlet pressure of 89 kPa. Injection started at a temperature of 87°C with secondary cooling for 4 min. The temperature was held for 2 min, then increased to 180°C at a rate of 5°C/min, held there for 1 min and then raised to 185°C at a rate of 1°C/min. The column was finally heated to 280°C at a rate of 20°C/min and held there for 2 min. The transfer line to the mass spectrometer was held at 280°C.

2.5. Detector parameters

Ionisation was achieved by electron impact at 70 eV. The source temperature was set to 250°C. Mass-to-charge ratios used in SIM mode for underivatised and derivatised samples are shown in Table 1.

Signal response of standards rapidly changed from one analysis to another for underivatised samples, which was presumably caused by the decomposition of the drugs on the column causing loss of column efficiency. Therefore, each series of samples was accompanied with standards (bracketing) and a calibration curve was calculated throughout.

2.6. Samples from LSSTPs

To determine the degree of drug degradation in sewage treatment plants, two LSSTPs were operated according to the modified OECD (Organisation of Economic Cooperation and Development) instruction 303A [18]. This instruction describes a method which simulates the degradational processes occurring in municipal sewage treatment plants with the help of LSSTPs. According to this method, our LSSTPs were inoculated with activated sludge from a municipal sewage treatment plant and ran with 12 l/day synthetic sewage over a period of 40 days for each drug degradation test. The synthetic sewage was adapted in its chemical oxygen demand and C/N/P-ratio to sewage water. To the influent of one of the LSSTPs, IF or CP was added at concentrations of 11 μg/l and 10 μg/l, which is the upper limit of the concentrational range estimated for sewage water. The second plant without drug addition served as a control. Samples of the effluent of the plant with additions of IF or CP were collected and analysed for drug concentration every second day.

2.7. Sewage water samples

Sewage water was sampled from the hospitals main sewage pipe. Approximately 4 l of a 24-h

Table 1
m/z-ratios used for detection and quantification of underivatised and derivatised IF, CP and TF (percentage of base peak (b.p.) is shown in brackets)

	m/z-ratios used for quantification (base-peak or quantifier ions)	*m/z*-ratios of qualifier ions and their relative abundance in % base peak
IF	175	56 (62%), 134 (72%), 211 (92%)
CP	175 (90%)	56 (b.p.), 147 (56%), 211 (38%)
1,2,3,5-TCB	108	–
IF-TFA[a]	150	154 (34%), 214 (16%), 307 (52%), 309 (16%)
CP-TFA[a]	307	150 (36%), 154 (34%), 214 (16%), 309 (34%)
TF	154	–

Dwell times were 100 ms for each ion at high resolution (span: 0.4 atomic mass units) for underivatised samples and 0.5 atomic mass units for derivatised samples.[a]IF-TFA, CP-TFA: Trifluoroacetylation products of IF and CP.

mixture were collected and partitioned into 1-l samples which were deep-frozen until analysis.

3. Results

3.1. Degradation studies in LSSTPs

Derivatisation of the samples proved to be unnecessary in the concentration range analysed in the LSSTPs. Retention times were 8.7 min, 25.5 min and 26.4 min for the internal standard TCB, IF and CP, respectively. Extraction efficiency determined by spiking the effluent of the control plant (no drug addition) with 10 μg/l of IF or CP, was found to be 99% (9.90 μg/l ± 0.31 μg/l; $n=3$) for IF and 72% (7.23 μg/l ± 0.08 μg/l; $n=3$) for CP. Extraction of the effluent of the control plant without spiking showed no interference of other sewage water components in the retention time of IF and CP. To assess the reproducibility (within-batch precision) of the method, the effluents from the plant with IF or CP addition were extracted in triplicate on a single day. The concentration of IF was determined to be 12.23 μg/l ± 1.15 μg/l and that of CP to be 11.90 μg/l ± 0.21 μg/l.

The mean recovery rate in the effluent over the whole time of the experiment (40 days, i.e. 20 samples) was 97% for IF and 86% for CP. These results show that a significant degradation of the two drugs did not occur during the course of the experiment.

3.2. Quantification of IF and CP in sewage water

In real sewage water a mixture of both drugs has to be expected. Since derivatisation of the drugs resulted in better resolution and signal response, the gas chromatographic separation was obtained after derivatisation with TFA. Retention times were 21.2 min, 24.2 min and 29.5 min for IF-TFA, CP-TFA and the internal standard TF, respectively. Full-scan mass spectra of derivatised IF, CP and the internal standard trofosfamide are shown in Fig. 1.

Due to a non-linear response for higher standard concentrations, calibration curves were approximated with two linear graphs (Fig. 2). The limit of detection was established at 7 ng/l for IF and 6 ng/l for CP. Identity of the drugs was confirmed by gas-chromatographic retention time and the relative abundance of selected qualifier ions (see Table 1).

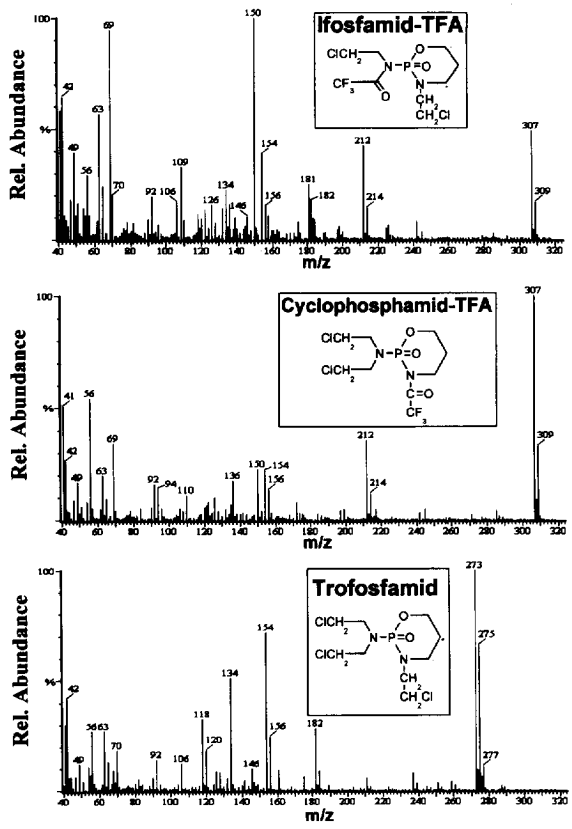

Fig. 1. Mass spectra of derivatised IF, CP and TF.

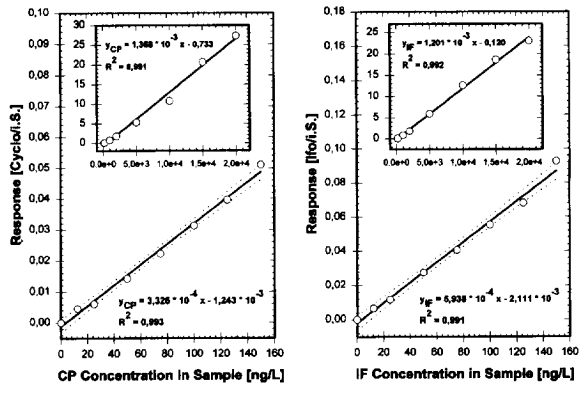

Fig. 2. Calibration curves based on authentic standards of derivatised IF and CP. Response was calculated as ratio from CP or IF quantifiers to TF quantifier.

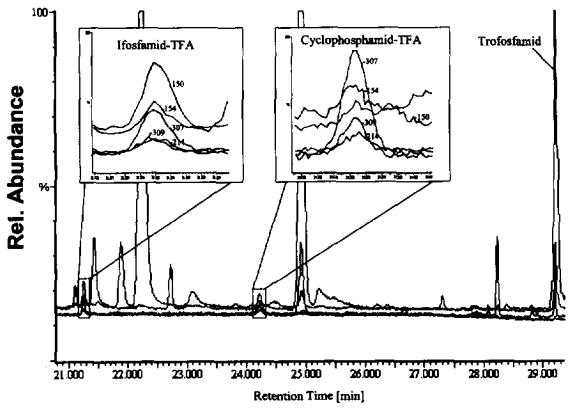

Fig. 3. SIM-chromatogram of a sewage water sample from a hospital (sample volume: 500 ml). The insets show the enlarged IF-TFA and CP-TFA peak at 21.2 min and 24.2 min, respectively. The numbers in the insets refer to the m/z-ratios of the quantifier and qualifier ions.

No interference with other sewage water components was observed. Furthermore non-hospital sewage water showed no peaks at the retention times of IF-TFA, CP-TFA and TF. Recovery rates from real sewage water samples were determined by spiking the samples with different concentrations of IF and CP standards (100 ng, 1 μg, 5 μg and 10 μg). Average recovery rates of 39% and 30% for IF and CP, respectively, were found (n=4). Additionally, spiking of samples further confirmed identity of the drugs by corresponding retention times of spiked and unspiked samples. Fig. 3 shows a representative chromatogram of a sewage water sample obtained from a hospital effluent. The hospital has 200 beds for cancer patients, where 80 beds are used for intensive chemotherapy. The consumption of CP was given as 1.6 kg for the year 1994. Precise data for IF were not available. However it was estimated as slightly lower than the consumption of CP. The concentrations of IF and CP in the displayed sewage water sample were determined to be 24 ng/l and 146 ng/l respectively.

4. Discussion

With the development of a sample preparation procedure and a GC–MS quantification method for the antineoplastics, IF and CP, it was possible to demonstrate that these drugs are not degraded in model sewage treatment plants. The appearance of excreted unmetabolised drugs in sewage water from a hospital was established.

Recovery rates were high for synthetic sewage effluent from LSSTPs. The concentrations determined in the triplicate extraction of the test plant's effluent (IF: 12.23 μg/l) are slightly higher (10%) than the dosage of IF (11 μg/l). For CP the determined concentration (11.90 μg/l) is also higher than the dosage (10 μg/l) and higher than expected from extraction efficiency data for CP. Drug concentration in the effluent of the LSSTPs is influenced by adsorption or desorption processes in the activated sludge. Sludge consistency may change from day to day, thereby causing changes in these processes.

In the case of real sewage water samples lower recovery rates are probably due to the more complex matrix. Real sewage water contains a high amount of dissolved organic compounds which interact with the analytes [19], thus lowering extraction efficiency. Dissolved organics also made the additional cleaning step with hexane and di-2-propyl ether necessary, which might account for additional losses.

The necessity of derivatisation of IF and CP for GC analysis is judged differently by various authors. Talha and Rogers [17] described artificial intramolecular alkylation of underivatised oxazaphosphorines during GC analysis, but nevertheless found a good correlation between derivatised and underivatised drugs. Similar results were reported by El-Yazigi and Cazemiro [15]. Mehta and Calvert [12], however, reported a low reproducibility of the above methods presumably due to decomposition of IF during GC analysis. In our case, derivatisation proved to be unnecessary at concentrations around 10 μg/l, as measured in the LSSTPs. Although only one drug is present in the LSSTP samples, baseline separation of IF and CP under the chosen chromatographic conditions was demonstrated by spiking an effluent sample with a standard mixture of IF and CP. However, in the sewage water samples, the lower concentrations of the drugs and the occurrence of other peaks between IF and CP necessitated a better resolution and signal response which was achieved with TFA derivatisation.

The determined concentrations of 24 ng/l IF and

146 ng/l CP in the sewage water sample are below the estimated sewage water concentration of 1 μg/l to 10 μg/l for both drugs by a factor of 7 to 400. The concentrations in the sewage water are influenced by three factors: firstly the number of cancer patients currently requiring administration of IF and CP, secondly the excretion rates of unmetabolised drugs, with high variations from patient to patient [1] and thirdly the amount of water consumed and shed into the sewage system diluting the excretion products. The time of sampling is obviously crucial to the concentrations determined. We expect high variances of IF and CP concentrations with a greater number of samples.

Because degradation of the two drugs was not observed in LSSTPs and their occurrence was confirmed in sewage water, we assume that the drugs in sewage water are also not degraded during their passage through a real sewage treatment plant. Investigations are currently under way to prove this hypothesis.

Acknowledgments

We are grateful to W. Weinmann and W. Schäfer for critical comments on this publication. We acknowledge the support of PWAB (Grant PA 94153) and ASTA Medica AG, Frankfurt, Germany.

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