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Short Communication

Determination of cyclophosphamide in urine by gas chromatography–mass spectrometry

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ABSTRACT

A sensitive gas chromatographic method for the determination of cyclophosphamide in urine is presented. After liquid–liquid extraction with diethyl ether and derivatization with trifluoroacetic anhydride, cyclophosphamide was identified and quantified with mass spectrometry. The method is suitable for the determination of cyclophosphamide at concentrations of more than 0.25 ng/ml, which enables the uptake of cyclophosphamide during occupational activities, such as the preparation and administration of antineoplastic agents in hospitals, to be measured. Simple preparation makes the method appropriate for routine analysis.

INTRODUCTION

Antineoplastic agents are widely used in cancer chemotherapy. When handling antineoplastic agents, pharmacy technicians and nursing personnel may face certain health risks. There is sufficient evidence that several antineoplastic agents are carcinogenic to humans, because secondary cancers are found in cancer patients treated with these drugs, and carcinogenic effects have been observed in non-cancer patients treated with these therapeutics for other purposes [1]. Special guidelines and extensive safety precautions were introduced in the Netherlands about ten years ago, to protect workers from contact with these toxic compounds [2]. Recently, these guidelines were amended [3].

In order to measure possible uptake of these compounds by workers, it is necessary to use sensitive detection methods. Non-specific detection methods, such as urinary mutagenicity, analysis of chromosome aberrations and sister chromatid exchange in blood lymphocytes, have been used for the determination of occupational exposure to antineoplastic agents [4,5]. These methods are suitable in cases when hospital workers had a minimum or a lack of protective measures against possible exposure to cytostatic drugs. Sensitive methods have scarcely been developed.

We have studied the amounts of drugs used in the Netherlands over the years 1986 to 1988 [6]. We found that cyclophosphamide (CP) was prepared and administered in Dutch hospitals most frequently. Therefore and because of CP's carcinogenic potency, we chose to concentrate of this drug.

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A literature review showed that the most suitable method for the detection of CP was derivatization with trifluoroacetic anhydride followed by gas chromatography (GC) with nitrogen–phosphorus, electron-capture, or mass-selective detection [7–10]. Also, a method without derivatization using GC with electron-capture detection has been published [11]. Unfortunately, some of these methods are not sensitive enough for the detection of exposure to the low levels of CP in urine expected during occupational activities under the regimen of the present Dutch guidelines [7,8], and others are time-consuming and not suitable for routine analysis [9,10].

This paper describes a GC method with liquid–liquid extraction, derivatization with trifluoroacetic anhydride and mass spectrometric (MS) detection. The method is sensitive and simple in practice, which makes it suitable for routine analysis.

EXPERIMENTAL

Materials and reagents

CP (Endoxan) and ifosfamide (IF) (Holoxan) were purchased from ASTA-Pharma (Bielefeld, Germany). Trifluoroacetic anhydride was obtained from Johnson Mattey (Karlsruhe, Germany). All other chemicals were of the highest purity obtainable.

Sample preparation

A 0.5-ml volume of 1 *M* Tris buffer (pH 8.0) and 100 μl of the internal standard IF (5 μg/ml in distilled water) were added to a 5-ml urine sample. After mixing, the samples were extracted twice with 20 ml of diethyl ether[a], and the ether layers were combined and evaporated under nitrogen at 30°C until a residue of *ca.* 2 ml was obtained. The solution was transferred to conical tubes with screw caps and further evaporated to dryness. Then 100 μl of ethyl acetate were added, and the solution was mixed until the residue was totally dissolved. Trifluoroacetic anhydride (100 μl) was added and, after mixing, the tubes were closed for derivatization for 20 min at 70°C. The samples were cooled to room temperature and evaporated to dryness under nitrogen at 40°C. The dried samples were dissolved in 100 μl of toluene, mixed and sonicated for 5–10 min. Vials were filled with sample and stored at −20°C until analysis.

Calibration

Calibration curves were constructed from the analysis of standard urine samples, which were freshly prepared by adding CP to blank urine. The CP concentrations of the standard urine samples were 0, 2, 10, 20, 50, and 100 ng/ml urine.

A control urine sample was obtained by adding CP to pooled blank urine (11.7 ng/ml). From this control urine sample, aliquots of *ca.* 10 ml were stored at −20°C. These aliquots were analysed in duplicate on every occasion when urine samples were assayed. The results from these determinations were used to calculate the inter- and intra-assay precision.

Gas chromatographic–mass spectrometric analysis

The samples were analysed on a Varian Saturn GC–MS ion-trap system with a Varian 8100 autosampler, which was controlled by a Compaq 386-20e personal computer (software version B). The on-column injection mode was used (SPI: septum equipped temperature programmable injector). Separation was carried out on a fused-silica capillary column (DB-5, 30 m × 0.25 mm I.D., film thickness 0.25 μm). The column was connected to a deactivated fused-silica retention gap (Varian, 5 m × 0.53 mm I.D.). The initial injector temperature was 110°C. After 1 min, the temperature was increased by 180°C/min to 280°C. After 8 min at 280°C, the temperature was decreased to the initial temperature by cooling with liquid carbon dioxide. The initial oven tem-

[a] Previously we used ethyl acetate combined with two clean-up steps. But greater amounts of solvents should be used and, after derivatization with trifluoroacetic anhydride, an additional extraction with *n*-hexane is needed to reach the same detection limit.

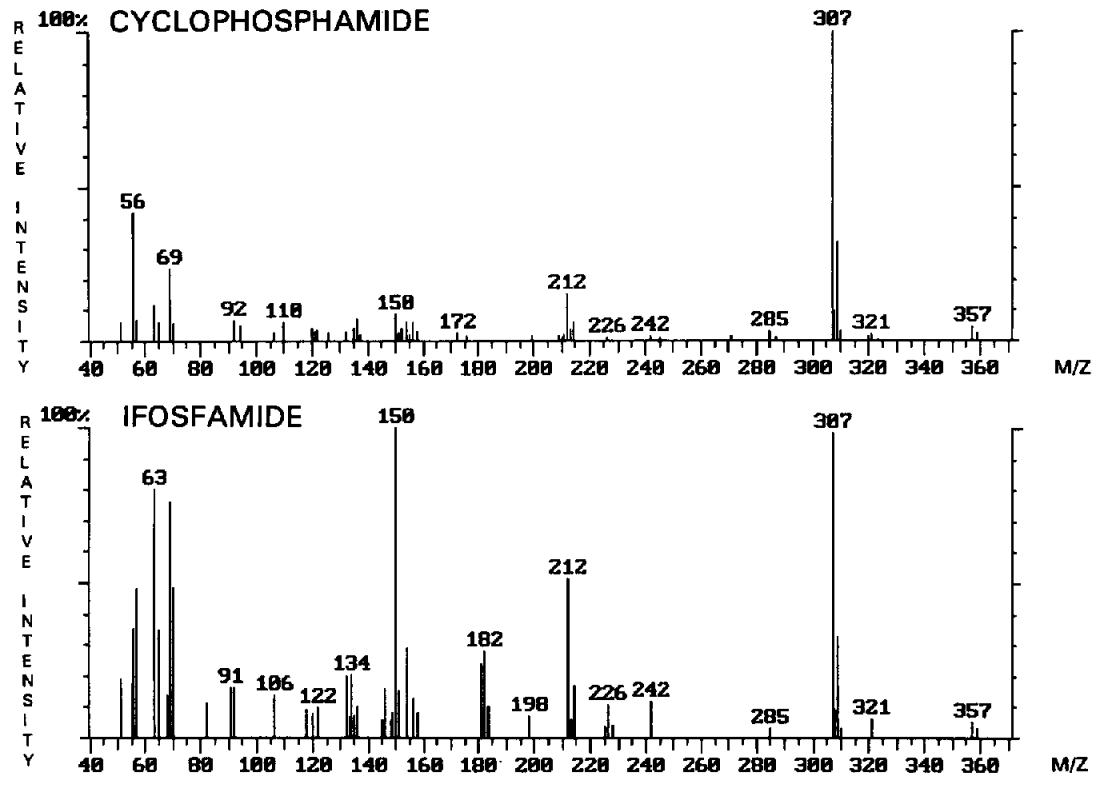

Fig. 1. Mass spectral fragmentation of N-trifluoroacetylated CP and IF (internal standard).

perature was 110°C. After 1 min, the temperature was increased by 15°C/min to 280°C, where it remained constant for 5 min. Helium was used as carrier gas (column inlet pressure 96 kPa). The interface temperature was 280°C. Electron impact (EI) was used as ionization mode. Identification was carried out by the combination of full-scan spectra (*m/z* 250–399) and retention times of CP and IF. The retention times of derivatized IF and CP were 9.40 and 10.20 min, respectively. Quantification of the N-trifluoroacetyl derivatives was performed on the selected-ion fragment *m/z* 307, which was abstracted from the full-scan spectra. By using a high background mass, the unwanted matrix ions were eliminated. For quantification, the CP/IF peak-area ratio was calculated.

RESULTS AND DISCUSSION

Mass spectra of N-trifluoroacetylated CP and IF are shown in Fig. 1. High relative intensity was found for *m/z* 307 ($M^+ + 1 - CH_3Cl$) for both compounds, because the molecular fragment *m/z* 356 was not stable under EI conditions [7,8]. Fig. 2 shows total and reconstructed (*m/z* 307) ion chromatograms of a blank urine sample spiked with CP and IF (A), a blank urine sample (B) and a urine sample from a CP-exposed pharmacy technician involved in the preparation of CP (C). CP and IF were completely separated, and no interference with other compounds was observed. When the selectivity was increased by the use of the reconstructed ion fragment (*m/z* 307) a higher sensitivity was obtained (Fig. 2A). The use of chemical ionization instead of EI did not result in a higher sensitivity.

The clean-up and derivatization procedure sounds similar to already published methods. The difference is the use of an ion-trap mass spectrometer instead of a quadrupole device with selected-ion monitoring (SIM). In previous unpub-

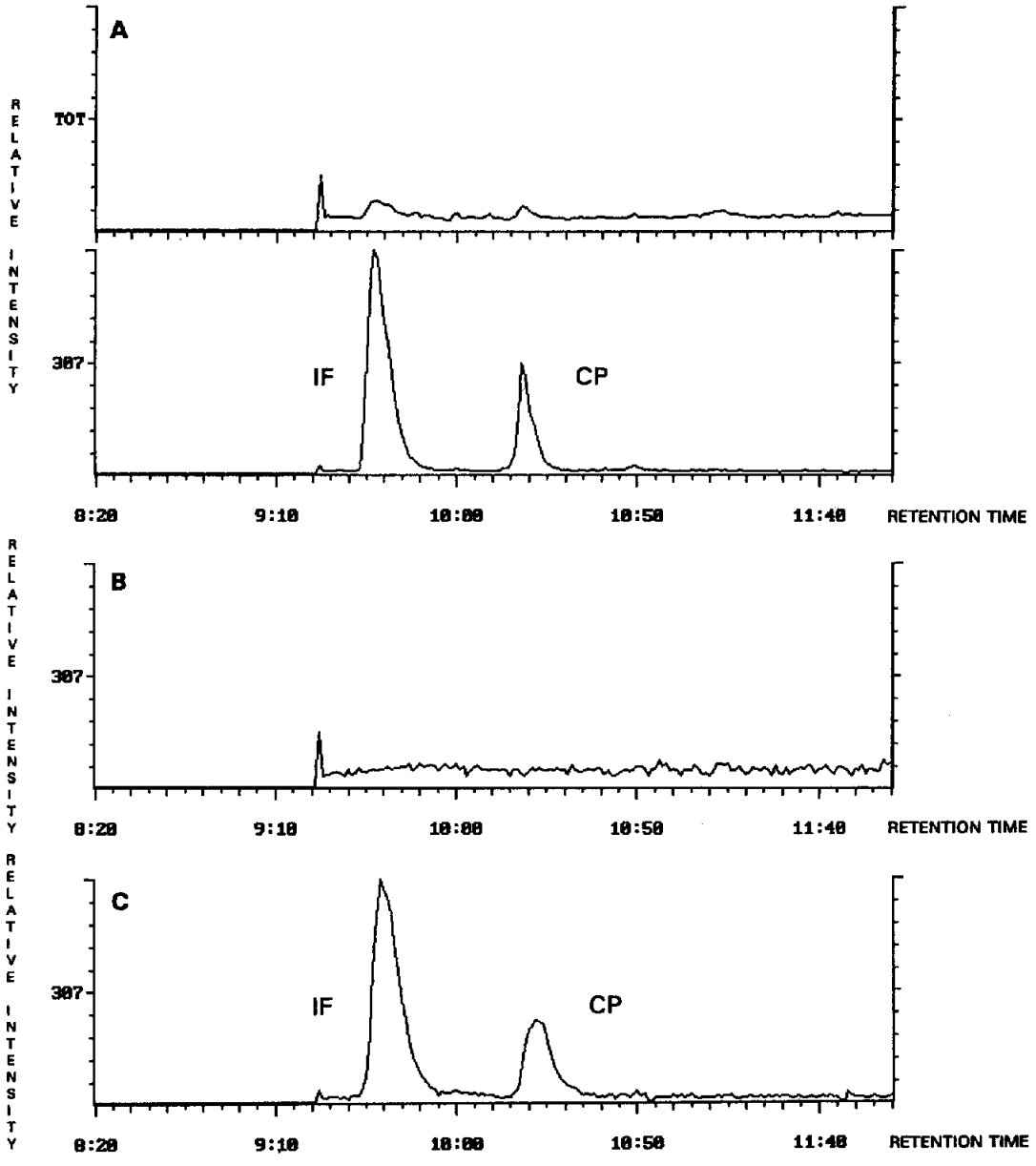

Fig. 2. Total and reconstructed (m/z 307) ion chromatograms. Filament and multiplier were switched on at 9:20 min. (A) A blank urine sample spiked with 10 ng/ml CP and 100 ng/ml IF (internal standard). (B) A blank urine sample. (C) A urine sample of a pharmacy technician involved in the preparation of antineoplastic agents, including CP. The concentrations of CP and IF were 18 and 100 ng/ml.

lished work we found that, in this particular case for the determination of CP in urine, the ion-trap spectrometer is more sensitive than a quadrupole device with SIM.

Thirty frozen control urine samples containing 11.7 ng/ml CP were analysed in duplicate during a period of three months. No significant loss of CP was observed, which means that the medium-term stability is good. The mean CP concentration of these analyses was 9.9 ng/ml. The coefficient of variation (C.V.), as a measure of the inter-assay precision, was 18%. The intra-assay

precision was 5.1%. The calibration curves were linear, with a coefficient of correlation of at least 0.99. The limit of detection was *ca.* 0.25 ng/ml urine (12.5 pg on column) with a signal-to-noise ratio of more than 3.

According to the International Agency for Research on Cancer, CP is a human genotoxic carcinogen (group 1). This means that exposure to this compound should be avoided because any detectable level is considered to be a hazard. This supports the need for a sensitive method for the determination of CP in urine.

The method presented in this study is sensitive and suitable for the detection of CP in urine at the low ng/ml level. This is the level we have found in workers occupationally exposed to this carcinogenic drug [12–14].

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