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Determination of Cyclophosphamide and Ifosphamide in Urine at Trace Levels by Gas Chromatography/Tandem Mass Spectrometry

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A specific and sensitive method based on gas chromatography/tandem mass spectrometry with on-column injection was developed to quantify simultaneously cyclophosphamide and ifosphamide in urine by using trophosphamide as an internal standard. The urine samples were extracted with diethyl ether and derivatization was performed with heptafluorobutyric anhydride. The detection limits of cyclophosphamide and ifosphamide in urine samples were 0.1 and 0.5 ng ml^{-1}, respectively, with a signal-to-noise ratio of 3 : 1. The sensitivity, the specificity and the low cost of the instrumentation involved make this method suitable for economical analysis on a large scale, such as for the biological monitoring of occupational exposure to cyclophosphamide and ifosphamide in production plants and in hospitals during their pharmacological use.

KEYWORDS: cyclophosphamide; ifosphamide; occupational exposure; biological monitoring; mass spectrometry

INTRODUCTION

Cyclophosphamide (CP) is an antineoplastic alkylating agent classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans.[1,2] Thus, workers involved in plant production, pharmacy technicians and nursing personnel handling this antineoplastic drug may run into health risks. Therefore, it is necessary to adopt specific and appropriate safety precautions to protect healthy individuals from any unnecessary hazard during the manufacture and handling of such a drug. At the same time, it is necessary to adopt suitable biological monitoring procedures to evaluate the possible uptake of CP in exposed workers.

In several studies, the concentration of CP in the urine of exposed workers has been used as a suitable marker in estimating the uptake of CP.[3–5] The level of non-metabolized CP excreted in the urine, in fact, represents the total uptake of the drug by different exposure routes even if it constitutes only a small fraction of the absorbed dose.

Among the specific methods for the detection of CP levels in urine, the most suitable has been based on gas chromatography (GC) coupled with nitrogen–phosphorus, electron-capture or mass-selective detection.[6,7] High-performance liquid chromatographic/ultraviolet (HPLC/UV) methods have been scarcely used; HPLC in conjunction with mass spectrometry (HPLC/MS) has been reported only as an off-line combination by using a field desorption ionization method.[8,9] Until now, GC/MS methods used for the quantification of CP at low levels in the urine of exposed people have detection limits of about 0.2 ng ml^{-1}. These methods require the preparation of suitable derivatives, such as trifluoroacetyl derivatives.[3,4,10] Recently, a sensitive and specific method for the determination of CP and ifosphamide (IP) in urine, based on HPLC/ionspray tandem mass spectrometry (HPLC/MS/MS), has been described.[11,12]

In this paper, we present a new method for the simultaneous trace determination of CP and IP in human urine. This procedure, based on cold on-column GC/MS/MS, is characterized by very high specificity and sensitivity because of the use of MS/MS and selected reaction monitoring (SRM). In addition, the use of trophosphamide (TP) as an internal standard allows the simultaneous determination of the CP and IP. The GC/MS/MS system used in setting up this procedure is less expensive, easier to use and requires less frequent routine maintenance than more sophisticated HPLC/MS/MS instrumentation; therefore, it seems to be more appropriate for routine analysis in monitoring occupational exposure and is economical.

EXPERIMENTAL

Chemicals and reagents

Cyclophosphamide (Endoxan) and ifosphamide (Holoxan) were purchased from ASTA-Pharma (Germany);

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Trophosphamide was kindly supplied by ASTA-Pharma. Heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL, USA) and anhydrous sodium sulfate from J. T. Baker Chemicals (Deventer, The Netherlands). Other chemicals were purchased from Merck (Darmstadt, Germany). All reagents were of the highest purity commercially obtainable.

Standard solutions and calibration

Standard urine samples were freshly prepared by spiking 5 ml spot urine from unexposed subjects (blank urine) with CP, IP and TP. Then samples were treated as described below. Calibration curves were constructed by varying the analyte concentration while the internal standard concentration remained constant. The CP concentration of the standard urine samples was 0, 1, 2, 5, 10 and 20 ng ml^{-1} and those of IP were 0, 2, 4, 10, 20 and 40 ng ml^{-1}. Each point on the calibration curves was an average value of four replicate measurements. Spiked urine samples were analysed by GC/MS/MS, according to the procedure described below. These samples were stored at −20 °C and used as controls when urine samples from exposed subjects were analyzed.

Sample collection and preparation

The collection of 24-h urine samples from hospital personnel started soon after the handling of the drug. In order to minimize the proliferation of bacteria, urine samples were acidified with 5% HCl and a 50 ml aliquot was stored at −20 °C.

Samples for analysis were prepared by adding to a 5 ml urine sample 500 μl of 1 M Tris buffer (pH 8) and 100 μl of 5 ng μl^{-1} TP aqueous solution. After mixing, the samples were purified from the matrix by liquid–liquid extraction with diethyl ether (2 × 20 ml); the ether layers were combined and residual water was removed with anhydrous sodium sulfate. Then samples were dried, dissolved in 100 μl of ethyl acetate and derivatized by adding 100 μl of heptafluorobutyric anhydride. After 20 min at 70 °C the reaction was stopped by evaporation to dryness. The residue was dissolved in 100 μl of isooctane, sonicated and stored at −20 °C until analysis.

Gas chromatographic tandem mass spectrometric analysis

Analyses were carried out on a Finnigan GCQ ion trap mass spectrometer equipped with a Trace MS gas chromatograph.

A DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) purchased from J & W Scientific (Folsom, CA, USA) was used for the GC separation. Samples were introduced with cold on-column injection. The temperature program used was as follows: 70–150 °C at 80 °C min^{-1}, 150–200 °C at 15 °C min^{-1}, then the temperature was increased at 5 °C min^{-1} up to 230 °C in order to perform a better separation and finally the oven was held at 300 °C for 3 min. Helium was used as the carrier gas with constant velocity of 40 cm s^{-1}. The retention times of IP, CP and TP were 5.56, 6.40 and 10.04 min, respectively. By using on-column injection no thermal decomposition was observed during the separation of the *N*-heptafluorobutyryl derivatives of CP and IP (HFB-CP, HFB-IP) and TP; in contrast, by using split/splitless injection a temperature-dependent decomposition of TP was recorded.

Mass spectral data were obtained under the following conditions: electron ionization (EI), 70 eV; source temperature, 200 °C; transfer line temperature, 275 °C; and emission current, 170 μA. Spectra were acquired in the MS/MS mode by using two different acquisition segments. The first segment went from 3.0 to 8.0 min. Ions with m/z from 406 to 410 were trapped for 12 ms and then excited by applying an excitation voltage of 1.75 V for 10 ms. Product ions were acquired in the ranges m/z 149–151. and 211–215. Quantification of HFB-CP and HFB-IP was performed on product ions at m/z 150, 212 and 214. Likewise, TP was characterized by acquiring product ions at m/z 118 and 120, with the ions at m/z 273 and 275 as precursors.

Limit of detection, precision and accuracy

The extraction recovery was estimated by comparison of standard solutions with blank urine samples from unexposed subjects, spiked with known amounts of CP and IP. Both spiked and standard samples were run in quadruplicate. The extraction yield was 80 ± 5% for CP and 67 ± 8% for IP. The detection limits, measured at a signal-to-noise ratio of 3, were 0.1 ng ml^{-1} for CP and 0.5 ng ml^{-1} for IP. The precision and accuracy were evaluated by determining the intra-and inter-assay reproducibility by determining CP and IP in quality control samples prepared at different levels of nominal concentrations. Five levels of concentrations were employed for intra-assay reproducibility (1.0, 2.0, 5.0, 10.0 and 20.0 ng ml^{-1} for CP and 2.0, 4.0, 10.0, 20.0 and 40.0 ng ml^{-1} for IP), and three levels for inter-assay reproducibility (1.0, 5.0 and 10.0 ng ml^{-1} for CP and 2.0, 10.0, 20.0 ng ml^{-1} for IP) on four different days.

RESULTS AND DISCUSSION

The structures of CP, IP and the internal standard TP are shown in Fig. 1. The presence of an acidic hydrogen in the molecules of both CP and IP requires its replacement by an appropriate derivatization process to produce thermally stable compounds, while the presence of a third chloroethyl group in the molecule of TP makes it unaffected by the same reaction. Among different perfluoroanhydrides used as derivatizing reagents we decided to utilize heptafluorobutyric anhydride because of the higher incremental mass of its derivatives with a consequent better specificity in quantitative analysis.[13] In addition, a contribution to the specificity of the method is assured by the presence in all of the compounds of chlorine atoms whose characteristic relative abundance is easily recognized.

EI full-scan mass spectra of HFB-CP, HFB-IP and TP are reported in Fig. 2. Both HFB-CP and HFB-IP show ions of high relative abundance at m/z 407 due to the loss

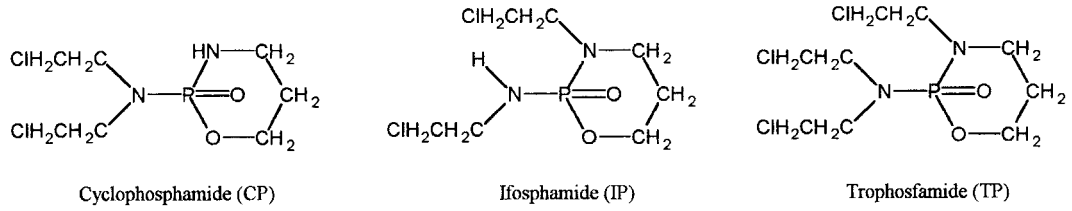

Figure 1. Structures of cyclophosphamide, ifosphamide and trophosphamide.

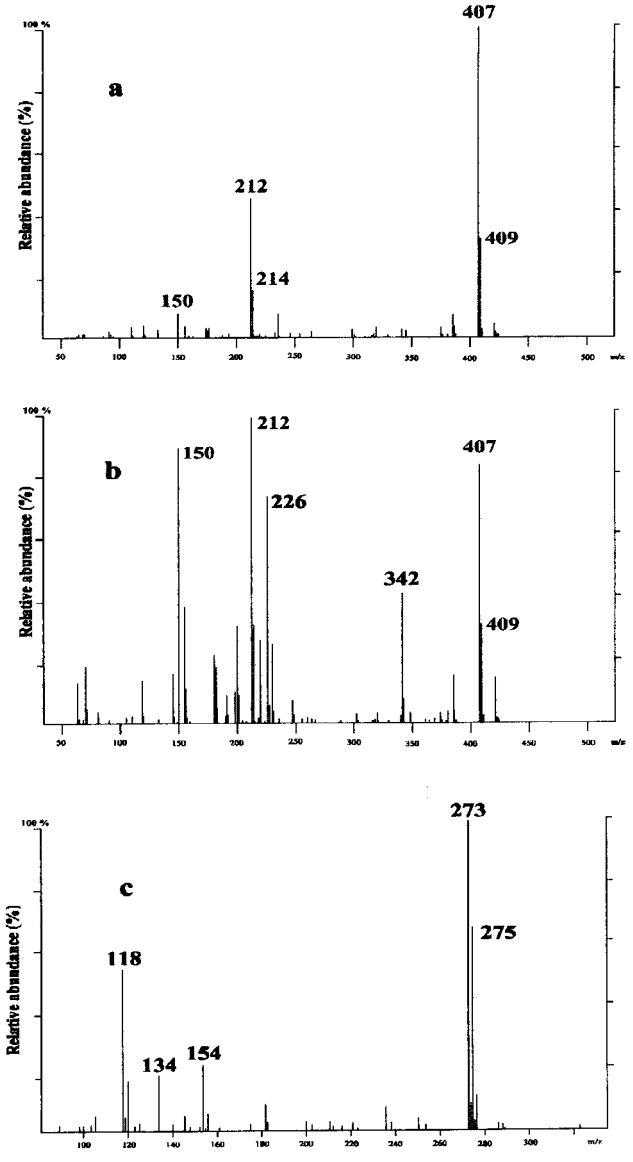

Figure 2. EI full-scan mass spectra of (a) cyclophosphamide, (b) ifosphamide and (c) trophosphamide.

of the radical fragment $CH_2Cl^{\bullet}$ from the low-abundance molecular ion at *m/z* 456. TP loses the same fragment from the very weak molecular ion at *m/z* 322, giving rise to the ion at *m/z* 273. Under the experimental conditions used, HFB-CP and HFB-IP are thermally stable compounds and in our experience they are stable even when analyzed using a GC/MS system equipped with a split/splitless injector.

No decomposition of CP and IP was observed during the synthesis of the derivatives. In contrast, thermal decomposition of CP, IP and TP occurs in the glass liner of a split/splitless injector and CP also decomposes in a cold on-column injector when analyzed without derivatization. The derivatization procedure and cold on-column injector prevent any loss of sample by decomposition, making unnecessary the use of more complex and expensive instrumentation, such as HPLC/MS and HPLC/MS/MS.

In order to observe the isotopic relative abundance in MS/MS experiments, mass ranges centered on *m/z* 408 and 274 were chosen to trap simultaneously ions at *m/z* 407, 409 and at *m/z* 273, 275 as precursor ions specifically related to the isomers HFB-CP and HFB-IP and to the internal standard TP, respectively. In the fragmentation pattern of CP and IP derivatives the ions at *m/z* 150 and 212, with its isotopic peak at *m/z* 214, occur whereas TP shows the base peak at *m/z* 118, followed by its isotopic peak at *m/z* 120 (data not shown). In order to reduce the background noise, product ions were acquired in small scan mass ranges centered on ions at *m/z* 150 and 213; then ions at *m/z* 150, 212 and 214 were extracted from the total ion current and the sum of their areas is used to quantify CP and IP.

The above-described MS/MS technique gives better results than the selected ion monitoring (SIM) procedure, both in sensitivity and in specificity. A comparison between SIM and MS/MS techniques in the analysis of a urine sample from a subject accidentally exposed to high concentrations of CP and IP is shown in Fig. 3. The SIM experiment was carried out by selecting the ion at *m/z* 407. In the SIM mode interference cannot be completely eliminated (Fig. 3(a)) and this could cause a misunderstanding in the identification of analytes. On the other hand, Fig. 3(b) shows the total ion current for the same sample but acquired in the MS/MS mode. In this case, the main product ion of the interference is at *m/z* 208 and it is completely eliminated by the extraction of ions at *m/z* 150, 212 and 214 from the total ion current (Fig. 3(c)). It is noteworthy that the proposed method allows the unambiguous identification of CP and IP because the trapping of the cluster of ions $[M - CH_2Cl^{\bullet}]^+$ produces in the MS/MS experiments both the ion at *m/z* 212 and the satellite signal at *m/z* 214, whose characteristic intensity ratio of 3 : 1, due to the presence of one chlorine atom, is retained.

The MS/MS technique was tested on samples from exposed workers for biological monitoring purposes. Calibration curves in the range 0–20 ng ml^{-1} for CP and 0–40 ng ml^{-1} for IP showed good linearity. These ranges of CP and IP concentrations include the average levels statistically found in urine samples from hospital personnel. Calibration curves were constructed by plotting on the abscissa the CP or IP urine concentration and on the ordinate the ratio between the areas of extracted

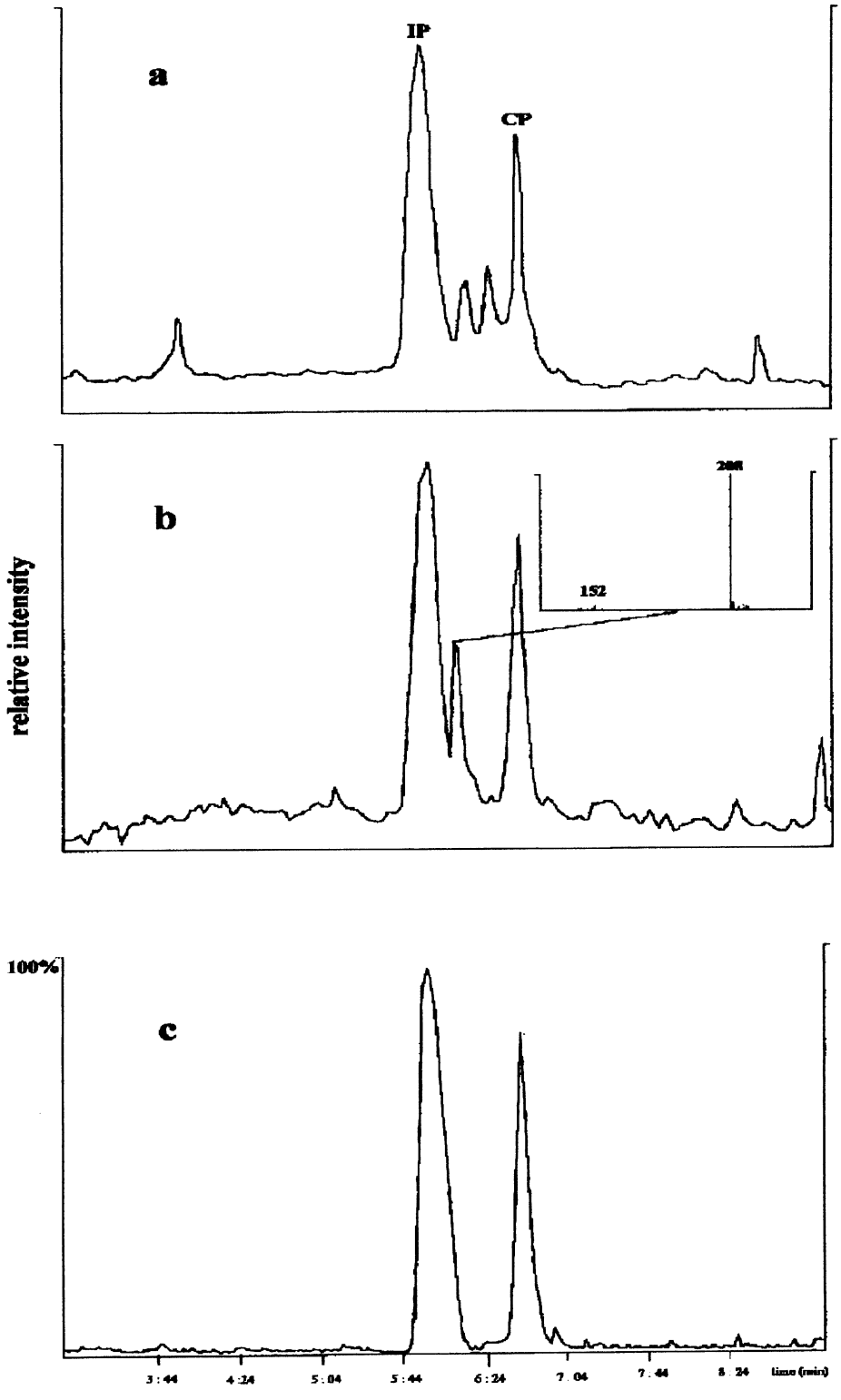

Figure 3. Urine sample from a worker accidentally exposed to high concentrations of CP and IP. (a) SIM experiment (*m/z* 407); (b) total ion chromatogram acquired in the MS/MS mode (trapping window, *m/z* 408 ± 2; acquisition range, *m/z* 145–155, 207–219); (c) chromatogram of extracted ions (*m/z* 150, 212, 214) from the total ion current of the MS/MS experiment shown in (b).

ions at *m/z* 212, 214 and 150 and those of the internal standard (*m/z* 118 and 120). The equations for the calibration curves were $y = 0.0514x + 0.0683$ for CP and $y = 0.06x + 0.1363$ for IP, with correlation coefficients of 0.9994 (CP) and 0.9949 (IP).

The detection limits were 0.1 and 0.5 ng ml^{-1} for CP and IP, respectively. This difference relies on the fact that CP and IP are isomers and therefore their chromatographic and mass spectrometric behaviors are different. In fact, the IP chromatographic peak is less sharp than the CP peak and the chosen precursor ion at *m/z* 407 is the base peak only in the CP mass spectrum.

A summary of intra-and inter-assay reproducibility is reported in Table 1.

Table 1. Evaluation of intra-and inter-assay reproducibility of CP and IP in quality control samples

Parameter	Intra-assay quality control ($n = 4$)									
	CP					IP				
Nominal concentration (ng ml^{-1})	1.0	2.0	5.0	10.0	20.0	2.0	4.0	10.0	20.0	40.0
X_m (ng ml^{-1})	0.90	2.11	4.90	9.73	20.06	1.86	4.16	10.71	19.61	39.72
S.D. (ng ml^{-1})	0.12	0.14	0.19	0.17	0.22	0.25	0.38	0.46	0.58	0.60
RSD (%)	13.3	6.6	3.8	1.7	1.1	13.4	9.1	4.2	2.9	1.5
Inaccuracy (%)	−10.0	5.5	−2.0	−2.7	0.3	−7.0	4.0	7.1	−1.9	−1.8

	Inter-assay quality control ($n = 16$)					
	CP			IP		
Nominal concentration (ng ml^{-1})	1.0	5.0	10.0	2.0	10.0	20.0
RSD (%)	10.1	5.3	1.5	11.5	7.1	2.4
Inaccuracy (%)	−7.8	−2.5	−1.8	−6.2	2.5	−2.1

CONCLUSIONS

The proposed method for the trace determination of CP and IP in urine of subjects occupationally exposed to these drugs achieves the highest levels of sensitivity and specificity possible at present because of the use of MS/MS. In fact, the advantage of SRM over SIM lies in the unambiguous identification of parent and product ions, linked in a specific fragmentation process, with the consequence of eliminating any possible interference, as shown in Fig. 3. From the experimental data a linear response over a wide range of concentrations can be deduced and the levels of precision and accuracy fall within the mean values reported for quantitative analysis.

The described method is comparable in terms of results to more sophisticated methods but it can easily be performed without any particular expertise in mass spectrometry. In principle it can also be applied to any other carcinogens excreted in biological fluids, in order to set up protocols for the biological monitoring of the human population exposed to them. The achievement of such a goal requires the analysis of samples on a large scale so the analytical protocol should be based on the use of procedures and instrumentation at the lowest cost possible to accomplish the greatest social benefit. In this respect, the method reported here will be applied in a large project of biological monitoring of pharmacy technicians and nursing personnel in hospitals in the Naples area; as it is based on the use of inexpensive GC/MS instrumentation it provides good value for money.

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