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Research Article

Rapid Method for the Determination of Cyclophosphamide and Ifosphamide in Urine at Trace Levels by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Introduction: The oxazaphosphorines cyclophosphamide (CY) and Ifosfamide (IF) are amongst the most widely used drugs in cancer chemotherapy. In both routine cancer therapy and experimental cancer research, many people might be exposed to CY and IF during pharmaceutical handling of the agents, so developing of a reliable analysis method for biomonitoring is necessary. Materials and Methods: A rapid and simple method for the simultaneous extraction of CP and IP from human urine has been developed using liquid-liquid extraction. The detection limits of cyclophosphamide and ifosphamide in urine samples were 0.5 and 1 ng ml^{-1}, respectively, with a signal-to-noise ratio of 3:1. The regression correlation coefficients (r^2) were over 0.99 in all experiments. Conclusion: This method is sensitive enough to determination of low levels of CP and IF in a range of urine concentrations relevant to performing low exposure assessment.

Keywords: cyclophosphamide; Ifosfamide; Urine; GC/MS

INTRODUCTION

Ifosfamide (IP) and its isomer cyclophosphamide (CP) are alkylating agents, both of which are active against a wide variety of tumours. The use of antineoplastic agents for the treatment of cancer and other non-neoplastic diseases is an increasingly common practice in hospitals. Over the last 20 years, several studies have reported environmental contamination with hazardous drugs in hospital pharmacies[1-4]. In addition, hazardous drugs were inadvertently absorbed, as determined by the presence of parent compounds and/or their metabolites in the urine of health care workers[5]. Due to the potential health risks of hazardous drugs, the increasing use of these drugs, and continuing environmental contamination, several studies have been published to establish exposure levels of hospital personnel preparing and administering of these drugs[6]. In several studies, the concentration of CP and IP in the urine of exposed workers has been used as a suitable marker in estimating the uptake of these two drugs. 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[7]. Several methods have been published on the quantification of CP and IP levels in urine, the most suitable has been based on gas chromatography (GC) coupled with nitrogen–phosphorus, electron-capture or mass-selective detection. These methods require the extraction of CP and IP prior to chromatography, which is a long and time wasting procedure. A rapid and simple method for the simultaneous extraction of CP and IP from human urine has been developed using liquid-liquid extraction. In addition, the use of trophosphamide (TP) as an internal standard allows the simultaneous determination of the CP and IP. The liquid-liquid extraction system used in setting up this procedure is less expensive and easier to use than solid phase microextraction procedure; therefore, it seems to be more appropriate for routine analysis in monitoring occupational exposure and is economical.

MATERIALS AND METHODS

Chemicals

Cyclophosphamide and ifosphamide were purchased from Sigma-Aldrich Chemie (Schnelldorf, Germany). Trophosphamide was from Santa Cruz Biotechnology (Dallas, Texas, U.S.A.). All other solvents and chemicals were of analytical-reagent grade.

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 analytes concentration while the internal standard

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Table 1: Back-calculated values of the calibration of the urine samples.

Compounds	Concentration (ng ml^{-1})	Mean concentration (ng ml^{-1}) (n=6)	Mean accuracy (%)	RSD (%)
Cyclophosphamide	2	2.23	110	9.09
	4	4.28	107	7.53
	10	9.93	99	4.42
	20	19.45	97	6.27
	40	39.22	98	7.36
Ifosphamide	2	2.11	105	7.51
	4	4.15	104	5.40
	10	9.82	98	6.71
	20	19.42	97	6.23
	40	39.61	99	7.29

Table 2: The accuracy and precision (intra- and inter-assay) for QC samples in urine

Compounds	Concentration (ng ml^{-1})	Intra-assay Mean concentration (ng ml^{-1})	Mean accuracy %(n=6)	RSD(%)	Inter-assay Mean concentration (ng ml^{-1})	Mean accuracy %(n=6)	RSD(%)
Cyclophosphamide	5	5.17	103	6.59	5.23	105	8.82
	15	14.89	99	5.50	15.34	102	4.46
	30	29.46	98	3.74	29.72	99	5.71
Ifosphamide	5	5.17	105	7.21	5.41	108	7.41
	15	14.92	99	5.09	14.72	98	6.40
	30	29.64	99	6.23	29.46	98	5.46

concentration remained constant. The CP and IP concentration of the standard urine samples was 2, 4, 10, 20 and 40 ng ml^{-1}. Each point on the calibration curves was an average value of three replicate measurements. Spiked urine samples were analysed by GC/MS, according to the procedure described below.

Extraction procedure

An aliquot of 5 ml urine for each sample was adjusted to pH 7, and100 µl of 0.5 µg ml^{-1} TP aqueous solution was added, mixed and extracted three times with 10 ml ethyl acetate. The organic layers were combined and evaporated to dryness under a gentle nitrogen stream. The residues were dissolved in 100 µl of isooctane and derivatized by adding 100 µl of trifluoroacetic anhydride. After 20 min at 70 °C the reaction was stopped by evaporation to dryness. The residue was dissolved in 100 µl of isooctane; the solution was shaken vigorously using vortex-mixer for 1 min. Finally, 2 µL of the resulting solution was injected into the GC/MS.

Instrumental and analytical conditions

The analysis was performed on a GC/MS Agilent 6890 plus gas chromatograph equipped with a 5973 quadrupole mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA). The gas chromatograph was fitted with a DB-5 ms capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness). The inlet was operated in splitless mode. The instrumental temperatures were set as follows: injector temperature 250 °C; initial oven temperature 70 °C, held for 1 min, increased to 250°C at a rate of 15 °C min^{-1}, held for 3 min and finally increased to 300 °C at a rate of 30 °C min^{-1}, held for 5 min. The temperature of the transfer line was maintained at 300 °C. Helium was used as carrier gas at 1 mL min^{-1} (constant flow). The source and quadrupole temperatures were kept at 230 and 150 °C, respectively. The electronic beam energy of the mass spectrometer was set at 70 eV. Qualification was performed by comparing the acquired mass spectra and retention times to reference spectra and retention times which were acquired by injection calibration standards under identical GC/MS conditions. The compounds were quantified using selected ion monitoring (SIM) mode.

Method validation

Method validation was conducted in accordance with the currently accepted US Food and Drug Administration Guideline for Industry[8,9].

RESULTS AND DISCUSSION

Optimization

Diethyl ether, Methyl tert-butyl ether and ethyl acetate (10 ml*3) were tested as the extraction solvent for liquid-liquid extraction system. The recovery for studied solvents was measured as the response of a processed spiked sample expressed as peak area and compared to that of pure standard solution at two concentration levels (4 and 10 ng ml^{-1}). Ethyl acetate gave a good recovery (70%) with the extraction time of 5 min. To minimize carryover the syringe was washed five times with 10µL methanol after each run.

Validation

Calibration data

The constructed calibration curve for urine samples consisted of five levels of spiked human urine in the concentration range of 1–50 ng ml^{-1}. The regression correlation coefficients (r^2) were over 0.99 in all experiments. The back-calculated values of the calibration points showed a good agreement with the theoretical concentrations, deviation between 1 and 10 % of the nominal concentrations was observed (n = 6) (Table 1).

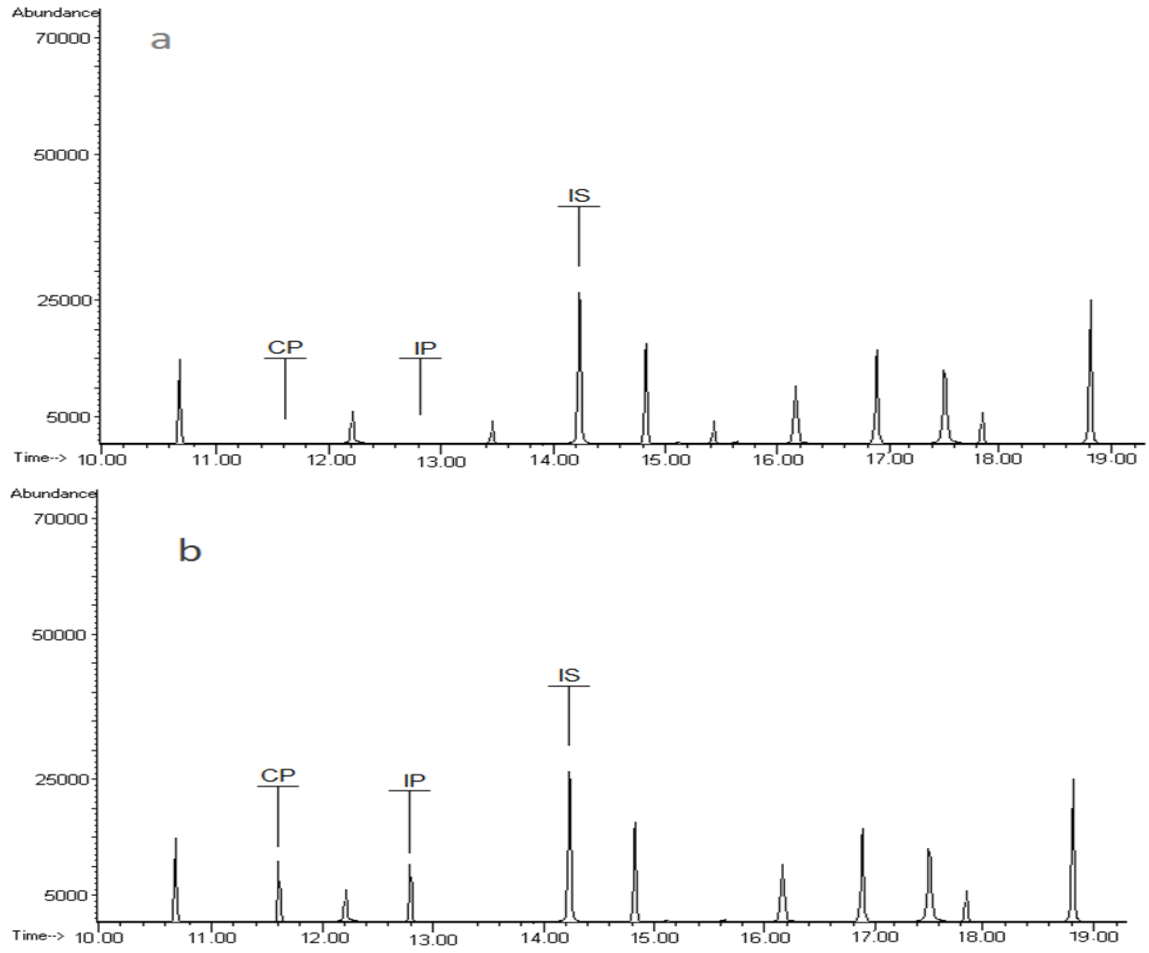

Figure 1: a) Representative mass chromatograms of blank human urine and b) spiked human urine at low QC (5 ng ml^{-1})

Accuracy and precision

The accuracy is determined as the percentage difference from the nominal concentration value of QC samples at three different concentration levels (Table 2). The accuracy

is a measure of the systematic error. The precision is a measure of random error and is determined by the percentage CV (RSD) of intra- and interday variations at three levels. The precision and the accuracy, intra- and interday assays, were determined by using three levels of concentrations, 5, 15 and 30 ng ml^{-1}. The QC samples were prepared in human urine samples and six QC samples at each level were used. The intra-assay precisions (RSD) at three different concentrations for QC samples were about 3.7–7.2% (n = 6). The inter-assay precisions (RSD) were 4.5–8.8% for both analytes in urine samples (n = 18). The accuracy varied from 98 to 108% for both intra assay and inter-assay (n = 18). The accuracy and precision data are summarized in Table 2.

Representative mass chromatograms of blank human urine and spiked human urine at low QC (5 ng ml^{-1}) are presented in Fig. 1.

LOD, LOQ and carryover

The determination method for LOD and LOQ was in the line of FDA-guidelines and as described by Shah et al.[8]. The LOD was 0.5 and 1 ng ml^{-1} for CP and IP, respectively. The LOQ for the analytes studied was calculated as 1 and 2 ng ml^{-1}. The carryover was tested by injecting methanol after the highest standard concentration.

CONCLUSION

According to the International Agency for Research on Cancer (IARC), CP and IP are human genotoxic carcinogens[10]. Several reports have discussed exposure to CP and IP during occupational activities and concluded that it should be avoided because any detectable level is considered to be hazardous[11-13]. In this work, a sensitive and reliable method for determination of CP and IP in human urine samples was developed. The method is based on using liquid- liquid extraction system. By using this system the method was simplified and the time-consuming procedures such as column passing and filtration steps were eliminated, therefore, it seems to be more appropriate for routine analysis in monitoring occupational exposure and is economical.

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