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QUANTITATION OF ANTICANCER DRUGS – CYCLOPHOSPHAMIDE AND IFOSFAMIDE IN URINE AND WATER SEWAGE SAMPLES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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Abstract

Objectives: Cyclophosphamide (CP) and ifosfamide (IF) are effective anti-cancer drugs but their genotoxicity can harm everyone contacting them occupationally or environmentally. Therefore, a sensitive method for monitoring their amounts in biological and environmental samples is needed. This has aimed to develop a method for analyzing these drugs in urine and water sewage samples. **Material and Methods:** The drug spiked samples were extracted, derivatized, and analyzed by gas chromatography–mass spectrometry and the analytical parameters were validated. **Results:** The method gave linear calibration curves at the concentrations of 0–190 nmol/l. It had the quantitation limit of 3.8 nmol/l and showed acceptable specificity, accuracy, recovery and precision. **Conclusions:** The developed method can be used reliably for monitoring CP and IF concentrations in urine and water sewage. The method will be applied for preventing health risk from occupational and environmental exposures to these drugs. Int J Occup Med Environ Health 2016;29(5):815–822

Key words:
Environment, Cyclophosphamide, Antineoplastic agents, Gas chromatography–mass spectrometry, Ifosfamide, Sensitive and specificity

INTRODUCTION

Cyclophosphamide (CP) and ifosfamide (IF) are alkylating agents commonly used to treat various cancers. However, their cytotoxic effects are not specific towards cancer cells, they harm normal cells causing life threatening adverse effects, including cancer, particularly CP has been classified as a human carcinogen [1], and the case of pregnant women these drugs can be teratogenic and fetotoxic [2–4]. Their toxicities have been reported not only the case of patients but also the case of health care workers occupationally contacting them [5–8]. Recently, the environmental contamination by these drugs from patient excretions and hospital waste products have raised concern [9–10]. Consequently, their concentrations in biological specimens and environment need to be monitored for health safety. Several methods have been developed for quantitation of these drugs; however, the best is liquid chromatography–mass spectrometry (LC-MS) [11–13] or liquid chromatography–tandem mass spectrometry (LC-MS/MS) [14,15], which were not available in our laboratory. The gas chromatography methods have been reported with

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and without derivatizations [16–19] but mostly showed low sensitivity which might be not suitable for preventive purposes. In this presentation, poor chromatographic behaviors of CP and IF have been revealed. The main goal of this study has been to develop a gas chromatography–mass spectrometry method for reliably measuring CP and IF concentrations in urine of people occupationally exposed to these drugs and in water sewage from environments expected to be contaminated by CP or IF.

MATERIAL AND METHODS

Chemicals and reagents

Cyclophosphamide monohydrate, ifosfamide, heptafluorobutylic anhydride (HFBA), and trifluoroacetic anhydride (TFAA) were purchased from Sigma-Aldrich chemical company (St. Louis, Missouri, USA). Cyclophosphamide-d4 and ifosfamide-d4 were obtained from Toronto Research Chemical Inc. (Ontario, Canada). The other solvents and chemicals were of analytical grade and were purchased from Merck KGaA (Darmstadt, Germany).

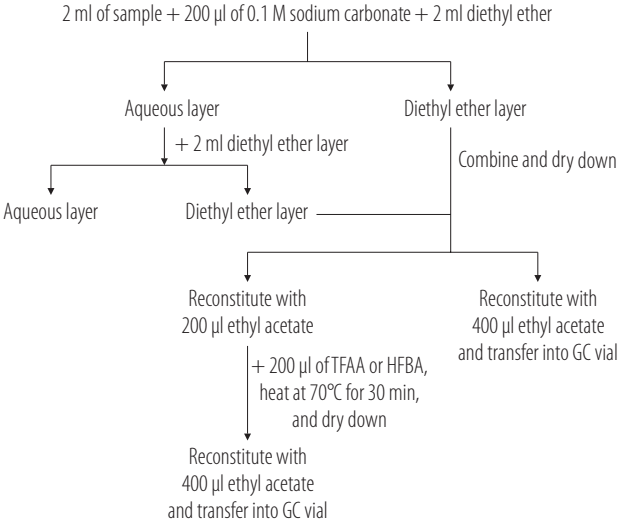
Instrumental conditions

The analyses were performed on 6890 gas chromatograph equipped with 5973N mass spectrometer and 7683 automatic liquid sampler (Agilent Technologies, USA). The Agilent MSD ChemStation software system and the National Institute of Standards and Technology (NIST) software system of MS library and chemical structures (software registration number BN 36CCD7AD and BN 35EACB87) on a personal computer were used to operate the instrument and to acquire data. Separation was carried out on a HP-5MS capillary column (J&W Scientific, USA). The initial oven temperature was set at 70°C for 1 min, and ramped to 250°C at a rate of 10°C/min. The final temperature was held for 1 min. For TFAA or HFBA derivatives, the initial oven temperature was set at 70°C for 1 min; then increased to 200°C at the rate of 10°C/min and to 240°C at the rate of 20°C/min. The final temperature was held for 1 min.

Sample preparation

The stock standard solutions (0.383 mmol/l) were prepared in methanol. Drug-free urine samples were collected from a healthy volunteer, centrifuged and pooled in a clean bottle. Five water sewage samples were collected from 5 different residential areas in Bangkok, located far away from health care centers. Water sewages were centrifuged and their sediments were discarded. Control samples and working standard solutions were prepared by adding the stock standard solution in urine to a certain concentration. Likewise, water sewages were spiked with the stock standard solution to a given concentration. The sample extraction was performed as indicated in the diagram below (Figure 1).

The CP-d_4 and IF-d_4 standard solutions (0.383 mmol/l) were used as the internal standards. Twenty µl of the internal standard were added into the sample prior to extraction and derivatization with TFAA. Trifluoroacetic anhydride derivatives of CP (TFA-CP) and IF (TFA-IF) were quantitated in selective ion monitoring (SIM) mode, the mass qualifier ions for TFA-CP were selected at the m/z 212, m/z 150 and m/z 136, with m/z 307 being the quantifier ion; the qualifier ions for



TFAA – trifluoroacetic anhydride; HFBA – heptafluorobutylic anhydride; GC – gas chromatography.

Fig. 1. Sample extraction and derivatization

TFA-IF were selected at the m/z 150, m/z 212 and m/z 181, with m/z 307 being the quantifier ion. The selected quantifier ions for TFA-CP-d_4 and TFA-IF-d_4 were at the m/z 311. All calculations were based on peak area ratios.

Method validation

The limit of detection was the lowest concentration of CP or IF correctly identified by NIST. The linearity was expressed as linear regression of the calibration curves. The limit of quantitation was the lowest concentration of drug giving measured results close to the added amount with the signal to noise ratio of 5:1. The accuracy and extraction recovery were determined by analyzed control samples at each concentration 5 times. Accuracy was indicated by averaging the percentages of differences between the measured value and the added concentration. The within-day and between-day precision were determined by analyzing 10 control samples of each concentration within the same day and in 10 consecutive days. The recovery and precision were expressed in mean ± uncertainty (U) at 95% confidence interval (CI); the coverage factor (k) = 2 [20]. Statistical analysis was performed using SPSS IBM Singapore Pte Ltd. (registration No. 1975-01566-C).

RESULTS

As shown in Figure 2, CP gave 2 separated peaks which were identified by NIST as (-)-cyclophosphamide.

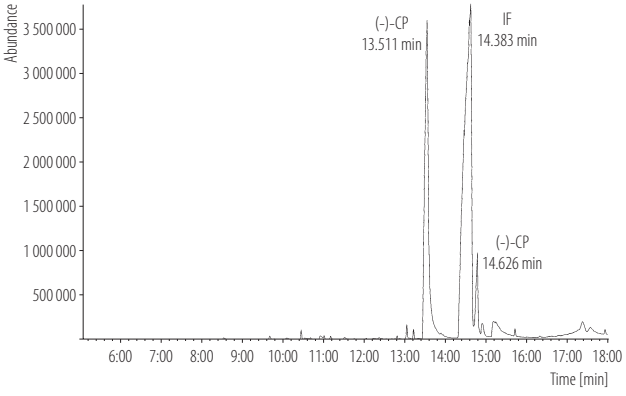
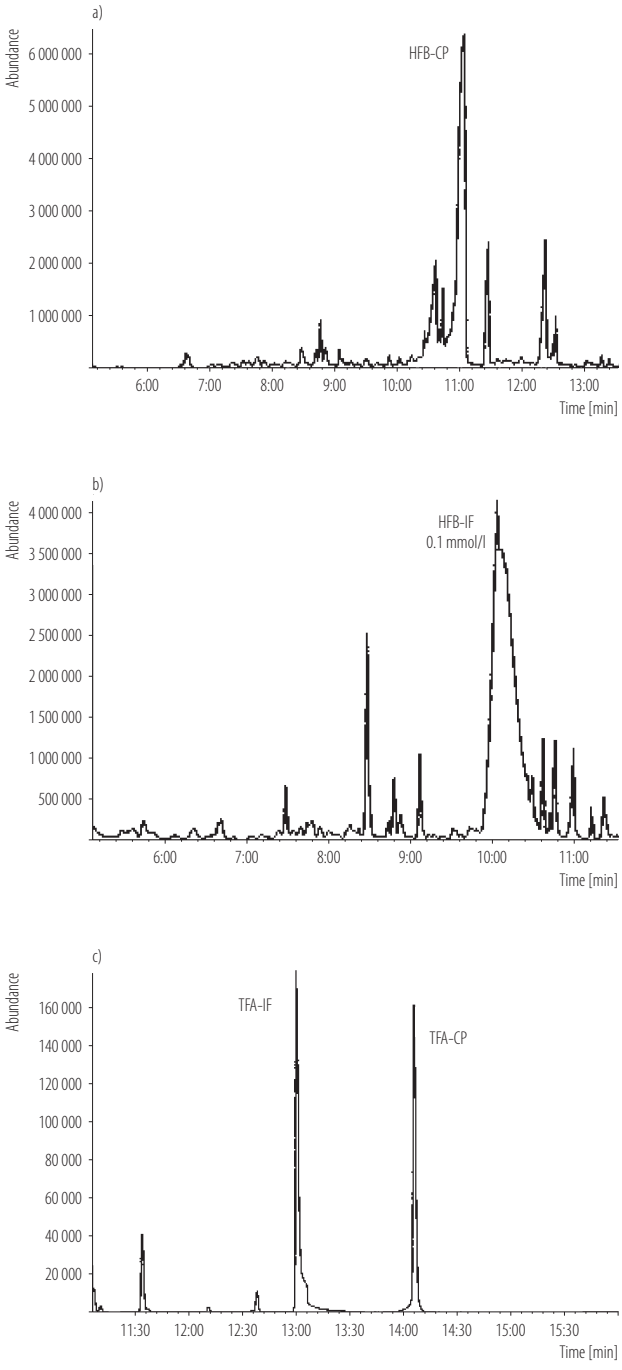


Fig. 2. Total ion chromatograms of cyclophosphamide (CP) and ifosfamide (IF)



HFB-CP – HFBA derivative of CP; HFB-IF – HFBA derivative of IF; TFA-IF – TFAA derivative of IF; TFA-CP – TFAA derivative of CP.

Fig. 3. Total ion chromatograms of heptafluorobutylic anhydride (HFBA) derivatives of a) cyclophosphamide (CP) and b) ifosfamide (IF), and c) trifluoroacetic anhydride (TFAA) derivatives of CP and IF

Table 1. Accuracy, recovery and precision of the gas chromatography–mass spectrometry in analyses of CP and IF in urine and water sewages

Drug spiked sample	Added concentration [nmol/l]	Accuracy (M of % differences) (N = 5)	Recovery ($M \pm U_r$) [nmol/l] (N = 5)	Precision ($M \pm U_p$) [nmol/l]	
				within-day (N = 10)	between-day (N = 10)
CP					
urine	0		0	0	0
	9.5	104	9.88±1.422	9.31±0.380	9.50±0.567
	38.0	102	38.76±3.723	38.32±1.134	38.71±1.964
water 1	0		0		
	9.5	100	9.50±1.202		
	38.0	106	40.28±3.875		
water 2	0		0		
	9.5	96	9.12±1.427		
	38.0	104	39.52±3.886		
water 3	0		0		
	9.5	104	9.88±1.590		
	38.0	110	41.80±2.687		
water 4	0		0		
	9.5	96	9.12±1.599		
	38.0	102	38.76±4.432		
water 5	0		0		
	9.5	104	9.88±1.565		
	38.0	104	39.52±3.875		
IF					
urine	0		0	0	0
	9.5	104	9.88±1.587	9.12±0.507	9.69±0.683
	38.0	104	39.52±5.098	37.62±0.761	38.11±1.964
water 1	0		0		
	9.5	104	9.88±1.423		
	38.0	108	41.04±4.954		
water 2	0		0		
	9.5	104	9.98±1.428		
	38.0	106	40.28±4.333		
water 3	0		0		
	9.5	104	1.03±1.873		
	38.0	102	38.76±3.723		

Table 1. Accuracy, recovery and precision of the gas chromatography–mass spectrometry in analyses of CP and IF in urine and water sewages – cont.

Drug spiked sample	Added concentration [nmol/l]	Accuracy (M of % differences) (N = 5)	Recovery ($M \pm U_r$) [nmol/l] (N = 5)	Precision ($M \pm U_p$) [nmol/l]	
				within-day (N = 10)	between-day (N = 10)
IF – cont.					
water 4	0		0		
	9.5	108	10.26±1.520		
	38.0	108	41.04±3.723		
water 5	0		0		
	9.5	96	9.12±1.414		
	38.0	98	37.24±4.465		

CP – cyclophosphamide; IF – ifosfamide; M – mean; N – number of analyzed samples; U_r – expanded uncertainty for extraction recovery; U_p – expanded uncertainty for precision.

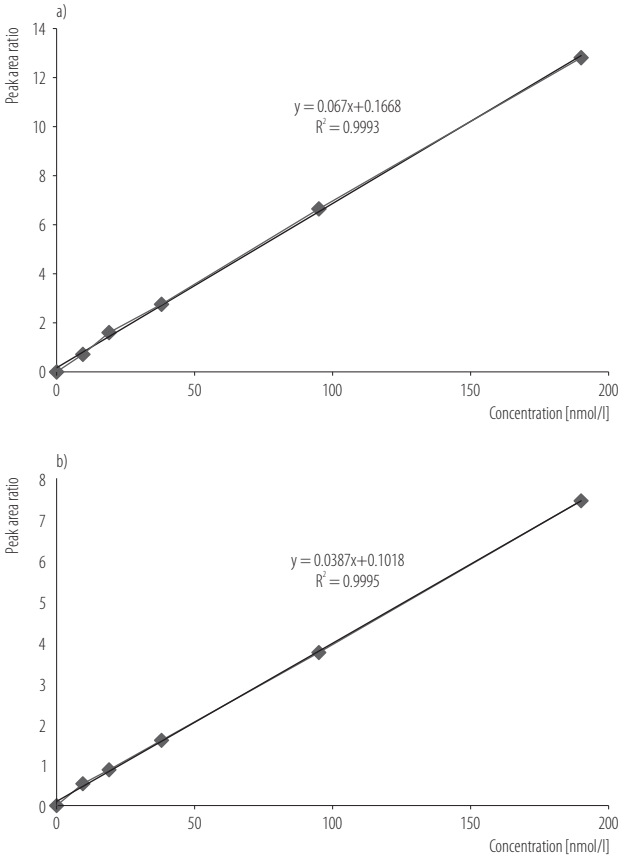


Fig. 4. Calibration curves of a) cyclophosphamide (CP) and b) ifosfamide (IF) after derivatization with trifluoroacetic anhydride (TFAA)

The IF peak was between these peaks. The chromatograms of HFBA derivatives showed high baseline noise (Figure 3). The peaks of TFA-CP and TFA-IF were well separated and low baseline noise was on the chromatogram (Figure 3). The calibration curves of both CP and IF were linear and passed zero with the $R^2 = 0.9993$ and 0.9995, respectively (Figure 4). Drug-free urine samples were triple analyzed and no CP or IF peak was found. Figure 4 demonstrates the calibration curves of CP and IF after derivatization with TFAA. The results from method validation are shown in Table 1.

DISCUSSION

According to previous reports [18,19], CP always gave 2 separated peaks on the chromatogram (Figure 2). The ratios of these 2 peaks were indeed inconsistent causing inaccurate quantitation. Though IF gave a single peak, its detection limit was equal to that of CP or at 380 nmol/l. Without derivatization, both CP and IF were poorly detected, possibly due to their low volatility, leading to ion suppression, i.e., reducing droplet formation, which in turn decreased the amount of charged ions in the gas

phase reaching the mass spectrometer [21]. The drug-free urine gave no detectable CP and IF peaks indicating high specificity.

As identified by NIST, the undesired peaks on the chromatograms of HFBA derivatives of CP (HFB-CP) and IF (HFB-IF) resulted from the column bleed (Figure 3). The samples were dried down after derivatization as recommended [22,23], but the column bleed revealed that remaining HFBA was not completely removed. Additionally, the peak of HFB-IF was aberrant, having long back tail. As reported [24], HFB-IF may have non-specific adsorption or interaction with the stationary phase leading to gradual elution. Because of the high baseline noise and the aberration of HFB-IF peak, the HFBA derivatization should not be a practical method for CP and IF quantitation either.

Trifluoroacetic anhydride derivatization obviously improved chromatographic behaviors of CP and IF. Both TFA-CP and TFA-IF presented a single sharp peak with high signal to noise ratios. Trifluoroacetic anhydride derivative of IF was well separated from the TFA-CP (Figure 3). Trifluoroacetic anhydride derivatization also enhanced the MS detection, supporting it to be a practical chemical derivatization for quantitation of CP and IF.

Drug-spiked plasma samples were studied but no studied solvent could well extract CP and IF from the plasma matrix. Urine and water sewage had similar matrix effect and could be extracted well with the propose procedure. An advantage of collecting urine from non-currently ill person is that an individual will willingly give his/her urine any time requested. Water sewage especially nearby a hospital or pharmaceutical plant is likely a source of these drugs derived from accumulating patient excretion and wastes, therefore, it should be a proper sample for monitoring the environmental contamination of these drugs.

The concentrations of CP and IF were determined after derivatization with TFAA. Their calibration curves passed the zero and were linear at the concentrations ranging from 0 to 190 nmol/l (Figure 4). The limits of quantitation of both drugs were 3.8 nmol/l. The formerly reported concentrations of these drugs in workers' urine were lower than 3.8 nmol/l [25,26]. Therefore, the proposed method may give a false negative result in these cases.

Because CP and IF are genotoxic, they have zero tolerance or no threshold level. By the meaning of zero tolerance, it should not be implied that their concentrations must be zero for health risk prevention. In fact, their adverse effects result from their accumulation in the body to the toxic levels, thus, the proposed method is still expected to be useful for prevention their toxicity in a healthy person. With its acceptable analytical parameters, the method will be further applied for monitoring CP and IF concentrations in urine and water sewage to prevent their adverse effects in occupationally and environmentally exposed people.

CONCLUSIONS

Demand for CP and IF analysis not only for therapeutic drug monitoring but also for prevention of occupational and environmental pollution is increasing despite the limitation of the available instrument. Chemical and physical properties of CP and IF are responsible for their poor chromatographic behaviors and quantitative parameters. This presentation supports the conclusion of the previous works that TFAA derivatization can improve the chromatographic behavior, molecular stability, and mass spectral detection of CP and IF. Using their stable isotope analogs as the internal standard could improve the analytical recovery, accuracy, and reliability. The method can detect levels of these drugs in urine and water sewage as low as 3.8 nmol/l and will be applied for prevention of both occupational and environmental toxicity.

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