A Direct Injection LC/ESI-MS/MS Analysis of Urinary Cyclophosphamide as an Anticancer Drug for Monitoring Occupational Exposure

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Abstract
A highly sensitive and reliable analytical method that uses liquid chromatography/electrospray ionization tandem mass spectrometry coupled with a simple on-line solid-phase extraction was developed to monitor occupational exposure by measuring cyclophosphamide in the urine of medical staffs who handle this anticancer drug. The quantitation limit is 3 pg/mL with a signal-to-noise ratio of more than 10, and the measurement range is 3 pg/mL to 3 ng/mL. Using this method, trace levels of cyclophosphamide have been detected in the urine of two pharmacists after handling this anticancer drug during more than 4 years exposure survey. This finding strongly suggests that it is very important to monitor the occupational exposure of medical staffs who handle anticancer drugs in order to assess the health hazard and control the use of these cytostatic drugs. These data also show that this analytical method can be successfully used to monitor occupational exposure by measuring the levels of residual cyclophosphamide in human urine.

Keywords: Anticancer drug, Cyclophosphamide, Exposure, LC/ESI-MS/MS, Medical staff

1. Introduction
Cyclophosphamide is an antineoplastic alkylating agent that is used in various chemotherapeutics such as cancer chemotherapies. Cyclophosphamide is a prodrug that exhibits antitumor activity after hepatic metabolism. This prodrug is used in combination with other anticancer drugs and is commonly incorporated into many cancer chemotherapeutic regimens. In 1979, Falck et al. [1] reported that the urine of nurses handling cyclophosphamide was mutagenic and noted the possibility that medical staff can be occupationally exposed to this agent. On the other hand, Staiano et al. [2] reported that the urine of eight pharmacists, who admix antitumor drugs, had no mutagenic activity, after controlling for smoking, medication, and alcohol intake. Bos et al. [3] reported that smoking has a synergistic effect by inducing enzymes involved in the mutagenic activation of cytostatic drugs, although the mutagenicity of urine concentrates from non-smokers does not increase with exposure. These conflicting reports suggest that evaluating mutagenicity is not sufficient to determine the occupational exposure of medical staff. Therefore, it is important to accurately assess the occupational exposure to cytostatic drugs by directly analyzing the levels that are absorbed into the body of medical staffs.
exposed workers.

Sessink et al. [4] reported an analytical method for measuring cyclophosphamide in human urine by gas chromatography/mass spectrometry (GC/MS). This method consisted of a liquid-liquid extraction step using diethyl ether, derivatization using trifluoroacetic anhydride, and GC/MS analysis. The selected ion monitoring analysis provided a highly sensitive analysis (detection limit: 0.25 ng/mL urine, 12.5 pg on column). The modified method was used to detect cyclophosphamide or ifosfamide in eight urine samples out of 25 pharmacy technicians and nurses [5]. Two of those subjects handled these antineoplastic agents, but six nurses and pharmacy technicians did not directly handle these agents during drug preparation and administration. This suggested that medical staff could be exposed to these sources in situations other than drug preparation and administration. In 2004, Kasel et al. [6] reported an analytical method using liquid chromatography/tandem mass spectrometry (LC/MS/MS) to quantify the concentrations of cyclophosphamide (detection limit: 5 ng/mL) and its metabolites, N-dechloroethylcyclophosphamide, 4-ketocyclophosphamide, and carboxyphosphamide, in urine. Although the method was reliable compared to the traditional GC/MS method, it was unable to detect trace amounts of cyclophosphamide in the urine of occupationally exposed medical staff.

In this study, we generated an analytical method to measure trace levels of urinary cyclophosphamide by LC/MS/MS equipped with electrospray ionization (ESI). Urine samples mixed with an internal standard (IS) solution can be injected directly into a simple column-switching system, which contains a trapping column for concentration of analyte.

2. Materials and methods

2.1. Chemicals

Cyclophosphamide monohydrate and d4-cyclophosphamide as an IS were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Toronto Research Chemicals Inc. (North York, Canada), respectively. Capcell Pak BB-H (150 mm × 2.1 mm I.D., 3 μm) was purchased from Shiseido Co., Ltd, (Tokyo, Japan), and TSKgel precolumn BSA-ODS/S (10 mm × 4.6 mm I.D., 50 μm) was obtained from Tosoh corporation (Tokyo, Japan). Ultra pure water was prepared using a PURELAB ultra apparatus (Organo Corporation, Tokyo, Japan). All other reagents were analytical grade, and solvents were HPLC or LC/MS grade. Cyclophosphamide monohydrate (11.6 mg) was dissolved in 10 mL of water-ethanol (1:1, v/v), and the solution was further diluted in water. The IS stock solution was prepared in the water-ethanol (1:1, v/v) and the concentration was adjusted to 4 ng/mL. These solutions were used as calibration standards and stock solutions for validation experiments and stored at 4°C.

2.2. LC/MS/MS analysis

LC/ESI-MS/MS was performed using an API 5000 tandem mass spectrometer (Sciex, Framingham, MA, USA) coupled with an LC-20AD system (Shimadzu Corporation, Kyoto, Japan). The 1.2 mL aliquots of samples were injected by an auto-sampler, and the analyte was trapped and concentrated at the inlet edge of TSKgel precolumn BSA-ODS/S (10 mm × 4.6 mm I.D., 50 μm) as a trapping column using formic acid-water (0.1:100, v/v) at a flow rate of 1 mL/min. The six-port valve position was switched after 3 min and the analyte was separated on Capcell Pak BB-H (150 mm × 2.1 mm I.D., 3 μm) as an analytical column using formic acid-water-acetonitrile (0.1:80:20, v/v/v) as the mobile phase at a flow rate of 0.3 mL/min. Thirty min later, the mobile phase was switched to formic acid-water-acetonitrile (0.1:5:95, v/v/v) for washing the analytical column for 6 min, and then, it was switched again to formic acid-water-acetonitrile (0.1:80:20, v/v/v) for conditioning column for 6 min. The column temperature was maintained at 40°C. The spray voltage was set at 5500 V. Vaporizer temperature was 650°C. Ion source gas 1, ion source gas 2, curtain gas, and collision gas were set at 35, 70, 20, and 6 units, respectively. Declustering potential, entrance potential, collision voltage, collision cell exit potential, and scan time were 50 V, 10 V, 31 V, 20 V, and 0.5 sec, respectively. The transitions of m/z 261 to 140 and m/z 265 to 140 on selected reaction monitoring (SRM) were used to monitor cyclophosphamide and d4-cyclophosphamide as an IS, respectively.

2.3. Analytical method validation

Since biological materials in urine affects the cyclophosphamide determination by LC/MS/MS, a stable isotope-labeled compound which minimized the influence of matrix was selected as the IS. The calibration standard solutions were prepared by diluting the stock solution in water to 0.054 ng/mL, 0.18 ng/mL, 0.54 ng/mL, 1.8 ng/mL, 5.4 ng/mL, 18 ng/mL, and 54 ng/mL. Seventy five μL of each calibration standard and 75 μL of the IS stock solution were used as calibration standards and stock solutions for validation experiments and stored at 4°C.

Intra-day and inter-day validation was performed using three different concentrations. Seventy five μL of the 0.54 ng/mL (30 pg/mL as urine concentration), 1.8 ng/mL (100
pg/mL), and 18 ng/mL (1000 pg/mL) stock solutions was added to 1350 μL of filtrated control urine with 75 μL of the IS solution (4 ng/mL), and treated with centrifugation. This procedure was repeated six times on the same day for intra-day validation and repeated once a day for six days for inter-day validation. The 1.2 mL aliquots of these preparations were injected into the LC/ESI-MS/MS system and the cyclophosphamide concentration was analyzed. The accuracy (R.E.) was calculated as \( \frac{\text{found concentration} - \text{theoretical concentration}}{\text{added concentration}} \times 100 \% \), and the precision was determined based on the coefficient of variation (R.S.D. %).

For investigation of stability of cyclophosphamide in urine, high (1000 pg/mL) and low (30 pg/mL) concentration samples (each n=3) were stored at −80°C for 4 hours, 24 hours, 1 month, and 3 months, and then analyzed cyclophosphamide concentrations after storage. The high and low concentration urine samples stored at −80°C (each n=3) were subjected to freeze and thaw stability test and then analyzed their cyclophosphamide concentrations. The stability of high and low concentrations urine samples added IS solution (each n=3) were investigated for 24 hours in auto-sampler adjusted the temperature to 4°C. In addition, the stability of high and low concentrations standard stock solutions also was investigated for 6 hours at room temperature.

2.4. Analysis of urinary cyclophosphamide

Pharmacists who engaged in preparation of antineoplastic drugs injectable solutions containing cyclophosphamide collected their urine (ca. 5 mL) after the procedure. The pharmacists who did not handle these preparations also collected their urine as a negative control. All of the urine samples were immediately frozen. The thawed urine samples were filtered (0.2 μm), and 1.35 mL aliquots were mixed with the 75 μL of the IS solution (4 ng/mL) and 75 μL of water, and was treated with centrifugation at 14000 × g for 1 min at 4°C. A volume of 1.2 mL supernatant was injected into the column-switching LC/ESI-MS/MS system. All samples were collected after the pharmacists provided informed consent according to the protocol adopted by the institutional review board of the Graduate School of Medicine at Tohoku University.

Fig. 1. Typical electrospray ionization mass spectrum of cyclophosphamide. Mass spectrometric conditions: introducing solvent, formic acid-water-acetonitrile (0.1:80:20, v/v/v); flow rate, 0.3 mL/min; ionspray voltage, 5500 V; declustering potential, 50 V; turbospray gas probe temperature, 650°C; curtain gas flow, 20 units; resolution, unit resolution mode.

Fig. 2. Product ion mass spectra of [M+H]+ including two 35Cl at m/z 261 (A), one 35Cl and one 37Cl at m/z 263 (B), and two 37Cl at m/z 265 (C). Mass spectrometric conditions: collision voltage, 31 V; collision gas, nitrogen at 6 units; resolution, unit resolution mode; the other conditions are the same as in Fig. 1.
3. Results and discussion

3.1. Mass spectrometry of cyclophosphamide

Cyclophosphamide has a favorable structure for ESI. We measured an ESI mass spectrum under the flow injection mode through a guard column (Inertisil ODS-3, 10 mm × 1.5 mm I.D., GL Sciences Inc., Tokyo, Japan). Cyclophosphamide (4 ng/2 μL) in water was injected into a flow of formic acid-water-acetonitrile (0.1:80:20, v/v/v) as the mobile phase at a flow rate of 0.3 mL/min. The obtained mass spectrum is shown in Fig. 1. A protonated molecule was detected at m/z 261 with sodium adduct and potassium adduct molecules at m/z 283 and m/z 299, respectively. Because cyclophosphamide has two chlorine atoms, the characteristic split patterns of a protonated molecule and its isotopic peaks were clearly detected. The intensity ratio of [M+H]+ : [M+H+2]+ : [M+H+4]+ was approximately 100:83:20 and was in close agreement with the theoretical ratio. Although the intensities of [M+H]+ increased when the ionspray voltage was changed to range from 3000 V to 5500 V, those of [M+Na]+ and [M+K]+ decreased at the declustering potential of approximately 50 V.

![Diagram](image)

Fig. 3. Estimated fragment pattern of a protonated molecule of cyclophosphamide on low-energy collision-induced dissociation.

The product ion mass spectrum of [M+H]+ at m/z 261 is shown in Fig. 2A. Product ions appeared at m/z 233, m/z 142, m/z 140, m/z 138, m/z 120, and m/z 106. The precursor ion at m/z 261 included two 35Cl, and the ions at m/z 263 and m/z 265 observed in the ESI mass spectrum (Fig. 1) have one 37Cl and two 35Cl, respectively. In the product ion mass spectrum of the precursor ion at m/z 263 (Fig. 2B), product ions were observed at m/z 235, m/z 144, m/z 142, m/z 138, m/z 120, m/z 108, and m/z 106. In the product ion mass spectrum of the precursor ion at m/z 265 (Fig. 2C), product ions were observed at m/z 237, m/z 146, m/z 144, m/z 138, m/z 120, and m/z 108. The common product ions at m/z 138 and m/z 120 were chlorine-free, and the product ions at m/z 233, m/z 142, and m/z 140 included two chlorine atoms. The product ion at m/z 106 included one chlorine atom. From these results, we assigned the structures of product ions and show them in Fig. 3. The product ion at m/z 140 was the most strongly produced, and the effect of collision energy on the intensity of the ion was investigated. Collision energy of 31 V gave intense product ion. At the appropriate condition, we could not observe the peaks corresponding to cyclophosphamide and d4-cyclophosphamide in healthy subjects (data not shown).

3.2. HPLC method development and validation

A previous study reported the presence of 10 ng of cyclophosphamide in 24-h urine [7]. The volume of 24-h urine from a normal healthy subject is estimated to be about 1,500 mL. Therefore, we have to measure at least 6.7 pg/mL of cyclophosphamide in the urine. Although several LC/MS/MS methods have been reported for measurement of cyclophosphamide in urine [7-9], their sensitivity was insufficient to monitor occupational exposure. The present method covered a linearity range of 3 pg/mL to 3000 pg/mL of concentrations in urine, and the correlation coefficient was more than 0.999. Intra-day (n=6) and inter-day (n=6) accuracy and precision were investigated at three different levels of 0.54 ng/mL (30 pg/mL urine), 1.8 ng/mL (100 pg/mL urine), and 18 ng/mL (1000 pg/mL urine), and the results are summarized in Table 1. The intra-day accuracy was in the range of 1.8% to 5.3%, and the intra-day precision was less than 2.5%. In the inter-day validation, the precision was less than 6.1%, and the accuracy was in the range of 3.5% to 7.4%. The 3 pg/mL of cyclophosphamide, which was considered as the lower limit of quantitation (LLOQ), had a signal-to-noise ratio of more than 10 as shown in Fig. 4B. Although the present method is an extremely simple analytical method which does not require even deproteinization, it was possible to measure with extremely high sensitivity compared with the method reported previously [4-6].

### Table 1. Assay performance for cyclophosphamide in human urine.

| Concentration (pg/mL) | Intra-day Observed (pg/mL) | Intra-day C.V. (%) | Intra-day R.E. (%) | Inter-day Observed (pg/mL) | Inter-day C.V. (%) | Inter-day R.E. (%) |
|-----------------------|---------------------------|-------------------|-------------------|---------------------------|-------------------|-------------------|
|                       |                           |                   |                   |                           |                   |                   |
| 30 pg/mL              |                           |                   |                   | 100 pg/mL                 |                   |                   |
|                       | Observed                  |                   |                   | Observed                  |                   |                   |
|                       | (pg/mL)                   |                   |                   | (pg/mL)                   |                   |                   |
|                       | C.V. (%)                   |                   |                   | R.E. (%)                  |                   |                   |
|                       | 30.6                      | 2.49              | 1.83              | 105                       | 2.37              | 4.83              |
|                       | 31.0                      | 5.37              | 3.48              | 106                       | 6.10              | 5.59              |
| 1000 pg/mL            |                           |                   |                   |                           |                   |                   |
|                       | Observed                  |                   |                   | Observed                  |                   |                   |
|                       | (pg/mL)                   |                   |                   | (pg/mL)                   |                   |                   |
|                       | C.V. (%)                   |                   |                   | R.E. (%)                  |                   |                   |
|                       | 1053                      | 1.66              | 5.33              | 1074                      | 2.75              | 7.39              |

Chromatography
### Table 2. Stability of cyclophosphamide.

| Condition | 30 pg/mL | 1000 pg/mL |
|-----------|----------|-------------|
|           | R.E. (%) | C.V. (%)    | R.E. (%) | C.V. (%) |
| (A) −80°C |          |             |          |          |
| 4 hrs     | -1.84    | 3.42        | -4.38    | 1.61     |
| 24 hrs    | -4.33    | 2.05        | -5.61    | 1.24     |
| 1 month   | 8.67     | 3.94        | 0.10     | 5.14     |
| 3 months  | 5.16     | 3.17        | 0.76     | 1.04     |
| (B) freeze and thaw | -2.15    | 0.46        | -2.16    | 2.02     |
| (C) auto-sampler at 4°C for 24 hrs | -0.02    | 2.48        | -7.37    | 2.11     |
| (D) stock solution at room temperature for 6 hrs | 1.08     | 3.83        | -4.28    | 1.28     |

**Fig. 4.** Typical SRM chromatograms of the urine sample spiked with 0 pg/mL (A), 3 pg/mL (B), and 10 pg/mL (C) of the standard solution with 50 pg/μL of d4-cyclophosphamide as an IS. Chromatographic and mass spectrometric conditions are described in the Experimental methods section.
The results of stability test are summarized in Table 2. Cyclophosphamide in the urine was stable at −80°C for 3 months at high or low concentrations and no change in content was observed even when freezing and thawing was repeated. The samples after preparation were stable in an autosampler (4°C) for 24 hours, and the content was not changed even when the stock solutions were left at room temperature for 6 hours. These all results suggest that the present method can accurately and reproducibly measure the amounts of urinary cyclophosphamide in high-sensitively.

3.3. Analysis of urinary cyclophosphamide

Figure 4 shows the SRM chromatograms of the urine sample spiked with 0 pg/mL (A), 3 pg/mL (B), and 10 pg/mL (C) of the standard solution. Although the retention time of the IS (d₄-cyclophosphamide) was nearly identical to unlabeled cyclophosphamide (tᵣ: 13.9 min), this standard is sufficient to correct variation in ionization, and adding d₄-cyclophosphamide (300 pg/75 μL) to the samples had no effect on the SRM chromatogram at m/z 261 to 140 (unlabeled monitoring transition).

To assess the personal occupational exposure, biological monitoring of urinary cyclophosphamide was performed once every month. Urine samples from a pharmacist who did not handle cyclophosphamide did not contain traces of cyclophosphamide (Fig. 5A). Almost all pharmacists who engaged in preparation of chemotherapeutic drugs had no positive results in any urine sample in exposure survey over 4 years. Only two pharmacists had once a positive result with urinary concentration of 7.88 pg/mL (Fig. 5B) and 13.6 pg/mL (approximately 11.8 and 20.4 ng/24-h urine). The results strongly suggest that pharmacists in our hospital are well protected from the exposure to antineoplastic drugs and monitoring chemicals associated with health hazards and safeguarding is very important to safely and adequately distribute chemotherapies. Carmustine, mustargen, ifosfamide, thiotepa, and cyclophosphamide demonstrated considerable vaporization at 37°C, and the former two demonstrated somewhat lower levels of vaporization at 23°C [10]. On the other hand, cyclophosphamide produced a minimal response at 23°C without dose response, and this suggested that cyclophosphamide vaporization at room temperature may be minimal. These facts suggest that significant sources of exposure for pharmacists and nurses handling cyclophosphamide are spills and aerosols produced from cyclophosphamide solution not vaporization. If that is true, we should prevent production of aerosol during preparation of injectable solution of all cytotoxic agents.

**Fig. 5.** Typical SRM chromatograms of the urine sample from a pharmacists who did not handle the cyclophosphamide (A) and a pharmacist who handled the cyclophosphamide in anticancer drug preparation (B). Chromatographic and mass spectrometric conditions are described in the Experimental methods section.
4. Conclusion

A highly sensitive analytical method of urinary cyclophosphamide to monitor occupational exposure was developed using LC/ESI-MS/MS with a simple on-line solid phase extraction. This method can be used to monitor trace levels of urinary cyclophosphamide in medical staffs to assess their exposure. Since traces of cyclophosphamide have been detected in urine from two pharmacists who handle this mutagenic agent, controlling exposure to this anticancer drug is important for occupational health and safety management. Therefore, this method is useful to maintain the health of medical staff and assess the workspace environment.

Acknowledgment

This manuscript is dedicated to late Mr. Kohei Abe who had contributed to go ahead with this study. The authors thank pharmacists for collaborating with this research.

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