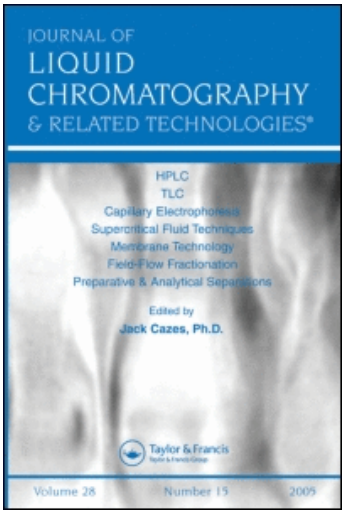

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Fox, P. A. , Lively, J. D. , Firth, J. W. , Woolfrey, S. G. and Greenslade, D.(1996) 'A Sensitive Assay for Cyclo-Phosphamide in Human Plasma Utilizing Mass Spectroscopy', Journal of Liquid Chromatography & Related Technologies, 19: 7, 1047 — 1059
To link to this Article: DOI: 10.1080/10826079608006301
URL: http://dx.doi.org/10.1080/10826079608006301

J. LIQ. CHROM. & REL. TECHNOL., 19(7), 1047–1059 (1996)

A SENSITIVE ASSAY FOR CYCLOPHOSPHAMIDE IN HUMAN PLASMA UTILIZING MASS SPECTROSCOPY

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ABSTRACT

This study describes a highly sensitive, solid-phase extraction-HPLC/MS/MS assay for cyclophosphamide in human plasma, with a minimum quantifiable level of 0.025 μg mL^{-1}. Plasma samples (0.5 mL) were mixed with 0.01 M ammonium acetate buffer (1.5 mL, pH 4.9) and internal standard (D4 deuterated compound, 50 μL, 5 μg mL^{-1}), and then extracted using Isolute CH(EC) cartridges (100 mg). Contaminants were washed from the cartridges with 0.01 M ammonium acetate:methanol (90:10, 1 mL), prior to the elution of the compounds of interest with 0.1 M ammonium acetate:methanol (50:50, 300 μL). An aliquot (200 μL) of the eluent was injected onto the chromatography system, which consisted of a LiChroCART 4-4 RP-select B, 5 μm cartridge precolumn, protecting a LiChrospher 60 RP-select B, 5 μm (250 x 4 mm id) cartridge analytical column.

The mobile phase was methanol:0.1M ammonium acetate buffer (pH 4.9, 60:40), run at a flow rate of 1 mL min^{-1}. Detection was by mass spectroscopy using a Finnigan MAT TSQ 700 triple stage quadrupole machine, fitted with an APCI interface. For quantification, the parent ions at $^{m}/_{z}$ 261 and 265 (cyclophosphamide and D4 internal standard respectively) were selected by quadrupole 1, collisonally dissociated in the octapole, and daughters ions at $^{m}/_{z}$ 120 and 124 then monitored via quadrupole 3. The calibration lines were linear over the range 0.025 to 1.0 μg mL^{-1}, with no evidence of any systematic deviation. The within- and between-day precision and accuracy were examined at five levels (0.025, 0.05, 0.5, 0.8 and 1 μg mL^{-1}, n=6), with values of <14% and within ±5% respectively. Loss of compound was not observed (0.05 and 0.8 μg mL^{-1}, n=6) after 3 freeze/thaw cycles, storage of the extracted samples for 24 h at room temperature and at 0 to 5°C, and after 4 weeks storage at -15 to -25°C. Diluting samples from 80 and 10 μg mL^{-1} to within the range of the standard curve was shown not to affect the assay. Although the recovery of cyclophosphamide and the internal standard was only 20%, the use of the deuterated compound ensured an accuracy and precision within the validation criteria. The utility of the assay was demonstrated using plasma from a patient who had received an IV infusion of cyclophosphamide at 1000 mg m^{-2}, given over 1 h.

INTRODUCTION

Cyclophosphamide is an alkylating agent which is used in the treatment of a variety of tumour types. The compound is activated in the liver by cytochrome P450 to phosphoramide mustard[1] and, as a result, its pharmacokinetics can be effected by agents which induce or inhibit P450 metabolism.[2] It is also now fairly well established that cyclophosphamide induces its own metabolism on repeated dosing.[3]

Several analytical methods have been developed for the measurement of cyclophosphamide in human plasma, although many of the early radiolabelled techniques[4] probably lacked specificity. The most commonly used methods have been gas chromatography with[5] or without[6] derivatization of the parent molecule.

HPLC has not been widely used to quantify cyclophosphamide in biological fluids because of its poor sensitivity. However, an HPLC/UV technique has been reported by Rustum and Hoffman[7] which had a detection limit of 0.3 $\mu g\ mL^{-1}$

We report a highly sensitive solid-phase extraction-HPLC assay for cyclophosphamide in human plasma using atmospheric pressure chemical ionization (APCI) mass spectrometry as the method of detection. The extraction stage is rapid because it utilizes solid-phase technology, and the method highly specific as a result of the detection method.

MATERIALS AND METHODS

Materials

Cyclophosphamide ([2-(bis-2-chloro-ethyl) aminotetrahydro-2H-1,3,2-oxaphosphorine-2-oxide]), was obtained from Sigma Chemicals Ltd, (Poole, UK) (Figure 1). The internal standard was a D4-deuteration of cyclophosphamide, (4,4, 6,6 tetradeuterated [2-(bis-2-chloro-ethyl) amino-tetrahydro-2H-1,3,2-oxaphosphorine-2-oxide]), generously supplied by the Cancer Research Unit, University of Newcastle-Upon-Tyne, UK (Figure 1). Cyclophosphamide and the internal standard were supplied as greater than 99% pure. Methanol was of HPLC grade and obtained from Rathburn Chemicals Ltd, (Walkerburn, UK). Ammonium acetate and acetic acid were of analytical grade and obtained from Fisons PLC, (Loughborough, UK) and BDH Ltd, (Poole, UK) respectively. Isolute extraction cartridges were obtained from International Sorbent Technology Ltd (Hengoed, Wales, UK). Human plasma was collected using sodium fluoride and potassium oxalate as the anticoagulant and stored frozen at -15°C to -25°C until required for analysis.

Preparation of Standards

Calibration samples were prepared by spiking control human plasma (0.5 mL) with cyclophosphamide (50 μL of the appropriate standard prepared in water) to give nominal concentrations of 0.025 to 1.0 $\mu g\ mL^{-1}$. Triplicate samples were run at the top (1.0 $\mu g\ mL^{-1}$) and bottom (0.025 $\mu g\ mL^{-1}$) levels.

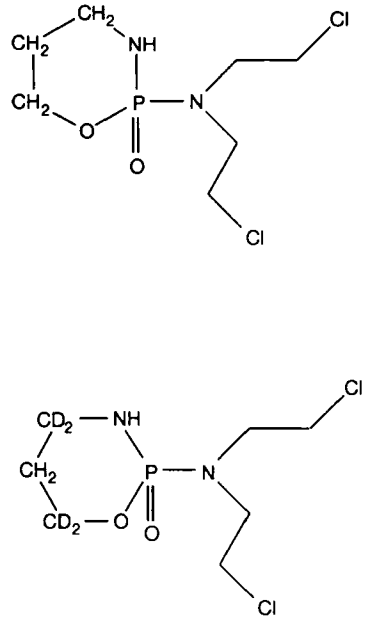

Figure 1. Chemical structure of cyclophosphamide and deuterated cyclophosphamide.

Validation samples were prepared at 5 levels by spiking human plasma (0.5 mL) with aliquots of cyclophosphamide (50 μL) to give nominal concentrations of 0.025, 0.050, 0.50, 0.80 and 1.0 μg mL^{-1}.

Instrumentation and Operating Conditions

The HPLC system consisted of a LiChroCART 4-4 RP-select B, 5 μm cartridge precolumn (Merck, Germany), protecting a LiChrospher 60 RP-select B, 5 μm (250 x 4 mm id) cartridge analytical column (Merck, Germany). A mobile phase of methanol:0.1 M ammonium acetate buffer (pH 4.9, 60:40) was pumped at 1.0 mL min^{-1} using a Hewlett Packard 1050 quaternary pump. The mass spectrometry system consisted of a Finnigan MAT TSQ 700 triple stage quadrupole mass spectrometer with an APCI/ESI interface. Data capture was achieved using the Finnigan MAT Instrument Control software (ICL, Version 7.2) and ICIS II (Version 7.0) application software running on a DEC 3100

workstation (ULTRIX Version 4.2a operating system). Samples were injected on to the system using a Gilson ASPEC XL autosampler.

The HPLC eluent was introduced into the APCI source of the mass spectrometer via an electronically actuated valve (Jones Chromatography, Clywd, UK), which diverted the first 3 minutes of each run to waste. Other conditions were as follows: vaporizer and capillary temperatures of 450 and 175°C respectively; a corona discharge of 5 μA; mass analyzers (quadrupoles 1 and 3) tuned to unit resolution (10% valley definition); argon collision gas pressure of 0.5 mtorr and a collision energy of -45 eV.

For quantification, the parent ions at $^{m}/_{z}$ 261 and 265 (cyclophosphamide and D4 internal standard respectively) were selected by quadrupole 1, collisonally dissociated in the octapole collision cell, and daughter ions at $^{m}/_{z}$ 120 and 124 then monitored via quadrupole 3. The scan time per dissociation was set to 0.5 s giving a cycle time of 1 s.

Assay Procedure

Plasma samples (0.5 mL) were mixed with 0.01 M ammonium acetate buffer (1.5 mL, pH 4.9) and internal standard solution (50 μL, 5 μg mL^{-1}), except for blank samples where 50 μL of water was substituted for the internal standard solution. Each sample was then extracted using Isolute CH(EC) cartridges (100 mg) preconditioned with methanol (2 mL) and 0.01 M ammonium acetate buffer (1 mL). Contaminants were washed from the cartridge with 0.01 M ammonium acetate:methanol (90:10, 1 mL), prior to the elution of the compounds of interest with 0.1 M ammonium acetate:methanol (50:50, 300 μL). An aliquot (200 μL) of the eluent was injected onto the LC/MS/MS system.

Validation Studies

The assay was validated by assessing accuracy and precision, both within and between runs. For the former, the accuracy (Eq 1) and precision (Eq 2) of the assay was examined at the 5 validation levels (n=6) in one run. In the case of the between-run assessment, one sample from each of the 5 validation levels was assessed in 6 separate runs. The stability of samples under various conditions (0.05 and 0.8 μg mL^{-1}, n=6) was also investigated, together with the effect of dilution from levels of 80 (n=4) and 10 μg mL^{-1} (n=6).

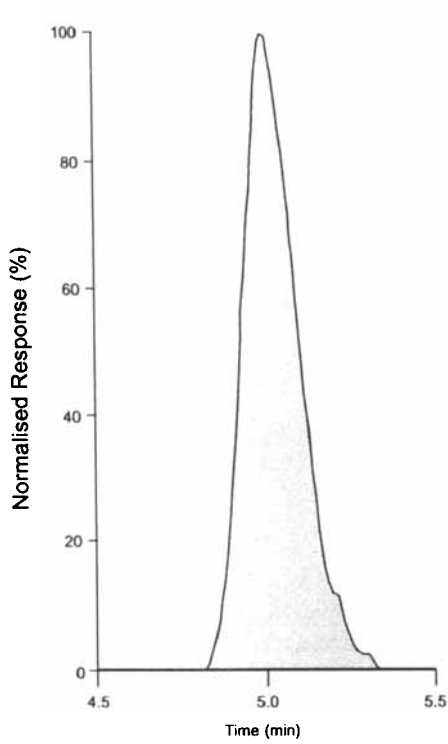

Figure 2. Typical mass chromatogram of a cyclophosphamide peak (1.05 μg mL^{-1}).

The acceptance criteria of the assay was based on Shah et al.[8] Acceptable mean accuracy and precision was defined as being within ±20% at the lowest validation level and ±15% at the remaining levels. In addition, no more than 2 samples at any one level could exceed the above limits, with overall a total of 75% of the individual samples being within specification. With the exception of the recovery experiment, these criteria were applied to the satellite studies using the ±15% limits.

$$\text{Accuracy (M\%D)} = \frac{\text{Observed conc.-expected conc. X 100}}{\text{Expected conc.}} \quad (1)$$

$$\text{Precision (CoV)} = \frac{\text{Standard deviation X 100}}{\text{Mean}} \quad (2)$$

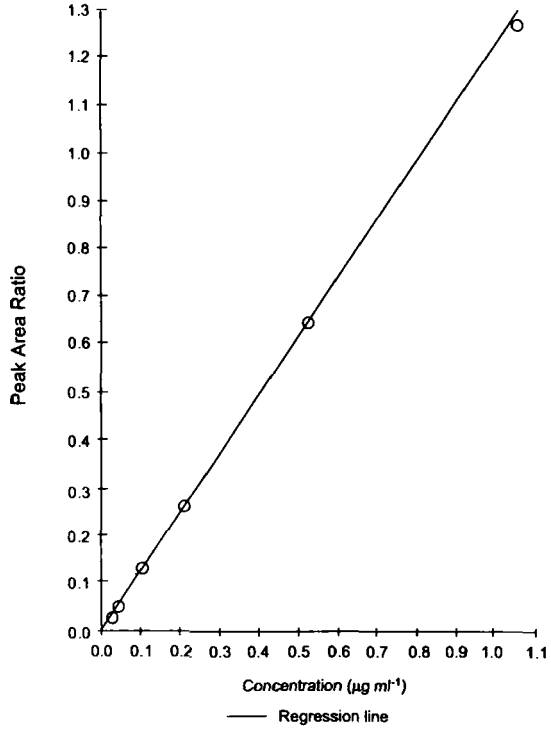

Figure 3. Typical calibration curve.

Utility of Method

To demonstrate the utility of the assay, the plasma concentrations of cyclophosphamide were measured in a cancer patient who was involved in a phase II drug interaction study. Cyclophosphamide was given as a 1 h intravenous infusion (1000 mg m^{-2}) 3 h after the administration of tirapazamine, a novel anticancer agent currently undergoing clinical assessment. For cyclo- phosphamide, plasma samples were obtained for up to 4.5 h following the start of the infusion and analyzed for parent compound as described above.

Table 1

Precision and Accuracy Data for the Assay of Cyclophosphamide in Human Plasma

Study	Parameter (n=6)	Observed Conc. ($\mu g\ mL^{-1}$) 0.025*	0.050*	0.500*	0.800*	1.00*
Within-Day	Mean ($\mu g\ mL^{-1}$)	0.0247	0.0520	0.523	0.843	1.07
	S.D. ($\mu g\ mL^{-1}$)	0.0014	0.0070	0.016	0.028	0.04
	CoV (%)	5.86	13.5	3.00	3.33	3.14
	M%D (%)	-4.26	0.87	1.49	2.25	3.98
Between-Day	Mean ($\mu g\ mL^{-1}$)	0.0258	0.0518	0.525	0.826	1.05
	S.D. ($\mu g\ mL^{-1}$)	0.0031	0.0031	0.023	0.029	0.04
	CoV (%)	8.14	6.04	4.41	3.46	3.76
	M%D (%)	0.00	0.55	2.01	0.22	1.59

*Nominal concentration; Individual accuracies were within the acceptance criteria.

RESULTS

The chromatograms for cyclophosphamide and the internal standard were free of interfering endogenous peaks, characteristic of this highly specific detection method (Figure 2). Retention times for both cyclophosphamide and the internal standard were in the region of 5 mins. The calibration lines were linear over the range 0.025 to 1.0 $\mu g\ mL^{-1}$, with no evidence of any systematic deviation (Figure 3).

The accuracy and precision of the assay was within the acceptance criteria. For the within-day data the mean precision was 5.86% at 0.025 $\mu g\ mL^{-1}$, 13.5% at 0.05 $\mu g\ mL^{-1}$, 3.00% at 0.5 $\mu g\ mL^{-1}$, 3.33% at 0.8 $\mu g\ mL^{-1}$ and 3.14% at 1.0 $\mu g\ mL^{-1}$ with mean accuracies of -4.26%, 0.87%, 1.49%, 2.25% and 3.98% respectively (Table 1).

Similar results were observed for the between-day data with a mean precision of less than 9% and a mean accuracy of within ±2% (Table 1).

Table 2

Freeze/Thaw and Dilution Data for the Assay of Cyclophosphamide in Human Plasma

Parameter	Observed Conc. ($\mu g\ mL^{-1}$) 2 Freeze/Thaw		3 Freeze/Thaw		Dilution	
	0.05*	0.80*	0.05*	0.80*	80*	10*
Mean ($\mu g\ mL^{-1}$)	0.0514	0.852	0.0509	0.861	84.4	10.8
S.D. ($\mu g\ mL^{-1}$)	0.0030	0.027	0.0035	0.026	5.66	0.2
CoV (%)	5.82	3.19	6.94	3.00	6.70	1.57
M%D (%)	-0.13	3.34	-1.13	4.47	6.63	5.28

n=6 except for dilution study at 80μ mL^{-1} where n=4

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 3

Stability of Samples After Extraction and Storage at 0 to 50°C for 24 h and Ambient Temperature for 24 h

Condition	Parameter (n=6)	Observed Conc. ($\mu g\ mL^{-1}$) 0.050*	0.80*
Fridge (0-5°C)	Mean ($\mu g\ mL^{-1}$)	0.0568	0.856
	S.D. ($\mu g\ mL^{-1}$)	0.0019	0.024
	CoV (%)	3.32	2.83
	M%D (%)	10.3	3,84
Ambient	Mean ($\mu g\ mL^{-1}$)	0.0574	0.874
	S.D. ($\mu g\ mL^{-1}$)	0.0032	0.030
	CoV (%)	5.51	3.45
	M%D (%)	11.4	6.05

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 4

Stability of Samples Stored at -15 to -250°C

Parameter (n=6)	Observed Conc. (μg mL^{-1}) 1 Week 0.050*	1 Week 0.80*	2 Week 0.050*	2 Week 0.80*	4 Week 0.050	4 Week 0.80*
Mean (μg mL^{-1})	0.0589	0.858	0.0545	0.833	0.0572	0.861
S.D. (μg mL^{-1})	0.0029	0.018	0.0020	0.031	0.0022	0.031
CoV (%)	4.93	2.11	3.60	3.72	3.81	3.55
M%D (%)	14.6	4.15	5.79	1.05	11.2	4.45

*Nominal concentration; Individual accuracies were within the acceptance criteria.

Table 5

Mean Recovery (± S.D.) of Internal Standard and Cyclophosphamide from Human Plasma

Component (n=6)	Recovery (%) 0.025*	0.50*	1.0*
I.S	23.9 ± 5.8	23.2 ± 6.2	25.4 ± 9.9
Cyclo	20.2 ± 5.1	21.6 ± 5.9	24.7 ± 10.0

*Nominal concentration

The stability of plasma samples at 0.05 and 0.8 μg mL^{-1} was examined under a variety of conditions. There was no evidence for any loss of cyclophosphamide, or any affect upon the quantification process, after 2 and 3 freeze/thaw cycles (Table 2), after storage of the extracted samples for 24 h at ambient temperature, after storage of the extracted samples at 0 to 5°C for 24 h and when kept in the freezer at -15°C to -25°C for up to 4 weeks (Tables 3 and 4).

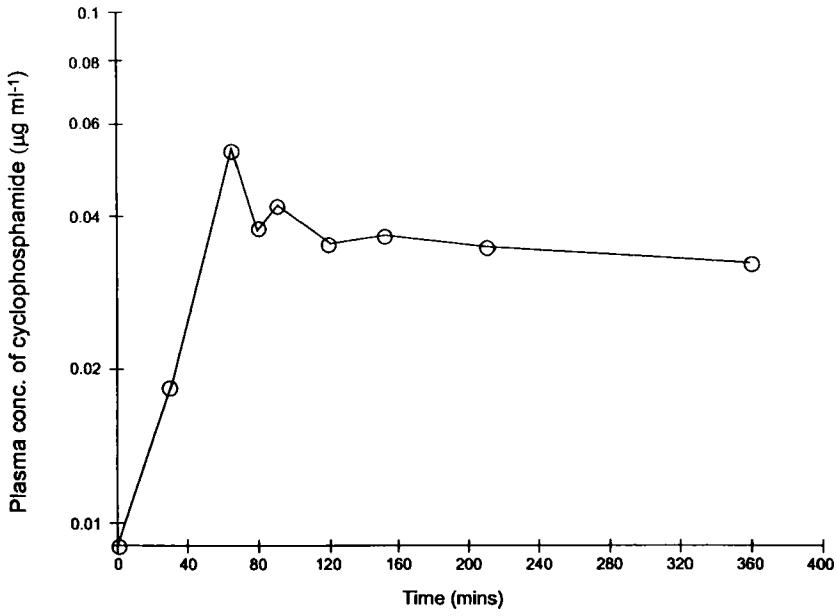

Figure 4. Plasma concentrations of cyclophosphamide following a 1 h intravenous infusion of 1000 mg m^2.

The recovery of cyclophosphamide and the internal standard was examined at the low, middle and high validation levels as part of the within-day validation. As would be expected, the recovery of both compounds was identical within the limits of the assay, being in the region of 20% (Table 5). Although the recovery was low, it did not have a significant effect upon the precision and accuracy of the assay.

The upper calibration range of the assay was low in relation to peak plasma concentrations that can be typically expected in adult patients (40-100 μg mL^{-1}) receiving a therapeutic dose of cyclophosphamide (1000-1500 mg m^2). In order to allow the analysis of such samples, the effect of dilution with control plasma was examined at cyclophosphamide concentrations of 80 and 10 μg mL^{-1}. Dilution of these samples was shown not to affect the quantification of cyclophosphamide with an accuracy and precision well within the acceptance criteria (Table 2).

Plasma samples from a patient treated IV with cyclophosphamide have been successfully analyzed using this assay, demonstrating the utility of the method (Figure 4).

DISCUSSION

This study reports the validation of a highly sensitive assay for cyclophosphamide in human plasma utilizing mass spectrometry as the method of detection. The assay has been developed to study the interaction of cyclophosphamide with other novel anticancer agents which may share the same metabolic pathway. The technique also has the potential for studying the pharmacokinetics of cyclophosphamide in situations where plasma volume is limited, such as paediatrics and small rodents. For the latter, however, further validation work would be required to ensure the performance of the assay.

The recovery of cyclophosphamide and the deuterated internal standard was low, being in the region of 20%. Such a low recovery is not always ideal and can introduce unacceptable assay variability. However, in the present study this was clearly not the case, most likely reflecting the use of a deuterated internal standard.

We did not attempt to identify the reason for the low recovery in terms of a poor extraction or retention by the solid-phase extraction cartridge. However, it is likely that the sensitivity of the assay could be improved even further by a modification of the solid-phase extraction procedure.

The short run time and simple extraction procedure for the current assay gives the potential for a high throughput of samples. Furthermore, detection by mass spectrometry affords this assay a high degree of specificity. This can be particularly important when dealing with cancer patients who are often treated with a variety of concomitant medications.

In conclusion, we have developed a highly sensitive LC/MS/MS assay for cyclophosphamide in human plasma. Over the range 0.025 to 80 $\mu g\ mL^{-1}$, the assay has been demonstrated to have an accuracy and precision within ±15%. The compound was stable in plasma after up to 3 freeze/thaw cycles and for storage for up to 4 weeks in the freezer. No compound loss was detected after the extracted samples were stored at room temperature and in the fridge for up to 24 h.

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Received September 10, 1995
Accepted September 22, 1995
Manuscript 3969