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High-performance liquid chromatographic determination of the enantiomers of cyclophosphamide in serum

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Abstract

A high-performance liquid chromatographic (HPLC) achiral–chiral coupled assay to measure the serum concentration of the enantiomers of cyclophosphamide is described. The *R*- and *S*-enantiomers of cyclophosphamide were quantified using a 5-cm-long C_1 Spherisorb 5-μm column, with switching of the eluent containing racemic cyclophosphamide onto a 10-cm-long α_1 acid glycoprotein column. The limit of determination was 1.25 mg l^{-1} for each enantiomer and the ratio of the enantiomers over the range 2.5 to 100 mg l^{-1} was 1. Serum enantiomer concentrations in blood samples taken from patients receiving 0.30 to 0.75 g m^{-2} of intravenous racemic cyclophosphamide could be measured at least three half-lives post dose. In six patients no significant difference in the clearance of *R*- and *S*-cyclophosphamide was found.

Keywords: Cyclophosphamide

1. Introduction

Cyclophosphamide and ifosfamide are alkylating agents which have been used in the treatment of both solid and haematological tumours for over 30 years. Both compounds are chiral pro-drugs which are administered to the patient in their racemic form. Preclinical data has demonstrated differences in the efficacy and toxicity of the enantiomers of ifosfamide and cyclophosphamide, with the *S*-enantiomer exhibiting a higher therapeutic index [1]. However, current knowledge of the pharmacokinetics and the pharmacodynamics of the enantiomers in man is limited, largely due to the lack of simple direct assays to quantify the enantiomers of ifosfamide and cyclophosphamide in serum and urine. An indirect method to resolve the enantiomers of cyclophosphamide, by a two-step pre-column derivatisation reaction to produce diastereoisomers which are then resolved on an achiral HPLC system has been described [2]. Plasma samples containing 1 to 50 mg l^{-1} cyclophosphamide were measurable. However, two derivatisations and two extractions were required prior to HPLC analysis. A simpler, direct method to quantify the enantiomers of cyclophosphamide and ifosfamide on a polyacrylamide chiral stationary phase has been reported [3]. However, baseline resolution for either compound, using this system, was not attained. Enantioselectivities for ifosfamide and cyclophosphamide of 1.45 and 1.21, respectively, have been achieved using a normal-phase achiral–chiral (DL-naphthylalanine CSP-

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Chiracel-OD) coupled HPLC method [4]. The limit of determination for racemic ifosfamide (Rac-Ifo) and cyclophosphamide (Rac-Cyc) was 20 mg l^{-1}. Using this method serum concentrations of the enantiomers of ifosfamide were determined in patients one half-life after a single 15-min infusion of 1.6 mg m^{-2} racemic ifosfamide. The serum concentration of cyclophosphamide enantiomers in patients was not reported. More recently, plasma concentrations of the enantiomers of ifosfamide and its 2- and 3-dechloroethyl metabolites, as low as 0.25 mg l^{-1}, have been determined using gas chromatography with mass spectrometric detection [5]. The limit of determination for the enantiomers of ifosfamide was 0.25 mg l^{-1}. However, this technique requires specialised equipment which is not routinely found in analytical laboratories. Furthermore, the enantiomers of cyclophosphamide were not resolved.

We have developed and validated a reversed-phase HPLC achiral–chiral coupled assay to determine the concentration of the enantiomers of ifosfamide in serum samples from clinical subjects [6]. Rac-Ifo was separated from its metabolites and interfering serum components on an achiral Spherisorb C_1 column. The eluent containing the enantiomers was then selectively transferred onto an α_1 acid glycoprotein column (Chiral-AGP) for quantification of the enantiomers. The limit of determination was 1.25 mg l^{-1} for each enantiomer. Serum concentrations in patients three to four half-lives after a 4 g intravenous bolus dose of racemic ifosfamide were measured. Preliminary evidence suggested that serum concentrations of the enantiomers of cyclophosphamide in patients receiving chemotherapy could be quantified using this method. We report here the validation of this assay for measurement of the enantiomers of cyclophosphamide in clinical subjects.

2. Experimental

2.1. Chemicals

All chemicals were of HPLC grade or analytical purity (BDH, Poole, UK). Rac-Cyc was purchased from Sigma (Poole, UK), and *R*- and *S*-Cyc were a gift from the department of tumour immunology, Academy of Sciences, Wrocław, Poland (Professor Radzikowski). The optical rotation for *R*- and *S*-Cyc, measured in a Bendix-NPL automatic polarimeter, was +3.06 (2.0%, w/v, methanol) and −2.83 (2.0%, w/v, methanol), respectively.

2.2. Preparation of standards

A 1 g l^{-1} solution of Rac-Cyc was prepared in acetonitrile–distilled water (10:90, v/v). The stock solution was prepared monthly, stored at 4°C prior to use and showed no degradation over that time period. From the stock solution, working standards were prepared by dilution with distilled water to yield nominal aqueous concentrations of 6.25, 12.5, 50, 125, 250 and 375 mg l^{-1}. A 40-μl volume of each working standard was added to 2 ml of blank serum and 2 ml phosphate buffer (0.025 *M*; pH 4) to yield final nominal serum concentrations of 2.5, 5.0, 20, 50, 100 and 150 mg l^{-1} of Rac-Cyc.

2.3. Sample preparation

Rac-Cyc was isolated from serum using 100 mg cyclohexyl (CH) solid-phase extraction cartridges (Varian, Harbor City, CA, USA). Serum standards were prepared as described above. For the extraction of Rac-Cyc from clinical samples 2 ml of serum were added to 2 ml of phosphate buffer (0.025 *M*; pH 4) and 0.4 ml of water (pH 4). The cyclohexyl column was conditioned with two 1-ml aliquots of methanol, followed by 1 ml of distilled water (pH 4). After vortex-mixing for 30 s, four 1-ml aliquots of the sample (spiked standard or clinical) were applied to the top of the sorbent bed, and allowed to elute slowly through the cartridge over 3 to 4 min. The column was dried by applying a full vacuum for 5 min and endogenous substances were then removed from the cartridge by washing with 1 ml of acetonitrile–distilled water (pH 4) (10:90, v/v). Finally, the analyte was eluted with 1 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen and the residue reconstituted in 0.25 ml of mobile phase. The extraction was performed on a Vac Elut station (Jones Chromatography, Mid Glamorgan, UK) and therefore up to ten samples could be extracted in parallel.

2.4. Instrumentation

Two separate systems were employed to quantify Rac-Cyc and its enantiomers. The coupled system which was used to quantify the enantiomers of cyclophosphamide was identical to that employed for ifosfamide [6]. Ambient temperatures were used throughout the study.

2.5. Chromatographic conditions

2.5.1. Achiral chromatography

Measurement of Rac-Cyc was accomplished using a separate system to that for the separation of the enantiomers. A 5-μm C_8 Spherisorb 15 cm×4.6 mm I.D. column (Anachem, Luton, UK) with a mobile phase of acetonitrile–phosphate buffer (0.025 *M*; pH 4) (25:75, v/v) at a flow-rate of 1 ml min^{-1} was used. UV detection at 195 nm was employed.

2.5.2. Enantioselective chromatography

Rac-Cyc was separated from interfering metabolites and serum components on a 5-μm Spherisorb C_1, 5.0 cm×4.6 mm I.D. column (packed in house). When the eluent fraction containing Rac-Cyc was detected, the switching valve was rotated and the eluent diverted to the chiral column [an α_1 acid glycoprotein (Chiral-AGP) (Chromtech, Stockholm, Sweden), 10.0 cm×4.0 mm I.D.], for the resolution of the *R*- and *S*-enantiomers. For both systems the mobile phase was acetonitrile–phosphate buffer (0.015 *M*; pH 4) (1:99, v/v) at a flow-rate of 1 ml min^{-1}, and UV detection at 195 nm was employed.

2.6. Validation studies

The recovery of Rac-Cyc from serum was estimated by comparing the peak height of aqueous cyclophosphamide standards after direct injection to the peak height of serum standards after extraction. Five extractions were performed at each standard Rac-Cyc concentration (2.5 to 150 mg l^{-1}).

Standard curves for Rac-Cyc were prepared from the spiked serum standards over the concentration range 2.5 to 150 mg l^{-1}. Standard curves were run in duplicate. Intra- and inter-day assay variation, expressed as the coefficient of variation in peak height, were determined from four repeats for each spiked standard over the concentration range 2.5 to 150 mg l^{-1}. Accuracy was determined by comparison of the measured to the nominal serum concentration.

The intra- and inter-day variation in the enantiomeric ratio for the chiral assay was determined for five Rac-Cyc serum standards at 2.5, 5, 20, 50 and 100 mg l^{-1}. The mean *S*/*R* peak-area ratio, expressed as a percentage of the theoretical value over the same concentration range, was also calculated.

2.7. Pharmacokinetics

Local Ethics Committee Approval was obtained prior to the study. Six patients (four female) who were prescribed 0.30 to 0.75 g m^{-2} racemic cyclophosphamide as an intravenous bolus gave their written informed consent to have a 10-ml blood sample taken pre-chemotherapy, and then at 1, 2, 5, 8, 14 and 20 h post dose. The blood was placed in a clean dry glass tube and allowed to clot. Following centrifugation at 1800 *g* for 10 min, the serum was removed and stored at −20°C prior to analysis. Serum concentrations of rac-, *R*- and *S*-cyclophosphamide were measured using the above system. The half-life was obtained from the slope of the logarithm of the serum concentration against time relationship, by linear regression. The clearance was obtained from the area under the curve (from zero to infinite time), with the residual area calculated by dividing the last measured concentration by the elimination rate constant, and the volume of distribution was calculated from the relationship between clearance and half-life.

3. Results and discussion

3.1. Extraction

The mean (C.V.) recoveries of Rac-Cyc from serum samples containing 2.5, 5, 20, 50, 100 and 150 mg l^{-1} were 99.8 (4.8), 101.7 (5.1), 99.5 (1.8), 93.0 (6.2), 90.6 (3.7), and 91.6 (2.7)%, respectively. The recovery of cyclophosphamide from serum for each standard was significantly greater than for ifosfamide ($p>0.05$) [6]. Ifosfamide is a more polar drug than

cyclophosphamide and is more likely to demonstrate weaker bonding to CH groups.

3.2. Chromatography

Chromatograms obtained from extracted blank and spiked serum samples containing 20 mg l^{-1} Rac-Cyc are presented in Fig. 1a and b, respectively. Calibration curves were linear over the concentration range examined with correlation coefficients >0.998. This system was used to quantify racemic cyclophosphamide.

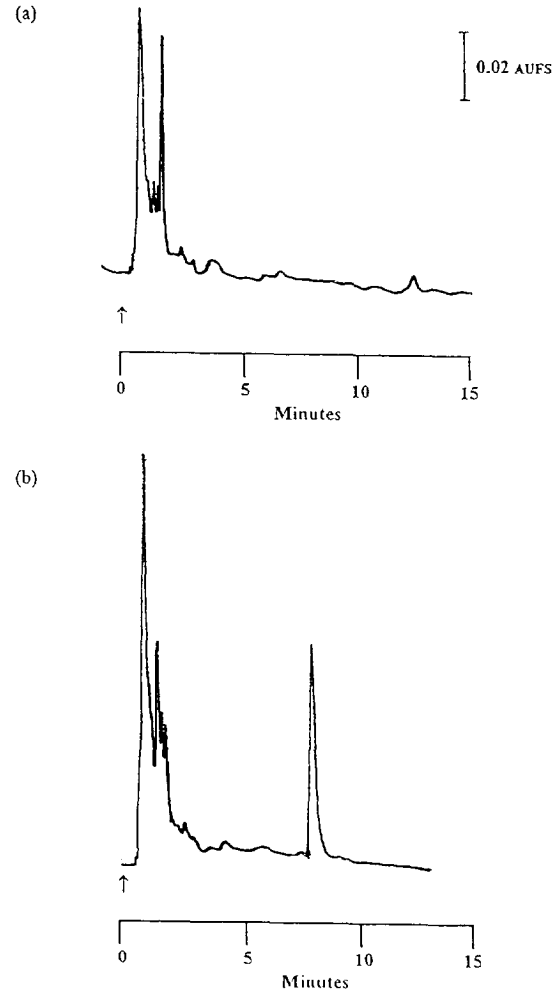

Fig. 1. Achiral chromatography. Chromatogram of (a) an extracted blank serum sample and (b) an extracted serum sample spiked with 20 mg l^{-1} racemic cyclophosphamide.

Earlier studies reported that for ifosfamide enantiomers a coupled system was required to overcome the problems of co-eluting metabolites and interfering serum components following direct injection onto the α_1 acid glycoprotein column. For these reasons the coupled system was used for the enantioselective cyclophosphamide method development. Identical chromatographic conditions to those employed for ifosfamide were used. Aqueous solutions of *R*- and *S*-cyclophosphamide (12.5 mg l^{-1}) were injected directly onto a Chiral-AGP column to determine the order of elution. The retention time (capacity factor) was 4.54 (2.4) and 8.48 (5.5) min, respectively with a mobile phase containing acetonitrile–phosphate buffer (0.015 *M*; pH 4) (1:99, v/v) at a flow-rate of 1 ml min^{-1}. The retention time of the cyclophosphamide enantiomers on the Chiral-AGP column was found to be the reverse of that observed for ifosfamide.

The accuracy, and inter-day and intra-day precision of the achiral and enantioselective methods are presented in Table 1 and Table 2, respectively. The limit of determination for both assays was 2.5 mg l^{-1} racemic cyclophosphamide. Serum concentrations as low as 0.625 mg l^{-1} for each enantiomer could be measured on the coupled system, however, the precision of the assay was less reliable at this concentration (inter-day C.V.~15%). Chromatographic parameters for the enantioselective assay are presented in Table 3, and a chromatogram of an extracted standard serum sample (50 mg l^{-1} racemic cyclophosphamide) on the Chiral-AGP column, following switching, is given in Fig. 2. The retention time of cyclophosphamide on the C_1 column was concentration dependent and this resulted in variable

Table 1
Accuracy and precision of the racemic cyclophosphamide assay

Nominal concentration (mg l^{-1})	Percentage of nominal concentration (mean)	Coefficient of variation (%)	
		Intra-day	Inter-day
2.5	104	6.3	8.7
5	100	3.0	6.6
20	103	1.5	4.2
50	97	0.3	1.1
100	97	0.6	5.7
150	102	2.9	6.9

Table 2
Accuracy and precision of the enantioselective assay

Racemic cyclophosphamide (mg l^{-1})	Percentage of theoretical *S/R* ratio (mean)	Coefficient of variation (%)	
		Intra-day	Inter-day
2.5	104	4.9	10.3
5.0	103	4.0	8.5
20	99	4.2	3.7
50	96	1.9	4.6
100	99	1.7	2.0

Table 3
Chromatographic parameters for cyclophosphamide serum standards on the coupled system

Racemic concentration (mg l^{-1})	Retention time (min)		*k'*		Enantio selectivity	Enantioselective resolution
	R-	*S*-	*R*-	*S*-	(α)	(R_{RS})
2.5	15.03	18.56	0.51	0.84	1.65	3.15
5.0	14.93	18.44	0.49	0.84	1.72	3.19
20	14.59	17.95	0.44	0.78	1.76	3.21
50	13.70	17.00	0.56	0.93	1.68	2.36
100	13.05	16.10	0.55	0.91	1.67	1.77

retention times for the enantiomers on the Chiral-AGP. The efficiency of the C_1 column was not adequate for quantitation of the racemate and therefore racemic cyclophosphamide was quantified on a separate system. The peak-area ratio of *R*- and *S*-cyclophosphamide from standard racemic serum samples was unity. Adequate baseline resolution for all standards was achieved.

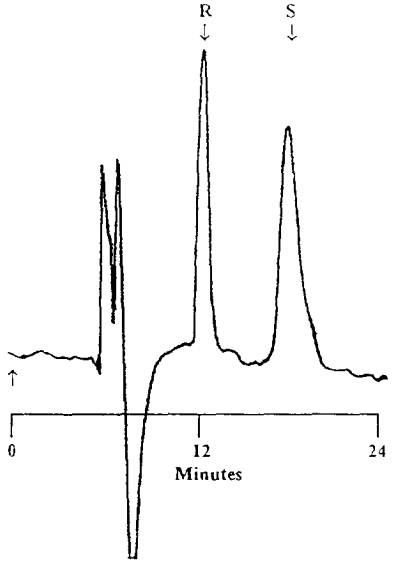

Fig. 2. Enantioselective chromatography. Chromatogram of an extracted serum sample spiked with 50 mg l^{-1} racemic cyclophosphamide.

3.3. Pharmacokinetics

Serum concentrations of *R*- and *S*-cyclophosphamide were measured up to 20 h post infusion in six patients (four female). Their mean (S.D.) age, weight and surface area was 52.8 (6.3) years, 73.7 (14.3) kg, and 1.79 (0.20) m^2 and the mean (S.D.) intravenous dose which they received was 0.55 (0.18) g m^{-2} racemic cyclophosphamide by intravenous bolus. The dose was determined by their body surface area, their clinical status and their particular dosage regimen. The mean (S.D.) serum concentration time profile for *R*- and *S*-cyclophosphamide is presented in Fig. 3. The mean (S.D.) serum concentration of *R*- and *S*-cyclophosphamide was 13.49 (4.10) and 12.89 (4.10) at 1 h, and 2.08 (1.20) and 2.25 (1.19) at 20 h post dose, respectively. The mean (S.D.) clearance, volume of distribution and half-life for *R*-cyclophosphamide was 0.049 (0.021) l h^{-1} kg^{-1}, 0.43 (0.07) l kg^{-1} and 6.82 (2.27) h, and for *S*-cyclophosphamide was 0.048 (0.020) l h^{-1} kg^{-1}, 0.45 (0.08)

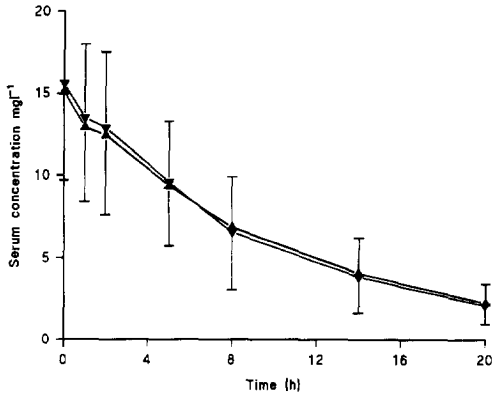

Fig. 3. Mean (S.D.) serum *R*- and *S*-cyclophosphamide concentration following an intravenous bolus dose of racemic cyclophosphamide to six patients.

$l\,kg^{-1}$ and 7.13 (1.84) h, respectively. Thus, in contrast to ifosfamide [7,8], cyclophosphamide does not appear to demonstrate stereoselective elimination.

4. Conclusion

The reversed-phase achiral–chiral HPLC coupled assay which has been developed for ifosfamide may also be used to measure the enantiomers of cyclophosphamide in patients receiving chemotherapy. The order of elution of the enantiomers of cyclophosphamide is different in that *R*-cyclophosphamide elutes first, whereas for ifosfamide the *S*-enantiomer has the shortest retention time. The method is linear, accurate and precise with a limit of determination for each cyclophosphamide enantiomer of 1.25 $\mu g\,l^{-1}$.

The mean (S.D.) serum concentration of *R*- and *S*-cyclophosphamide 1 h after the intravenous administration of 0.3–2 $g\,m^{2}$ of the racemate was 13.49 (4.10) and 12.89 (4.10) $mg\,l^{-1}$, respectively. The mean (S.D.) half-life of *R*- and *S*-cyclophosphamide was 6.82 (2.27) and 7.13 (1.84) h. Serum concentrations of the enantiomers therefore may be determined for at least three half-lives after administration of an intravenous bolus dose of the racemate to patients.

The similar clearance of *R*- and *S*-cyclophosphamide of 0.049 (0.021) and 0.048 (0.020) $l\,h^{-1}\,kg^{-1}$ suggests that there is no difference in their elimination from the body. Studies in adults and children have indicated that the body recognises the enantiomers of ifosfamide as two separate drugs [7,8]. Analysis of the pharmacokinetics of the enantiomers of cyclophosphamide up to 20 h post dose suggests that, in contrast to its structural isomer, cyclophosphamide does not demonstrate stereoselective clearance. However, the metabolism of cyclophosphamide is complex. Individual metabolic routes may be preferential for one enantiomer but this effect may be compensated for by other pathways.

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