ELSEVIER

Journal of Chromatography B, 654 (1994) 152–158

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

Enantiomeric separation of *R*- and *S*-ifosfamide and their determination in serum from clinical subjects

S.A. Corlett, H. Chrystyn*

Pharmacy Practice, Postgraduate Studies in Pharmaceutical Technology, School of Pharmacy, University of Bradford, Bradford BD7 1DP, UK

(First received October 11th, 1993; revised manuscript received December 15th, 1993)

Abstract

A method to measure racemic, *R*- and *S*-ifosfamide concentrations from the serum of patients receiving ifosfamide chemotherapy has been developed. The racemic ifosfamide concentrations are quantified on a separate system and then the ratio of the enantiomers is determined using an achiral–chiral coupled system. Racemic ifosfamide is separated on the achiral system using a C_1 spherisorb stationary phase and the eluent containing analyte is selectively transferred to the chiral system for separation of the two enantiomers by an α_1 glycoprotein column. On both systems the mobile phase is 1% acetonitrile in 0.015 *M* phosphate buffer (pH 4) at a flow-rate of 1 ml/min. The retention times of *S*- and *R*-ifosfamide were 11.6 and 13.0 mins, respectively, with a resolution factor of 1.53. Serum concentrations at least three to four half-lives post-infusion were detected by this method. In ten patients, following a mean ± S.D. 1-h infusion of 3.9 ± 0.32 g racemic ifosfamide, the mean ± S.D. clearances of *R*- and *S*-ifosfamide were 0.061 ± 0.013 and 0.072 ± 0.014 l h^{-1} kg^{-1}.

1. Introduction

Ifosfamide and cyclophosphamide are alkylating agents which have the same molecular weight and contain the same phosphorous chiral centre. The difference in the molecular structure is that one of the two 2-chloroethyl groups of cyclophosphamide is transferred from the extracyclic to the endocyclic nitrogen of the oxazaphosphorine ring. This small difference in the chemical structure is thought to be responsible for the differences which are observed in the metabolic profiles of the congeners. Both are extensively metabolised and their pharmacodynamic effects (efficacy and side effects) are due to the metabolites. Ifosfamide exhibits a slower rate of ring hydroxylation and a greater proportion of the dose is oxidised at the side chain to yield dechloroethyl metabolites and chloracetaldehyde. Chloracetaldehyde has been implicated in the characteristic neurotoxicity which is observed with ifosfamide therapy. Studies in man have indicated that the metabolism of ifosfamide is stereospecific. Limited data, in children, suggests that *S*-ifosfamide is eliminated faster from the body than *R*-ifosfamide [1,2]. Ifosfamide is used extensively in the treatment of solid and haematological malignancies. It has been shown to be more active than cyclophosphamide when

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(93)E0446-W

used in testicular tumours, soft tissue sarcomas and some types of lung cancer [3].

A method to separate the enantiomers of cyclophosphamide in plasma by derivitisation to form diastereoisomers and achiral HPLC analysis has been described [4]. The resolution of ifosfamide using this method has not been reported. Blaschke and Maibaum [5] have quantified the enantiomers of ifosfamide by direct injection of the racemic mixture onto a polyacrylamide chiral stationary phase but baseline resolution was not obtained. An achiral–chiral coupled column switching method has reported baseline resolution of the enantiomers of ifosfamide but the sensitivity was low with a reported detection limit of 20 mg/l [2] which is equivalent to serum concentrations reported approximately one half-life after a single 1.6 mg/m^2 intravenous dose of ifosfamide [2]. This method used a covalent D,L-naphthylalanine column to quantify the total drug concentration, coupled to a Chiral OD column based upon cellulose-tris(3,5-dimethylphenylcarbamate) for determination of the enantiomeric composition. A gas chromatographic method which employs a chiral column with mass spectroscopic detection has been reported for ifosfamide [6], but the retention times were long, baseline separation was not completely achieved and the concentration of the analyte is not reported. An enantioselective gas chromatographic method with mass spectrometric detection to determine the enantiomers of ifosfamide and its 2- and 3-dechloroethylated metabolites in plasma and urine has been recently described [7]. This technique is accurate and precise and can detect plasma levels of each enantiomer as low as 0.25 mg/l in plasma. However, it requires expensive specialised equipment which is not routinely found in laboratories.

We have developed a reversed-phase achiral–chiral coupled high-performance liquid chromatographic (HPLC) method to determine the concentration of *R*- and *S*-ifosfamide in clinical samples. Ifosfamide is separated from its metabolites and interfering serum components on an achiral C_1 column. The eluent containing racemic ifosfamide is then selectively transferred onto an α_1 glycoprotein column for quantification of the enantiomers. The assay is sufficiently sensitive to measure serum racemic concentrations of 2.5 mg/l, which are typically obtained at least 24 h after a 4-g intravenous dose.

2. Experimental

2.1. Chemicals

All chemicals were purchased from BDH (Poole, UK) and were of Analar or HPLC grade (as appropriate). Ifosfamide, 2-dechlorethyl ifosfamide, 3-dechloroethyl ifosfamide, 4-ketoifosfamide, carboxyifosfamide, acrolein and 2-mercaptoethanesulfonic acid (mesna) were donated by Asta Medica, Germany. *R*- and *S*-ifosfamide were prepared by semi-preparative high-performance liquid chromatography [2]. The optical rotation measured in a Bendix-NPL automatic polarimeter (Type 143) (Bendix, UK) was +61.0 (0.2% w/v; methanol) and −61.3 (0.2% w/v; methanol) for *R*- and *S*-ifosfamide, respectively.

2.2. Sample preparation

An extraction method using cyclohexyl (CH) cartridges to isolate racemic ifosfamide from serum has been described [8]. Burton and James report an extraction yield of 99.7 ± 9.9% (mean ± S.D.). Using this method we obtained an experimental extraction yield of only 22.2 ± 5.9% and therefore the method of Burton and James [8] was modified to increase our recovery.

Prior to extraction 0.2 ml of aqueous standard was added to both 1 ml of blank serum and 1 ml of 0.025 *M* phosphate buffer pH 4 to produce spiked serum concentrations of 5–150 mg/l racemic ifosfamide. For clinical samples 0.2 ml of distilled water pH 4 (adjusted with phosphoric acid) was added to both 1 ml of patient serum and 1 ml of 0.025 *M* phosphate buffer, pH 4. After vortex-mixing for 30 s, two 1-ml aliquots were removed for extraction.

Ifosfamide was extracted from serum using a Vac Elut (Jones Chromatography, Mid Glamorgan, UK) station with 100-mg CH Bond Elut

cartridges (Varian, Harbor City, CA, USA). Up to ten cartridges could be extracted at one time. The cartridges were conditioned with two 1-ml aliquots of methanol followed by 1 ml of distilled water pH 4. A sample (spiked or clinical) was then applied to a cartridge in two 1-ml aliquots. The sample was allowed to elute through the cartridge over 2 to 3 min and the column was then dried by applying a full vacuum for 5 min. After drying, the column was washed with 1 ml of 10% acetonitrile in distilled water (pH 4) to remove endogeneous material. The analyte was eluted with 1 ml of methanol, evaporated to dryness under a stream of nitrogen and the residue reconstituted in 0.25 ml of mobile phase.

2.3. HPLC equipment and analytical method

The achiral–chiral coupled system used to determine the ratio of ifosfamide enantiomers is shown in Fig. 1. This system is similar to that used for the separation of *R*- and *S*-warfarin from the serum of anticoagulated patients [9]. Samples were introduced onto the system via a Rheodyne 7125 injection valve fitted with a 20-μl loop. The HPLC instrumentation consisted of two Gilson Model 307 pumps (Anachem, Luton, UK), and Applied Biosystems Model 759A ultraviolet detectors (Anachem) detectors 1 and 2. Output from detector 1 was linked to a Shimadzu CR-6A integrator (Dyson Instruments, Haughton-le-Spring, UK), integrator 1, and output from detector 2 linked to a computer with the signal integrated by Gilson 712 controller software (Anachem), integrator 2. The achiral and chiral systems were coupled using a six port Rheodyne 7030 valve (Anachem) and switching was controlled by Gilson 712 controller software. A 5-μm spherisorb C_1, 5.0 cm × 4.6 mm I.D. column (packed in house) was used for the achiral separation of racemic ifosfamide from interfering metabolites and serum components. The eluent containing ifosfamide was then selectively transferred onto an α_1 acid glycoprotein (Chiral–AGP) (Chromtech, Stockholm, Sweden), 10.0 cm × 4.0 mm I.D. column for the resolution of the *R*- and *S*-ifosfamide enantiomers. A third column was used to provide back

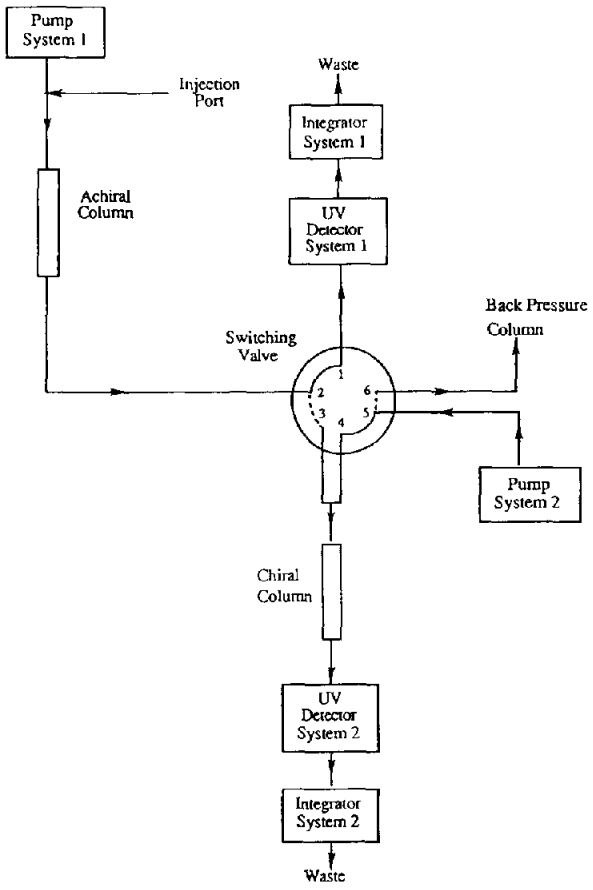

Fig. 1. Schematic design of the coupled system.

pressure to pump 2 during switching in order that it might be operated continuously. For both systems the mobile phase used was 1% acetonitrile in 0.015 *M* phosphate buffer pH 4 at a flow-rate of 1 ml/min, and UV detection at 195 nm was employed.

To quantify racemic ifosfamide a separate system was employed. A Gilson Model 302 pump with a Model 802C manometric module (Anachem) was used with a Rheodyne model 7125 injector valve fitted to a 20-μl loop, a Gilson Model 115 UV detector (Anachem), set at 195 nm, and a Shimadzu CR6A integrator. The stationary phase was a spherisorb 5-μm C_8, 15.0 cm × 4.6 mm I.D., column (Anachem) and the mobile phase was 25% acetonitrile in 0.025 *M* phosphate buffer pH 4 at a flow-rate of 1 ml/min.

Ambient temperatures were used throughout the study.

2.4. Patient study

Ten patients receiving ifosfamide as part of their therapeutic management for small cell lung carcinoma gave written informed consent to have blood samples drawn from a heparinised indwelling cannula at 0, 1, 2, 4, 6, 12, 15, 18 and 24 h after an intravenous dose of racemic ifosfamide. The patients received a standard 4-g dose (Yorkshire Region Small Cell Study Protocol), irrespective of their weight and body surface area, as a short term infusion over 1 h. Those patients with a poor performance status on study entry received a reduced dose of 3 g. Blood samples were allowed to clot, centrifuged for 10 min at 3000 rpm and the serum frozen at −20°C prior to analysis.

3. Results and discussion

The mean recovery of ifosfamide from serum samples containing 2.5, 5, 25, 50, 100 and 150 mg/l was 87.8(4.1), 85.3(3.8), 90.2(4.5), 89.4(4.3), 90.2(5.4) and 88.6(4.2)% respectively. Figures in brackets give coefficient of variation.

Aqueous solutions of *R*-ifosfamide, *S*-ifosfamide and the ifosfamide metabolites were injected directly onto a Chiral-AGP with a mobile phase containing 1% acetonitrile in 0.015 *M* phosphate buffer pH at a flow-rate of 1 ml/min. 2-Dechloroethyl ifosfamide, 3-dechloroethyl ifosfamide and acrolein were eluted with the solvent front. The capacity factor (k) of 4-ketoifosfamide, carboxyifosfamide, ifosfamide mustard, *S*- and *R*-ifosfamide was 5.4, 11.8, 11.4, 14 and 16.2, respectively. These correspond to retention times of 3.2, 6.4, 6.2, 7.5 and 8.6 min. The achiral metabolites carboxyifosfamide and ifosfamide mustard produced broad tailing peaks which interfered with the quantification of *S*-ifosfamide.

An achiral–chiral coupled column switching system was developed to overcome the problem of co-eluting metabolites and to limit the possibility of poisoning the chiral-AGP column by endogenous compounds. Assay optimisation revealed that an achiral system using a 5 cm × 4.6 mm I.D. C_1 spherisorb column was compatible with the above conditions for the chiral system. The switching time was optimised at 42 s. Fig. 2a demonstrates that racemic ifosfamide (serum sample containing 50 mg/l) was retained with a capacity factor of 10.7 (retention time 6.5 min) on the achiral system. The same sample switched onto the Chiral-AGP column separated *S*- and *R*-ifosfamide with retention times of 11.6 and 13.0 min, respectively, as illustrated in Fig. 2b. The respective capacity factors were 0.66 and 0.86. Baseline resolution of the enantiomers, resolution factor of 1.53 ± 0.06 (mean ± S.D., $n = 12$), over the racemic ifosfamide range of 5–150 mg/l was achieved. Carboxyifosfamide and ifosfamide mustard were analysed on the coupled system and no interfering peaks were observed to elute with the same retention time of *R*- or *S*-ifosfamide. Table 1 describes the accuracy and precision of the method for spiked serum samples containing between 5 and 150 mg/l of racemic ifosfamide. The detection limit of the assay could be reduced to 2.5 mg/l by extracting double the quantity of serum. The retention of racemic ifosfamide on the achiral C_1 system was concentration dependent and therefore the switching time was determined for each sample. The efficiency of the C_1 system was poor and therefore a second method was used to quantify the serum concentrations of racemic ifosfamide.

The method of Burton and James [8] was modified using a 15.0 cm × 4.6 mm I.D. C_8 spherisorb column and a mobile phase containing 25% acetonitrile in 0.025 *M* phosphate buffer pH 4 at a flow-rate of 1 ml/min. During assay optimisation, baseline separation of ifosfamide and the internal standard cyclophosphamide [7] could not be achieved. Various internal standards were identified but by the law of propagation of error [10] none proved to increase the precision of the assay and, therefore, the assay was calibrated using external methods. Fig. 3a, a chromatogram of a racemic ifosfamide (20 mg/ml) after injection onto the C_8 column shows that

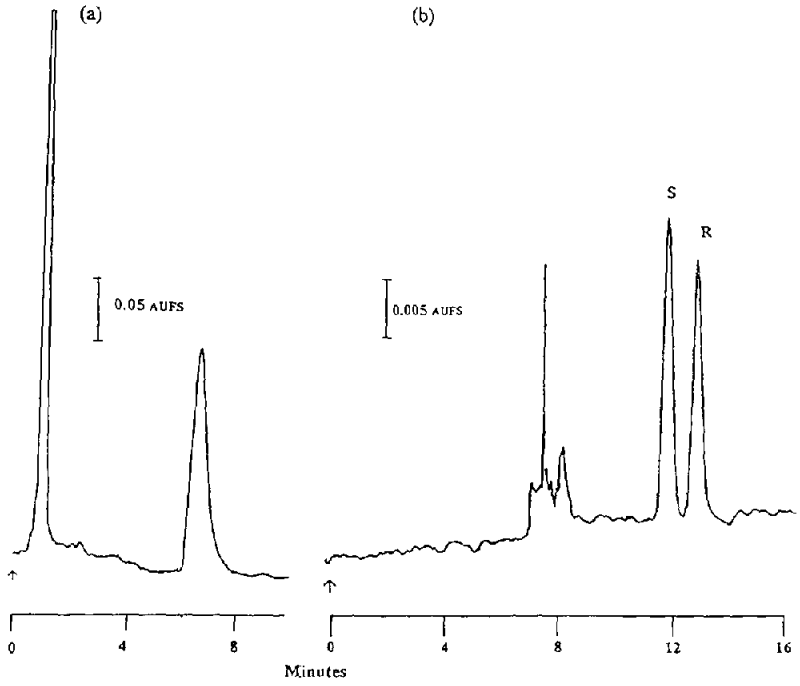

Fig. 2. Chromatogram of (a) racemic ifosfamide (50 mg/l), and (b) *R*- and *S*-ifosfamide (25 mg/l of each) from a serum sample using the coupled achiral–chiral system.

ifosfamide was retained with a capacity factor of 7.2. No interfering peaks were found to co-elute with the ifosfamide from a blank serum sample as revealed by Fig. 3b. Using identical chromatographic conditions aqueous solutions of the ifosfamide metabolites were analysed. The capacity factors of 4-ketoifosfamide and carboxyifos-

Table 1
S/*R* peak-area ratio after switching racemic ifosfamide from C_1 on to the AGP column

Racemic concentration (mg/l)	Percentage of theoretical *S*/*R* ratio (mean)	Coefficient of variation (%)	
		Intra-day	Inter-day
2.5	106	10.3	10.9
5	104	10.0	11.2
15	97	4.5	4.1
25	100	4.4	4.5
50	100	3.7	2.4
100	102	3.8	1.9

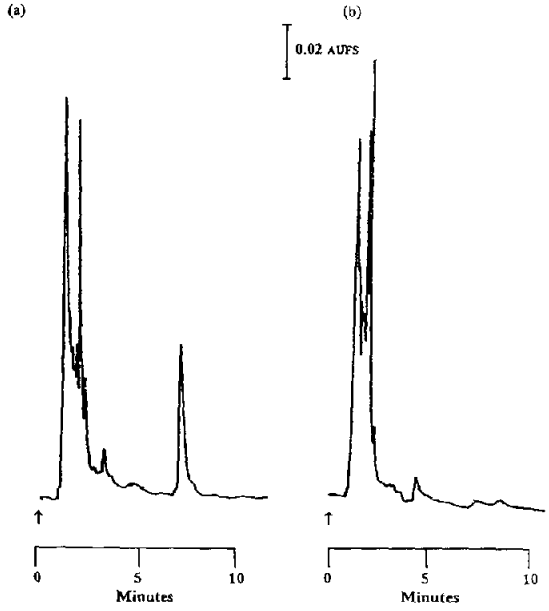

Fig. 3. Chromatogram of (a) racemic ifosfamide, from a serum sample containing 20 mg/l, and (b) an extracted blank serum sample using the C_8 system.

Table 2
Accuracy and precision of the racemic ifosfamide assay

Nominal concentration (mg/l)	Percentage of measured concentration (mean)	Coefficient of variation (%)	
		Intra-day	Inter-day
2.5	112	4.1	11.3
5	111	3.9	14.6
10	104	1.8	7.7
25	98	3.0	8.0
50	96	3.6	5.4
100	102	3.8	2.1
150	100	1.6	1.3

famide were 4.4 and 2.4 and did not, therefore, interfere with the quantification of racemic ifosfamide. All other metabolites were not retained on the column. The accuracy and precision of the method are shown in Table 2.

Mean ± S.D. serum concentrations of racemic, *R*- and *S*-ifosfamide following a mean ± S.D. 1-h intravenous infusion of 3.90 ± 0.32 g of racemic ifosfamide are shown in Fig. 4. The mean ± S.D. age and weight of the ten patients was 61.5 ± 7.60 years and 65.2 ± 10.7 kg, respectively. The stereoselective elimination of the two isomers described in Fig. 4 are highlighted by the mean ± S.D. clearance rates (calculated from the area under the curve data) of 0.061 ± 0.013 and

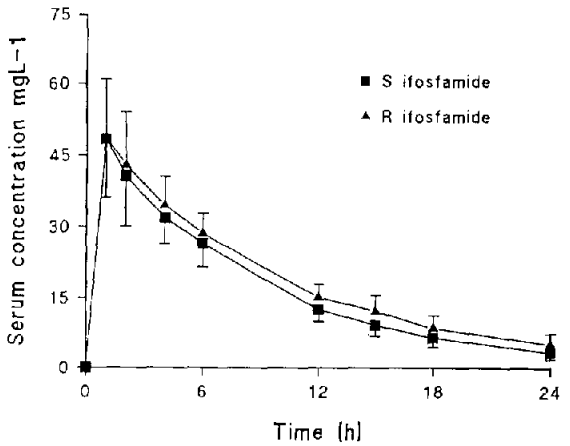

Fig. 4. Mean ± S.D. serum *R*- and *S*-ifosfamide concentrations following an intravenous dose of racemic ifosfamide to ten patients.

0.072 ± 0.014 l h^{-1} kg^{-1} for *R*- and *S*-ifosfamide. The respective half-lives were 7.30 ± 1.91 and 6.20 ± 1.54 h and volumes of distribution were 0.62 ± 0.17 and 0.63 ± 0.14 l kg^{-1}. The mean serum concentrations of racemic, *R*- and *S*-ifosfamide at the end of the infusion were 97.1, 48.7 and 48.4 mg/l, respectively.

4. Conclusion

The method developed is accurate for the measurement of the *R*- and *S*-ifosfamide for serum concentrations over three to four half-lives after the intravenous administration of a racemic dose. The limit of detection for each enantiomer is 2.5 mg/l compared to average values of 48.7 and 48.4 mg/l for *R*- and *S*-ifosfamide, respectively, obtained 1 hour after intravenous injection of 4 g of racemic ifosfamide. The C_1 column used in the achiral system was not suitable for accurate measurement of racemic ifosfamide and, therefore, a separate system was necessary. Nevertheless, sufficient sample is available from a 10-ml blood sample to make the required number of injections and up to 40 samples can be analysed in one day. Preliminary results indicate that the same system and conditions can be used for the separation and measurement of *R*- and *S*-cyclophosphamide from the serum of patients receiving racemic cyclophosphamide.

Pharmacokinetic analysis revealed that the body identifies *R*- and *S*-ifosfamide as two separate drugs. The clearance (and half-life) of *S*- was faster than of *R*-ifosfamide but there was no stereoselective difference in their distribution. Further studies are in progress to identify the pharmacokinetics and pharmacodynamics of the two enantiomers of ifosfamide.

5. Acknowledgements

S.A.C. was the recipient of a SERC award. The authors would like to thank the assistance of

Dr. I.W. Wainer, Montreal General Hospital (Montreal, Canada) and also Mrs. Frances Dawson for typing the manuscript.

6. References

[1] I.W. Wainer, C.F. Stewart, C.L. Young, D. Masurel and H. Frank, *Abstracts of the American Society of Clinical Oncology Annual Meeting, New Orleans, LA, May 22–24, 1988.*
[2] D. Masurel and I.W. Wainer, *J. Chromatogr.*, 490 (1989) 133.
[3] M.J. Moore, *Clin. Pharmacokinet.*, 20 (1991) 194.
[4] J.F. Stobaugh, J.M. Reid and L. Sternson, *Pharm. Res. (Suppl.)*, 5 (1988) 5.
[5] G. Blaschke and J. Maibaum, *J. Chromatogr.*, 366 (1986) 329.
[6] G. Blaschke and U. Koch, *Arch. Pharm. (Weinheim)*, 319 (1986) 1052.
[7] C.P. Granville, B. Gehrcke, W.A. König and I.W. Wainer, *J. Chromatogr.*, 622 (1993) 21.
[8] L. Burton and C.A. James, *J. Chromatogr.*, 431 (1988) 450.
[9] S.D. McAleer, H. Chrystyn and A.S. Foondun, *Chirality*, 4 (1992) 488.
[10] P. Haefelfinger, *J. Chromatogr.*, 218 (1981) 73.