*Journal of Chromatography*, 431 (1988) 450–454
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4311

## Note

# Rapid method for the determination of ifosfamide and cyclophosphamide in plasma by high-performance liquid chromatography with solid-phase extraction

LINDA C. BURTON* and C.A. JAMES

*Department of Clinical Pharmacology, United Medical and Dental Schools, Guy's Hospital, London Bridge, London SE1 9RT (U.K.)*

(First received January 11th, 1988; revised manuscript May 17th, 1988)

Ifosfamide [3-(2-chloroethyl)-2-(2-chloroethylamine)tetrahydro-2H-1,3,2-oxazaphosphorine oxide] and its isomer cyclophosphamide [2-(bis-2-chloroethyl)aminotetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide] are alkylating agents, both of which are active against a wide variety of tumours. Cyclophosphamide is also used as an immunosuppressive agent. Ifosfamide differs chemically from cyclophosphamide in the transfer of one 2-chloroethyl group from the mustard nitrogen atom to the ring nitrogen atom [1] (Fig. 1). This results in differences in the physiochemical properties of the two compounds and leads to different pharmacological and toxicological properties [2]. Both ifosfamide and cyclophosphamide are prodrugs that require activation by hepatic microsomal enzymes to form alkylating metabolites.

The usual method for the determination of cyclophosphamide and ifosfamide in biological fluids has been gas chromatography [3–10]. This usually requires the preparation of trifluoroacetyl derivatives prior to chromatography, although

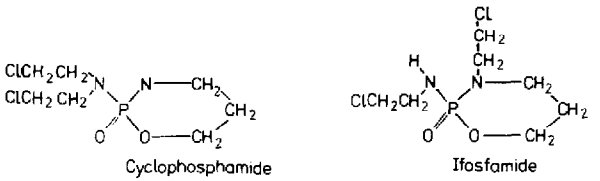


Fig. 1. Structures of ifosfamide and cyclophosphamide.

0378-4347/88/$03.50 

analysis of underivatised ifosfamide [11] and cyclophosphamide [3] by gas chromatography has been reported.

High-performance liquid chromatography (HPLC) has been employed for the analysis of cyclophosphamide in pharmaceutical preparations [12,13], although these methods were reported as not having sufficient sensitivity to measure cyclophosphamide in biological samples. More recently determination of ifosfamide [14] and cyclophosphamide [15,16] in serum or plasma has been demonstrated using HPLC with detection at low UV wavelengths. However, two of these methods lack an internal standard [14,16] and two of these three methods also involve solvent extraction and drying down procedures [14,15]. The extraction of cyclophosphamide [15] in particular is relatively complex.

A rapid and simple method for the extraction of ifosfamide and cyclophosphamide from plasma has been developed using solid-phase extraction columns containing a cyclohexyl bonded-silica sorbent. Ifosfamide and cyclophosphamide are not administered simultaneously, and can therefore be conveniently used as internal standards for one another.

## EXPERIMENTAL

### *Chemicals*

Ifosfamide and cyclophosphamide were gifts from Boehringer Hospital Division (Bracknell, U.K.). All other chemicals were obtained from BDH (Poole, U.K.) and were of analytical grade, except for the acetonitrile, which was of a "Far UV" HPLC grade, and water, which was produced from a Milli-Q water purification system (Millipore, Harrow, U.K.).

### *Instrumentation*

The HPLC system consisted of Shimadzu SPD-6A UV spectrophotometric detector (Dyson Instruments, Hetton, U.K.), a spectroflow 400 pump (Severn Analytical, Shefford, U.K.) and a Shimadzu CR3A computing integrator (Dyson Instruments). Samples were introduced into the system by either a Rheodyne 7125 injection valve (Severn Analytical) or a Spectra-Physics SP8780 XR autosampler (Spectra-Physics, St. Albans, U.K.).

The column used was a 5-$\mu$m Merck LiChrosorb $C_8$ RP-Select B (25 cm $\times$ 4.0 mm) equipped with an interchangeable precolumn (4 mm $\times$ 4 mm) of the same packing material (BDH).

The cyclohexyl Bond Elut columns (100 mg per 1.0 ml size) and the Vac Elut station were obtained from Jones Chromatography (Hengoed, U.K.).

### *Assay conditions*

The mobile phase consisted of acetonitrile–0.025 *M* phosphate buffer pH 4.0 (25:75). The flow-rate was 1.0 ml/min. The detector wavelength was set at 200 nm. The autosampler cycle time was 12.5 min. The analytical column was operated at ambient temperature.

*Sample preparation*

The cyclohexyl extraction columns were prepared on the Vac Elut station using reduced pressure (40 kPa) to draw various solutions through the columns. The cyclohexyl columns were first conditioned by perfusing them with two 1-ml aliquots of methanol and 1 ml of 0.01 *M* phosphate buffer pH 4.0. A 250-$\mu$l volume of plasma was mixed with internal standard solution (50 $\mu$l of 200 $\mu$g/ml cyclophosphamide or 40 $\mu$l of 200 $\mu$g/ml ifosfamide) and 750 $\mu$l of 0.01 *M* phosphate buffer pH 4.0, and then passed through the columns. The columns were then washed with 1 ml of a mixture of acetonitrile–0.01 *M* phosphate buffer pH 4.0 (10:90). The isolates were eluted with 500 $\mu$l of a mixture of acetonitrile–0.025 *M* phosphate buffer pH 4.0 (40:60) into either an autosampler vial or a 1.5-ml polypropylene microcentrifuge tube. Amounts (20 $\mu$l) of this solution were introduced into the HPLC system, either by the autosampler or via an injection valve. All plasma was centrifuged at 10 000 *g* for 3 min (Eppendorf microfuge, Anderman, Kingston-upon-Thames, U.K.) prior to extraction to prevent blocking of the columns by particulate matter.

*Preparation of calibration curve*

Calibration curves were prepared by spiking blank plasma with 200–5 $\mu$g/ml ifosfamide or 100–5 $\mu$g/ml cyclophosphamide. The appropriate internal standard was added to all plasma samples and standards. Peak heights were measured by the integrator and used to calculate peak-height ratios.

## RESULTS AND DISCUSSION

Linear calibration curves were obtained up to a concentration of 200 $\mu$g/ml for ifosfamide and 100 $\mu$g/ml for cyclophosphamide. The inter- and intra-assay coefficients of variation and the recovery from plasma are within acceptable limits [17] for both compounds (Table I).

The use of solid-phase extraction greatly simplifies and accelerates the preparation of samples prior to chromatography. In particular, the solvent extraction procedures necessary for most other techniques and the need to form derivatives for gas chromatography are avoided. At least 40 samples and standards can be processed in a working day using this method.

Previous HPLC methods have either not included an internal standard [16] or used an unrelated compound [17]. Ifosfamide and cyclophosphamide are structurally closely related and consequently make almost ideal internal standards for one another. However, careful choice of HPLC column packing material was required to ensure separation. In initial experiments with a $\mu$Bondapak $C_{18}$ column (10 $\mu$m, 30 cm$\times$3.9 mm; Millipore) and a LiChrosphere $C_8$ endcapped column (5$\mu$m, 25 cm$\times$4 mm; BDH) adequate resolution between ifosfamide and cyclophosphamide was not obtained. Satisfactory peak shapes and baseline separation were, however, found with a LiChrosorb $C_8$ Select B column (Fig. 2).

Using gas chromatography, deviations from linearity have been found at higher ifosfamide concentrations (50 $\mu$g/ml) both in this laboratory [18] and by Mehta

TABLE I

INTRA-ASSAY AND INTER-ASSAY REPRODUCIBILITY AND RECOVERY OF IFOSFAMIDE AND CYCLOPHOSPHAMIDE FROM PLASMA

| Compound | Concentration added (μg/ml) | n | Concentration measured (mean ± S.D.) (μg/ml) | Coefficient of variation (%) | Recovery (mean ± S.D.) (%) |
|---|---|---|---|---|---|
| *Intra-assay* | | | | | |
| Ifosfamide | 5 | 8 | 5.4 ± 0.1 | 2.0 | 89.5 ± 4.5 |
| | 20 | 8 | 18.9 ± 0.4 | 2.1 | 98.5 ± 1.7 |
| | 30 | 8 | 30.4 ± 0.4 | 1.4 | 108.7 ± 2.5 |
| | 50 | 8 | 47.8 ± 1.7 | 3.6 | 110.9 ± 5.4 |
| | 100 | 8 | 99.6 ± 2.3 | 2.8 | 90.8 ± 4.6 |
| Cyclophosphamide | 5 | 8 | 5.1 ± 0.2 | 3.3 | 104.3 ± 9.2 |
| | 10 | 8 | 9.7 ± 0.1 | 1.1 | 114.3 ± 4.4 |
| | 20 | 8 | 20.1 ± 0.3 | 1.4 | 97.6 ± 5.3 |
| | 30 | 8 | 29.5 ± 0.4 | 1.2 | 92.8 ± 3.8 |
| | 50 | 8 | 50.0 ± 1.4 | 2.8 | 100.3 ± 8.3 |
| *Inter-assay* | | | | | |
| Ifosfamide | 5 | 6 | 5.1 ± 0.4 | 8.6 | |
| | 20 | 6 | 19.7 ± 1.1 | 5.5 | |
| | 30 | 6 | 29.5 ± 2.4 | 8.2 | |
| | 50 | 6 | 50.5 ± 3.4 | 6.8 | |
| | 100 | 6 | 102.2 ± 4.9 | 4.8 | |
| Cyclophosphamide | 5 | 6 | 5.1 ± 0.3 | 6.1 | |
| | 10 | 6 | 9.8 ± 0.5 | 4.9 | |
| | 20 | 6 | 20.2 ± 0.5 | 2.3 | |
| | 30 | 6 | 29.9 ± 1.3 | 4.4 | |
| | 50 | 6 | 50.8 ± 1.3 | 2.6 | |

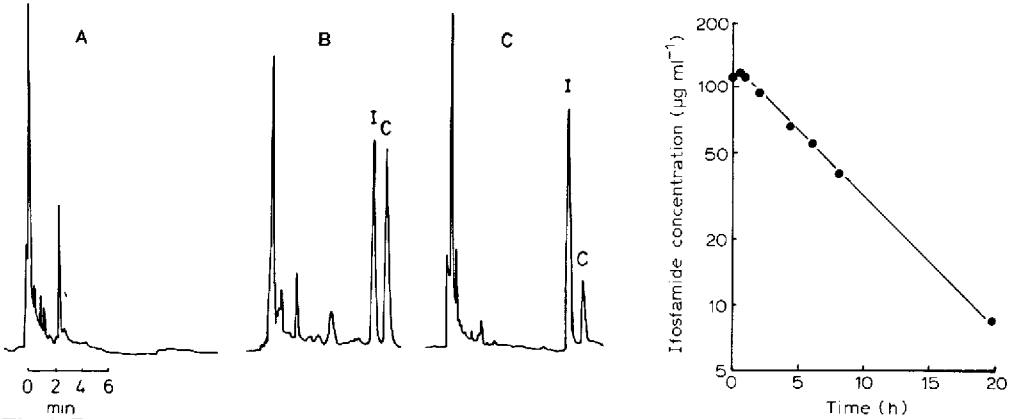


Fig. 2. Representative chromatograms from plasma. (A) Blank plasma: (B) plasma spiked with 30 μg/ml cyclophosphamide and 32 μg/ml ifosfamide; (C) plasma obtained from a patient who received 5 g/m$^2$ ifosfamide. Sample contains 115 μg/ml ifosfamide. Peaks: I = ifosfamide; C = cyclophosphamide. Detection sensitivity was 0.01 a.u.f.s.

Fig. 3. Semi-logarithmic plot of plasma ifosfamide concentration against time from a patient who received 5 g/m$^2$ ifosfamide as a 3-h intravenous infusion. Half-life of ifosfamide was 5.0 h.

and Calvert [10]. This new method has a good linear range, up to at least 200 μg/ml ifosfamide, without any need for dilution.

The detection limit using 250 μl of plasma is 1 μg/ml. This was sufficient for all pharmacokinetic studies so far undertaken using this method, although sensitivity could be improved by using a larger sample size if required. An example of a plot of plasma ifosfamide concentration against time from a patient receiving 5 g/m$^2$ ifosfamide is shown in Fig. 3. No problems of interference have been encountered when estimating ifosfamide or cyclophosphamide in patients who have received combination chemotherapy regimens. Similarly no interference has been found from commonly used anti-emetic drugs including prochlorperazine, dexamethasone, metoclopramide or domperidone. This method is currently being used to investigate further the pharmacokinetics of ifosfamide following long infusions of this drug.

ACKNOWLEDGEMENTS

We are grateful to Dr. Peter Harper for allowing us access to his patients and to the Cancer Research Campaign for financial support.

REFERENCES

1 W.P. Brade and J. Engel, Drugs Today, 20 (1984) 491-496.
2 W.P. Brade, K. Herdrich and M. Varini, Cancer Treat. Rev., 12 (1985) 1-47.
3 A. El Yazigi and C.R. Martin, J. Chromatogr., 374 (1986) 177-182.
4 T. Facchinetti, M. D'Incalci, G. Martelli, L. Caltoni, G. Belvedere and M. Salmona, J. Chromatogr., 145 (1978) 315-318.
5 F.D. Juma, H.J. Rogers, J.R. Trounce and I.D. Bradbrook, Cancer Chemother. Pharmacol., 1 (1978) 229-231.
6 M.S.B. Nayar, L.Y. Lin, S.H. Wan and K.K. Chan, Anal. Lett., 12 (B8) (1979) 905-915.
7 F.D. Juma, H.J. Rogers and J.R. Trounce, Br. J. Clin. Pharmacol., 10 (1980) 327-335.
8 N. van den Bosch and D. de Vos, J. Chromatogr., 183 (1980) 49-56.
9 M.R. Holdiness and L.R. Morgan, Jr., J. Chromatogr., 275 (1983) 432-435.
10 A.C. Mehta and R.T. Calvert, J. Chromatogr., 421 (1987) 377-380.
11 M.R.Z. Talha and H.J. Rogers, J. Chromatogr., 311 (1984) 194-198.
12 T.T. Kensler, R.J. Behme and D. Brooke, J. Pharm. Sci., 68 (1979) 172-174.
13 L.R. Wantland and S.D. Hersh, J. Pharm. Sci., 66 (1979) 1144-146.
14 J.M. Margison, P.M. Wilkinson, T. Cerny and N. Thatcher, Biomed. Chromatogr., 1 (1986) 101-103.
15 R.W. Hardy, C. Erlichman and S.J. Soldin, Ther. Drug Monit., 6 (1984) 313-318.
16 A.B. Rustum and N.E. Hoffman, J. Chromatogr., 422 (1987) 125-134.
17 M.J. Cardone, J. Assoc. Anal. Chem., 66 (1983) 1257-1282.
18 L.D. Lewis, personal communication.