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Gas chromatographic determination of ifosfamide in microvolumes of urine and plasma

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ABSTRACT

In oncology, particularly in pediatric malignancies, high doses (5–10 g/m^2) of the oxazaphosphorine ifosfamide play an important role in the treatment of sarcomas. Pharmacokinetic data of ifosfamide and its metabolites in these cases are scanty. Considering the special demands of the determination of ifosfamide in plasma of young children, a very sensitive capillary gas chromatographic method, requiring only 50 μl of plasma, has been developed. This bioanalysis of ifosfamide shows good linearity and accuracy in the concentration range 10 ng to 100 μg per ml of plasma and 25 ng to 1 mg per ml of urine. The absolute limits of detection in plasma and urine are 2 ng/ml and 5 ng/ml, respectively. The stability of various solutions of ifosfamide and trofosfamide was tested and proved to be satisfactory, except for ifosfamide in plasma and urine kept in the refrigerator. The validity of the method for pharmacokinetic purposes is shown in the case of one patient.

INTRODUCTION

Ifosfamide [IF, 3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine 2-oxide] is a structural analogue of cyclophosphamide (CP), a drug widely used in cancer chemotherapy (Fig. 1). IF as a single agent or in

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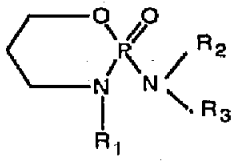

	R_1	R_2	R_3
Ifosfamide	CH_2CH_2Cl	CH_2CH_2Cl	H
Cyclophosphamide	H	CH_2CH_2Cl	CH_2CH_2Cl
Trofosfamide	CH_2CH_2Cl	CH_2CH_2Cl	CH_2CH_2Cl
2-dechloroethylifosfamide	CH_2CH_2Cl	H	H
3-dechloroethylifosfamide	H	CH_2CH_2Cl	H

Fig. 1. Structures of ifosfamide, cyclophosphamide, trofosfamide, 2-dechloroethylifosfamide and 3-dechloroethylifosfamide.

combination chemotherapy is often part of the treatment of various cancers. The drug is inactive *per se*; it needs to be activated by liver enzymes to 4-hydroxyifosfamide, which is in equilibrium with aldoifosfamide. This compound is metabolized to ifosfamide mustard, a strongly alkylating product [1,2]. During the metabolism of IF some toxic metabolites are formed, of which acrolein is believed to be responsible for the urotoxic properties of IF [3,4] and chloroacetaldehyde for the neurotoxic symptoms [5]. Because of the formation of these highly toxic metabolites the importance of therapeutic drug monitoring is clear.

In Ewing's sarcoma and soft tissue sarcoma, the response rate and proportion of patients surviving with complete remission have been increased by 15–20% with an ifosfamide-containing regimen [2,6]. Also, in pediatrics IF plays an important role in the treatment of sarcomas, often with the application of high doses (5–10 g/m^2).

For the determination of IF in plasma a number of gas chromatographic (GC) [7–19] and high-performance liquid chromatographic (HPLC) methods [20–23] are available. However, in the case of GC only a few investigators use a capillary column [13,18,19] with the advantage of lower limits of detection and better resolution. Because of the supposed thermal instability of CP and IF, a derivatization method was thought obligatory [8,10–12,14,19]. However, for the last several years it has proven to be possible to determine IF and CP underivatized [13,15–18,24].

The aim of our study was to develop a simple and sensitive method requiring only microvolumes of plasma in order to make it applicable for pharmacokinetic drug monitoring in children. The method described here needs only 50 μl of plasma, making it suitable for IF determination in a few droplets of blood taken from the fingertip.

EXPERIMENTAL

Chemicals

IF, trofosfamide (TF), 2-dechloroethylifosfamide (2-DCIF) and 3-dechloroethylifosfamide (3-DCIF) were kindly donated by Asta Pharma (Frankfurt, Germany).

Analytical-reagent-grade sodium hydroxide and ethyl acetate were purchased from Merck (Darmstadt, Germany) and were of the highest purity available. Ethyl acetate was distilled before use. All other chemicals were of analytical grade and used as received. Throughout the study distilled water was used.

Instrumentation

A gas chromatograph (HRGC 5300, Carlo Erba Instruments, Milan, Italy) equipped with a split–splitless injector and nitrogen–phosphorus-selective flame ionization detection (NP-FID) was used. Separation was achieved on a capillary column (CP-Sil 8 CB, 25 m × 0.32 mm I.D., film thickness 0.12 μm, Chrompack, Middelburg, Netherlands) with the application of the following temperature programme. The starting temperature of the column was 120°C and was raised to 200°C at 40°C/min. The column temperature was held at 200°C for 8 min.

The chromatograph was operated under the following conditions: temperature at the injection site, 175°C; temperature of the detector, 275°C; carrier gas, helium, at a flow-rate of 3 ml/min; flow detector gases, air at 300 ml/min, hydrogen at 30 ml/min, make-up gas, helium at 30 ml/min; split injection, where the split depends on the concentration in the sample. The detector was connected to a Model DP 700 integrator (Carlo Erba Instruments).

For GC measurements with mass spectrometric (MS) detection a Finnigan 700 ion trap detector (Finnigan MAT, San José, CA, USA) was used, operated in the chemical ionization mode with isobutane as the chemical ionization reagent gas. The manifold temperature was maintained at 200°C and the temperature of the transfer line was held at 260°C. The detector was operated in the automatic reaction control (ACR) mode.

Plasma procedure

The internal standard used was TF. Solutions of TF and IF in water with various concentrations were employed.

A 50-μl sample of plasma was transferred into a polypropylene Eppendorf cup. An equal amount of water, 10 μl of a 1 *M* sodium hydroxide solution and 25 μl of a TF solution were added. The cups were closed and the solutions were vortex-mixed for 15 s. Next, 500 μl of ethyl acetate were added. The mixture was placed on a vortex mixer for 1 min followed by centrifugation at 3000 *g* for 1 min. The organic layer was transferred into a 700-μl glass vial (Chromacol, London, UK) and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The dry residue was dissolved in 20 μl of ethyl acetate and the vial

closed with a natural rubber–PTFE crimp cap. A 1-μl aliquot of the IF solution was injected into the gas chromatograph under split conditions. For the concentration range 1–1000 ng IF per ml of plasma, a split of 1:10 was used; for the concentration range 1–100 μg IF per ml of plasma the split had to be 1:100.

Samples for the calibration curves were composed of 50 μl of drug-free human plasma, to which different amounts of an IF solution in water were added. This quantity was supplemented with distilled water to make up to 100 μl. Calibration samples were treated identically to the corresponding patient samples, yielding a final solution of 135 μl, which was then equally processed.

Urine procedure

Urine samples were treated in the same way as plasma samples. In the whole concentration range (10 ng to 1 mg IF per ml) 50 μl of urine were used. For the different concentration ranges, the dry residue was dissolved in different quantities of ethyl acetate:

For 10 ng to 100 μg IF per ml: the residue was dissolved in 20 μl of ethyl acetate.

For 100 to 1000 μg IF per ml: the organic layer was separated from the urine layer and 1 μl of this layer was injected into the gas chromatograph, without prior evaporation and reconstitution.

For the concentration range 10 ng to 1 μg IF per ml a split ratio of 1:25 was used, and for the range 1–1000 μg the split ratio was 1:100.

Chemical stability

Standard solutions of IF and TF in water at concentrations of 10 and 100 ng/ml, and in ethyl acetate at a concentration of 10 μg/ml, were kept in the refrigerator (4°C) for eight weeks, after which time they were analyzed.

Before analysis, solutions of IF in plasma at concentrations of 100 ng/ml and 10 μg/ml, and in urine at concentrations of 10 and 300 μg/ml, were left at room temperature for 5 h and stored in the refrigerator (4°C) and in the freezer (−20°C) for four and eight weeks. Plasma and urine samples of the same concentrations were also kept at room temperature for 2 h after preparation of the sample. Then the samples were stored in the freezer (−20°C) for four weeks. Before analyzing the samples, they were kept at room temperature for another 2 h.

The stability of plasma samples containing 100 ng/ml and 10 μg/ml IF after 30 min in a water bath with a temperature of 60°C was tested, as this procedure has been advocated for inactivation of viruses [25].

GC–MS

GC–MS studies were performed with a solution of 1 μg/ml IF in ethyl acetate, of which 1 μl was injected using a split ratio of 1:10. The same temperature programme as described above was applied.

Patient samples

The method described above was used to determine the plasma concentration of IF in a seven-year-old patient treated for soft tissue sarcoma with a 24-h infusion of increasing dose on three consecutive days (day 2, 2 g/m^2; day 3, 3 g/m^2; and day 4, 4 g/m^2) with concomitant administration of sodium 2-mercaptoethanesulphonate (mesna). On day 1 carboplatin (500 mg/m^2) was given by infusion over 4 h.

Samples were taken on day 1, before starting the infusion of carboplatin, and on days 2, 3 and 4, 5 min before starting the (new) infusion of IF and 3.5 h after the start of the (new) infusion. The last sample was taken on day 5, when the infusion was stopped. The results are graphically presented in Fig. 2.

RESULTS AND DISCUSSION

The GC methods presently available for the determination of underivatized IF have the disadvantages of needing rather large plasma samples (at least 1 ml [15]) or having high detection limits (100 ng/ml [15]). The lowest sample volume reported for the determination of underivatized CP in plasma is 100 μl [26], but this implies a detection limit of 25 ng/ml CP. The lowest detection limit reported for the determination of derivatized CP in plasma is 10 ng/ml using 200 μl of plasma [13].

Extraction

Burton and James [23] described an HPLC method for IF-containing plasma samples using solid-phase extraction (SPE). A liquid–liquid extraction (LLE) was

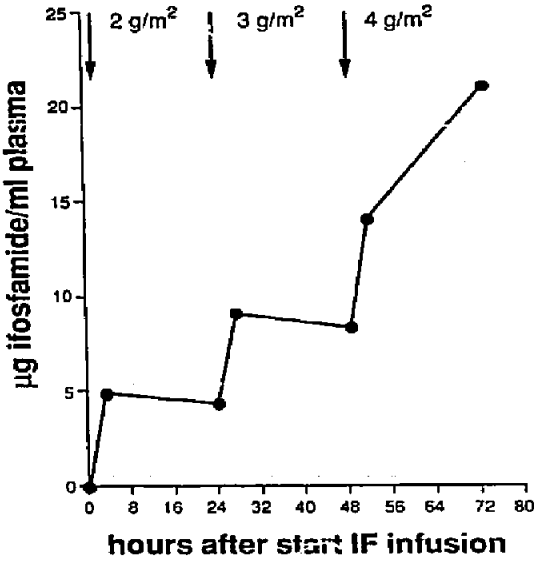

Fig. 2. Concentration–time curve in plasma of a patient receiving a three-day continuous infusion with increasing doses of IF.

preferred for this assay because of the low cost without loss of recovery. Moreover, SPE appeared to be as time-consuming as LLE.

The use of ethyl acetate as the extraction solvent brings about the danger of coeluting many endogenous plasma and/or urine compounds. However, the use of NP-FID diminishes the interference of coeluted compounds to such an extent that for urine a split injection of 1:25 suffices to eliminate all possible interfering urine components.

Chromatography

Fig. 3 shows a typical chromatogram for the assay of IF in a patient plasma sample. Fig. 4 shows a chromatogram from a spiked urine sample. The retention times for IF and TF are 4.65 and 7.35 min, respectively. Unfortunately, the drug-free plasma sample showed a component peak at the retention time of IF. It was not possible to separate this peak from the IF peak by varying the chromatographic conditions. When using a split ratio of at least 1:10 for plasma samples the interfering plasma peak is absent.

Two metabolites of IF appear in the plasma chromatogram. By comparing the GC properties of 2-DCIF and 3-DCIF (Fig. 1) with those of the two compounds eluted at 2.91 min (M1) and 3.24 min (M2), it could be concluded that 2-DCIF and M1 and 3-DCIF and M2 possess identical GC properties. These observations are not inconsistent with the assumption that M1 is 2-DCIF and M2 is 3-DCIF. These metabolites are currently under investigation, and this subject will be dealt

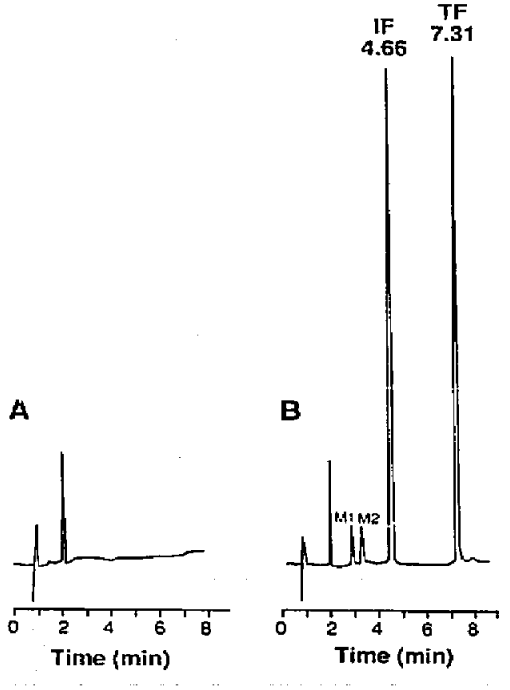

Fig. 3. Chromatogram of (A) a drug-free plasma sample and (B) a patient's plasma sample containing 14 μg of IF per ml of plasma (split 1:100). Two metabolites of IF are also shown (M1, M2).

with in a subsequent paper. In urine samples these same metabolites could also be detected.

Figs. 3 and 4 show that the retention times of IF and TF differ to such an extent that an isothermally operated gas chromatograph may be considered. When IF is the only compound of interest, it may even be the method of first choice. However, in an early stage of the research, it seemed possible to determine simultaneously two metabolites of IF when applying the advocated temperature programme and carrier gas velocity. The possibility of analyzing more than one compound in one run was preferred to a reduction in the time of analysis. The studies using GC–MS showed that the peak at 4.65 min consists of a compound with a molecular mass of 260 and contains two chlorine atoms (Fig. 5A). The spectrum of TF, eluted at 7.35 min, also showed the pattern of the intact molecule (Fig. 4B). These results prove that unchanged IF and TF were eluted by GC; IF and TF proved to be more stable than CP under GC conditions. The observation that unchanged IF is eluted by GC is consistent with the findings of Lambrechts *et al.* [24].

Validation

For the determination of IF by means of a GC method, CP is very often used as the internal standard. Lambrechts *et al.* [24] and De Bruijn *et al.* [27] have already reported the intra-alkylation of CP during the GC procedure, leading to two peaks for CP. Owing to the uncertainty surrounding the thermal instability

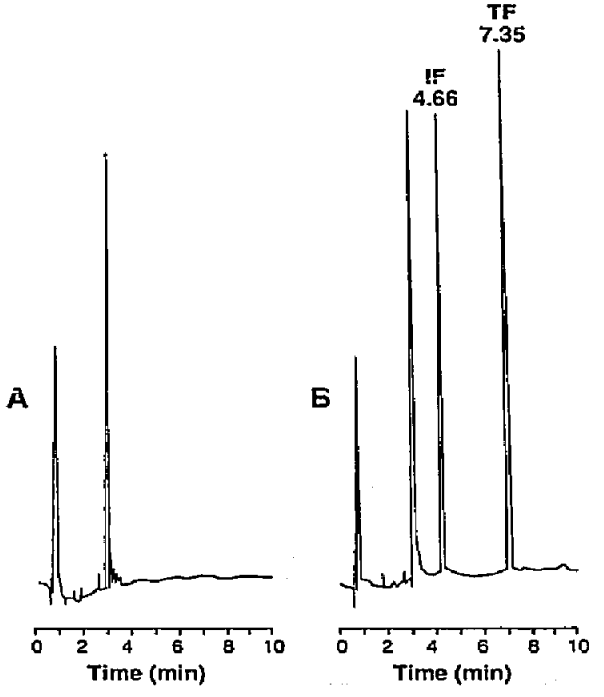

Fig. 4. Chromatogram of (A) a drug-free urine sample and (B) a spiked urine sample containing 500 ng of IF per ml of urine (split 1:25).

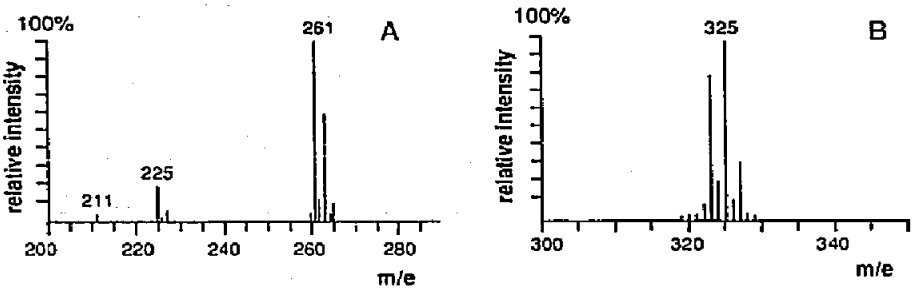

Fig. 5. GC–MS profiles of IF (A) and TF (B).

of CP, the thermally more stable oxazaphosphorine TF was chosen here as the internal standard.

The results of the calibration graphs for the various concentration ranges in plasma and urine are shown in Tables I and II, respectively.

Percentages recoveries of known quantities of IF in plasma and urine were calculated for each calibration curve at a concentration in the middle of the graph by measuring the absolute quantity of IF recovered, using a calibration curve of IF in ethyl acetate. The recovery of IF from plasma and urine in the various calibration ranges is shown in Table III.

The accuracy, intra- and inter-assay precision were determined in the middle of the various decades of the whole concentration range for plasma and urine and are summarized in Tables IV and V, respectively.

To determine the intra-assay precision, five analyzed samples with a concentration in the middle of the calibration graph and measured on the same day were compared. To determine the inter-assay precision a sample with a concentration in the middle of the decade under study was quantified on five subsequent days.

The limit of detection (signal-to-noise ratio of 3:1) when using a split ratio of 1:10 and an injection volume of 1 μl appears to be 2 ng IF per ml of plasma and 5 ng IF per ml of urine, using a 50-μl sample.

The presented procedure appears to be a suitable method of determining IF

TABLE I

EQUATIONS OF CALIBRATION CURVES FOR THE ANALYSIS OF IF IN PLASMA (n = 10)

Concentration range	Curve fit	Correlation
1–10 ng/ml	$y=0.35(\pm0.04)+0.15(\pm0.01)x$	$r^2=0.9982$
10–100 ng/ml	$y=0.46(\pm0.16)+0.06(\pm0.002)x$	$r^2=0.9993$
100–1000 ng/ml	$y=0.01(\pm0.001)+0.002(\pm2\cdot10^{-5})x$	$r^2=0.9993$
1–10 μg/ml	$y=-0.03(\pm0.03)+0.10(\pm0.002)x$	$r^2=0.9991$
10 –100 μg/ml	$y=0.01(\pm0.018)+0.01(\pm3\cdot10^{-4})x$	$r^2=0.9992$
Overall curve	$y=-0.03(\pm0.04)+0.26(\pm0.002)x$	$r^2=0.9998$

TABLE II

EQUATIONS OF CALIBRATION CURVES FOR THE ANALYSIS OF IF IN URINE (n = 10)

Concentration range	Curve fit	Correlation
10–100 ng/ml	$y=0.05(\pm0.018)+0.01(\pm6\cdot10^{-4})x$	$r^2=0.9991$
100–1000 ng/ml	$y=0.003(\pm0.002)+0.001(\pm2\cdot10^{-5})x$	$r^2=0.9994$
1–10 μg/ml	$y=-0.004(\pm0.002)+0.014(\pm2\cdot10^{-4})x$	$r^2=0.9991$
10–100 μg/ml	$y=-0.09(\pm0.017)+0.021(\pm3\cdot10^{-4})x$	$r^2=0.9996$
100–1000 μg/ml	$y=0.05(\pm0.019)+0.002(\pm3\cdot10^{-5})x$	$r^2=0.9994$
Overall curve	$y=-0.09(\pm0.09)+0.03(\pm4\cdot10^{-4})x$	$r^2=0.9995$

levels in biological fluids. The reported detection limits of IF of 2 ng/ml in plasma and 5 ng/ml in urine have been established using the lowest split ratio possible.

Because this method of analysis covers a large concentration range, it was not possible to use a single IF and TF stock solution. For every decade of the concentration range, IF solutions of four different concentrations and a single TF solution were used. The IF solutions were prepared from two stock solutions. In this way it was possible always to add the same amount of the TF solution and no more than 50 μl of an IF solution.

Chemical stability

The concentrations of IF and TF in water and ethyl acetate and the concentrations of IF in plasma and urine, used to test the chemical stability of IF and TF, were chosen in accordance with the concentrations of IF in patient samples and the solutions of IF and TF used for the determination of IF. The solutions of IF and TF in water showed no loss of IF after eight weeks. The loss of IF from plasma and urine solutions was not significant under the investigated storage conditions except for the storage at 4°C; after four weeks the maximal loss of IF from plasma solutions was 10%, whereas no loss of IF from urine solutions was

TABLE III

RECOVERY OF IF FROM PLASMA AND URINE (n=10)

Concentration	Recovery from plasma (%)	Recovery from urine (%)
5 ng/ml	92.5 ± 10.0	N.D.[a]
50 ng/ml	91.3 ± 5.7	94.0 ± 5.2
500 ng/ml	86.9 ± 4.7	92.8 ± 4.2
5 μg/ml	86.4 ± 1.9	84.4 ± 2.4
50 μg/ml	94.9 ± 2.7	88.9 ± 1.9
500 μg/ml	N.D. [a]	86.2 ± 3.1

[a] N.D. = not determined.

TABLE IV

ACCURACY, INTRA- AND INTER-ASSAY PRECISION OF THE DETERMINATION OF IF IN PLASMA ($n=5$)

Concentration	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
5 ng/ml	85.9	2.2	4.8
50 ng/ml	96.3	2.5	2.8
500 ng/ml	101.2	2.6	2.9
5 μg/ml	99.2	1.2	2.0
50 μg/ml	95.2	2.3	2.5

seen. After eight weeks at 4°C *ca.* 15% of IF was lost from the plasma samples and *ca.* 10% from the urine samples. Patient samples should therefore be kept in the freezer to prevent any loss of IF.

Patient samples

The plasma sample taken before starting the IF infusion and after the carboplatin infusion showed no interfering peaks of carboplatin. Nor does mesna appear in the chromatogram. The graph of the patient samples shows a normal pattern except for the last measurement, which is higher than expected. This may indicate that the IF present is too much for the microsomal liver enzymes to cope with, resulting in saturation of the enzymatic system and a high plasma concentration of IF.

CONCLUSION

The presented method is simple, fast and economic because of a low-cost extraction procedure and GC without prior derivatization. The method is suit-

TABLE V

ACCURACY, INTRA- AND INTER-ASSAY PRECISION OF THE DETERMINATION OF IF IN URINE ($n=5$)

Concentration	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
50 ng/ml	101.9	5.9	5.9
500 ng/ml	98.9	1.6	1.9
5 μg/ml	100.0	1.7	1.8
50 μg/ml	98.1	0.9	1.3
500 μg/ml	99.4	1.9	2.1

able for the determination of IF in children because only very little plasma is needed. With detection limits of 2 ng/ml IF in plasma and 5 ng/ml IF in urine, it can be used for kinetic measurements. With this method of analysis two metabolites of IF, possibly 2-DCIF and 3-DCIF, can be detected in plasma. Studies are in progress to complete the identification and analysis of these and other metabolites.

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REFERENCES

1 N. Brock, *Cancer Res.*, 49 (1989) 1.
2 L. Y. Dirix and A. T. van Oosterom, *Semin. Oncol.*, 16 (1989) 39.
3 N. Brock, J. Stekar and F. Pohl, *Arzneim.-Forsch.*, 29 (1979) 659.
4 P. J. Cox, *Biochem. Pharmacol.*, 28 (1979) 2045.
5 M. P. Goren, R. K. Wright, C. B. Pratt and F. E. Pell, *Lancet*, ii (1986) 1219.
6 H. Jürgens, J. Treuner, K. Winkler and U. Göbel, *Semin. Oncol.*, 16 (1989) 46.
7 L. M. Allen and P. J. Creaven, *Cancer Chemother. Rep.*, 56 (1972) 721.
8 C. Pantarotto, A. Bossi, G. Belvedere, A. Martini, M. G. Donelli and A. Frigerio, *J. Pharm. Sci.*, 63 (1974) 1554.
9 T. Facchinetti, M. D. D'Incalci, G. Martelli, L. Cantoni, G. Belvedere and M. Salmona, *J. Chromatogr.*, 145 (1978) 315.
10 B. Whiting, S. H. K. Miller and B. Caddy, *Br. J. Clin. Pharmacol.*, 6 (1978) 373.
11 F. D. Juma, H. J. Rogers, J. R. Trounce and I. D. Bradbrook, *Cancer Chemother. Pharmacol.*, 1 (1978) 229.
12 M. S. Balachandran Nayar, L. Y. Lin, S. H. Wan and K. K. Chan, *Anal. Lett.*, 12 (1979) 905.
13 N. van den Bosch, O. Driessen, A. Emonds, A. T. van Oosterom, P. J. A. Timmermans, D. de Vos and P. H. T. J. Slee, *Methods Find. Exp. Clin. Pharmacol.*, 3 (1981) 377.
14 M. R. Holdiness and L. R. Morgan, Jr., *J. Chromatogr.*, 275 (1983) 432.
15 M. R. Z. Talha and H. J. Rogers, *J. Chromatogr.*, 311 (1984) 194.
16 G. Blaschke and U. Koch, *Arch. Pharm.*, 319 (1986) 1052.
17 A. El-Yazigi and C. R. Martin, *J. Chromatogr.*, 374 (1986) 177.
18 E. A. de Bruijn, U. R. Tjaden, A. T. van Oosterom, P. Leeflang and P. A. Leclercq, *J. Chromatogr.*, 279 (1983) 279.
19 A. C. Mehta and R. T. Calvert, *J. Chromatogr.*, 421 (1987) 377.
20 R. W. Hardy, C. Erlichman and S. J. Soldin, *Ther. Drug Monit.*, 6 (1984) 313.
21 J. M. Margison, P. M. Wilkinson, T. Cerny and N. Thatcher, *Biomed. Chromatogr.*, 1 (1986) 101.
22 A. M. Rustum and N. E. Hoffman, *J. Chromatogr.*, 422 (1987) 125.
23 L. C. Burton and C. A. James, *J. Chromatogr.*, 431 (1988) 450.
24 H. Lambrechts, K. A. Van Cauwenberghe, G. Pattyn, A. T. van Oosterom, E. Gheuens and E. A. de Bruijn, *J. High Resolut. Chromatogr.*, 13 (1990) 567.
25 B. Spire, D. Dormont and F. Barré-Sinoussi, *Lancet*, i (1985) 188.
26 E. A. de Bruijn, Y. Geng, J. Hermans and O. Driessen, *Int. J. Cancer*, 45 (1990) 935.
27 E. A. de Bruijn, U. R. Tjaden and P. A. Leclerq, *Eur. Chromatogr. News*, 2 (1988) 16.