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Review

# Cyclophosphamide and related anticancer drugs

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**Abstract**

This article presents an overview of the methods of bioanalysis of oxazaphosphorines, in particular, cyclophosphamide, ifosfamide, and trofosfamide as well as their metabolites. The metabolism of oxazaphosphorines is complex and leads to a large variety of metabolites and therefore the spectrum of methods used is relatively broad. The various methods used are shown in a table and the particularly important assays are described. 

*Keywords:* Reviews; Oxazaphosphorines; Cyclophosphamide; Ifosfamide; Trofosfamide

## Contents



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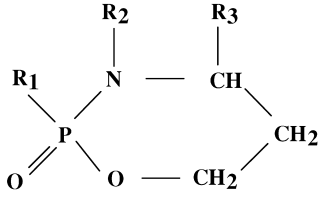
## 1. Introduction

Even today, 40 years after its introduction, cyclophosphamide (2-[bis-(2-chloroethyl) amino]-tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide) is one of the most widely used cytostatics and is a constituent of many polychemotherapy regimens. There have been more than 35 000 scientific publications on this agent, which demonstrate the wide interest it has received. The two most commonly used oxazaphosphorines are cyclophosphamide and ifosfamide, although other bifunctional mustard analogues, e.g. trofosfamide and mafosfamide, are also used therapeutically. The structure of the oxazaphosphorines is shown in Fig. 1.

Both ifosfamide and cyclophosphamide are widely used in the treatment of non-Hodgkin lymphomas and a variety of bone and soft tissue sarcomas [1]. In comparison with many other anticancer drugs, cyclophosphamide exhibits relatively little non-hematopoeitic toxicity. High-dose therapy forms have been used in both adults and children [2]. In contrast to cyclophosphamide, ifosfamide shows increased nephrotoxicity and therefore its clinical use in paediatric patients is dose-limited. Ifosfamide has to be given with at least an equimolar dose of the uroprotective agent mesna. This prevents hemorrhagic cystitis, thought to be caused by the toxic metabolites acrolein and chloroacetaldehyde. The pharmacological profile of these drugs is important and in order to achieve this information reliable methods for determination of oxazaphosphorines and their metabolites are necessary when determining optimal dosages of oxazaphosphorines.




| Substance | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Cyclophosphamide | $ClCH_2CH_2$ <br> N — <br> $ClCH_2CH_2$ | H- | H- |
| Mafosfamide | $ClCH_2CH_2$ <br> N — <br> $ClCH_2CH_2$ | H- | $^{-}O_3S$-$CH_2$-$CH_2$-S- |
| Ifosfamide | H <br> N — <br> $ClCH_2CH_2$ | Cl-$CH_2$-$CH_2$- | H- |
| Trofosfamide | $ClCH_2CH_2$ <br> N — <br> $ClCH_2CH_2$ | Cl-$CH_2$-$CH_2$- | H- |

Fig. 1. Structure of oxazaphosphorines.

In this article an overview is given of the methods of bioanalysis of oxazaphosphorines and their metabolites. Several reviews on the pharmacology and determination of oxazaphosphorines have been published (Moore [3], Lind and Ardiet [4], Yule et al. [5], Kaijser et al. [6] and Malet-Martino et al. [7]). They provide excellent summaries of pharmacokinetics, metabolism and analysis of oxazaphosphorines.

## 2. Metabolism

The metabolism of oxazaphosphorines can be divided into three stages: activation, toxification, and deactivation. The initial activation is based on hydroxylation of the ring at C-4 by mixed function oxidases in the liver (cytochrome P-450) to give 4-hydroxyoxazaphosphorines. We will describe the metabolism of oxazaphosphorine using the example of ifosfamide. Investigations of the activation pathway have indicated that the CYP enzymes CYP 3A4 and 3A5 are able to catalyse the formation of 4-hydroxy metabolites [8,9]. 4-Hydroxyifosfamide exists in equilibrium with the tautomeric form, aldoifosfamide (ALDOIF), as shown in Fig. 2. Aldoifosfamide is either dehydrogenated to inactive metabolite carboxyifosfamide, or spontaneously decomposes to form pharmacologically active isophosphoramide mustard and acrolein. Inactive metabolites are produced by oxidation of 4-hydroxyifosfamide to 4-ketoifosfamide or reaction with glutathione to form 4-thioifosfamide. Acrolein is a toxic and very active substance. It reacts with free sulfhydryl and/or free amino groups of proteins. Marinello et al. [10] reported that acrolein-induced destruction of cytochrome P-450 is caused by the alkylation of critical sulfhydryl groups located in cytochrome P-450.

Another inactivating reaction is the *N*-dealkylation of chloroethyl side chains which yields 2- and 3-dechloroethylifosfamide (2- and 3-DCl) and chloroacetaldehyde. This reaction is much more significant in the case of ifosfamide than cyclophosphamide, accounting for up to 50% of dose [11]. Chloroacetaldehyde is formed as a by-product of this reaction and it has been suggested that chloroacetaldehyde causes nephro- and neurotoxicity [12].

## 3. Analysis of oxazaphosphorines on biological materials

Sensitive and specific analytical methods have been developed for the oxazaphosphorines and their metabolites and Table 1 shows an overview of these. In the following sections of the text various aspects of oxazaphosphorine bioanalysis are discussed.

### *3.1. Oxazaphosphorines*

#### *3.1.1. Gas chromatographic approach*

Ifosfamide (IF), cyclophosphamide (CP) and trofosfamide (TR) are chemical analogues and therefore the analytical methods for their determination are similar. The method mostly used for the determination of oxazaphosphorines is gas chromatographic separation with nitrogen–phosphorus (NP)-detection. The advantage of NP-detection (NPD) is high selectivity and sensitivity as well as a small solvent peak in comparison with flame ionization detection, and a relatively large linear range. Gas chromatography with nitrogen–phosphorus detection has been compared to positive ion electron-impact ion-trap mass spectrometry (GC–MS) [38] and GC–NPD proved to be superior in sensitivity, detection range, accuracy, and precision. Therefore GC–NPD is the method of choice for fast underivatized determination of IF, 2-DCl, and 3-DCl in human plasma. A previous report suggested that decomposition of underivatized IF occurs on an SE30 column [98], however, Talha and Rogers [50] could not confirm this fact. Many methods do not use a derivatization step (as Table 1 shows). Trifluoroacetic anhydride (TFAA) is a substance usually used for derivatization and it reacts with oxazaphosphorines [13,32,49]. During the GC analysis of underivatized CP an additional peak in the chromatogram is often observed due to decomposition of CP. Van den Bosch and de Vos [32] studied the decomposition of CP on a 3% Versamid 900 column applying various experimental conditions. The decomposition reaction is an intramolecular alkylation of the NH group by one of the chloroethyl substituents in the nor-nitrogen mustard part of the CP molecule. Another derivatization procedure is the formation of *N,N*-dimethylformamide dimethyl acetals [16]. In addition, heptafluorobutyric anhydride has also been

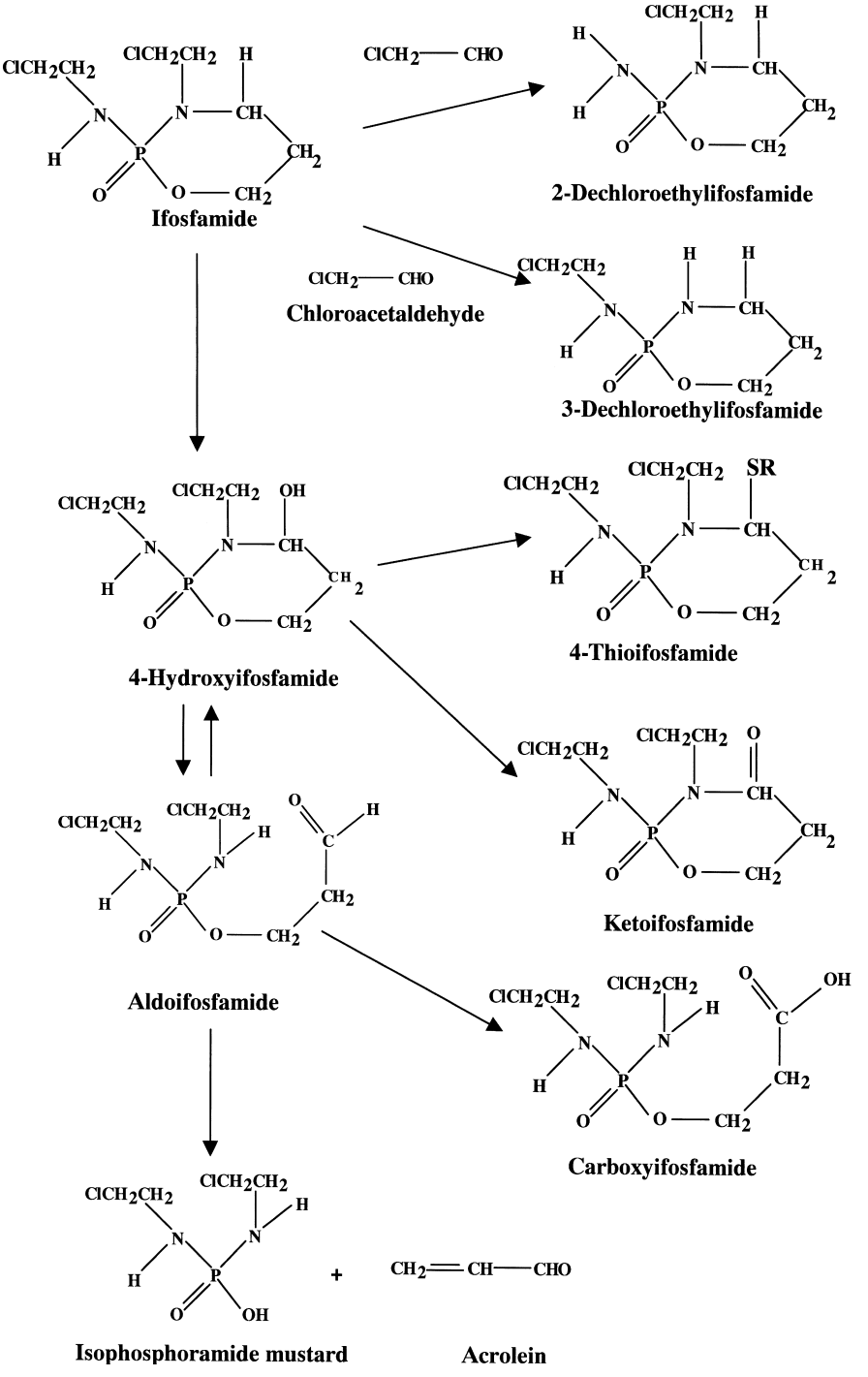



Fig. 2. Metabolism of ifosfamide.

Table 1
Methods for the analysis of oxazaphosphorines and their metabolites in various biological materials

| Compound | Method | Clean-up | Derivatization | Assay limits | Material | Ref. |
|---|---|---|---|---|---|---|
| CP, KCP, 3-DCl | GC–NPD | LLE | TFAA | 10, 0.6, 0.75 μ*M* (Q) | Plasma | [13] |
| ALCOCP, CXCP, PM | HPLC–MS | | | 1.5, 1.5, 3 μ*M* (Q) | Plasma | [13] |
| CXCP, CXIF | NMR | | | | Urine | [14] |
| CP, 3-DCl | GC–MS | LLE | | 50, 250 ng/ml (Q) | Plasma | [15] |
| CP, NNM, KCP, CXCP | GC–NPD | LLE | BCS, DFDA | 0.1–0.5 nmol/ml (D) | Microsomal incubation | [16] |
| 3-DCl | GC–NPD | SPE | | 1 ng/ml (D), 5 ng/ml (Q) | Microsomal incubation | [17] |
| CP, IF | Color. determ. | | Nitrous acid | | Pure or in dosage form | [18] |
| CP and hydrolysis products | NMR | | | | Buffered solutions | [19] |
| CP, 3-DCl, KCP, CXCP, | GC–MS | SPE | TFAA | Up to 600 pg/ml | Plasma | [20] |
| ALCOCP, NNM, NCOO | | LLE | Diazomethane | | | |
| CP | GC–MS | LLE | TFAA | 0.25 ng/ml | Urine | [21] |
| CP, KCP, CXCP, PM, NNM | TLC–PD | SPE | NBP | 1 μg/ml (D) CP, PM, NNM<br>0.5 mg/ml KCP, CXCP | Urine | [22] |
| CP, IF | HPLC–UV | SPE | | 1 μg/ml (D) | Plasma | [23] |
| CP | HPLC–UV | | | 0.3 μg/ml (D) | Blood, plasma | [24] |
| CP, KCP | GC–MS | | | | | [25] |
| CP | GC–NPD | SPE | | 10 ng/ml (D) | Plasma | [26] |
| CP | HPLC–UV | SPE | | 300 ng/ml | Serum | [27] |
| CP | GC–MS | LLE | TFAA | 0.2 μg/ml (Q) | Plasma | [28] |
| CP | Polarographic | | Nitrous acid | 10 μg/ml | Pure or in dosage form | [29] |
| CP, KCP, CXCP | GC–ECD or NPD | LLE | | | Plasma | [30] |
| CP, KCP, CXCP | HPLC off-line<br>FDMS | LLE | | $4\times10^3$–$10^5$ lower than UV-det. | Urine | [31] |
| CP | GC–NPD | LLE | TFAA | 10 ng/ml | Plasma | [32] |
| CP, IF, TR | GC–MS | | | | Urine | [33] |
| OHIF, OHCP | TLC off-line MS | | BMC, TCA | | Urine | [33] |
| CP, IF, TR, KCP, CXCP, CA | TLC–photometric<br>determination | | PBH | 0.25–0.5 μg | Urine | [34] |
| CP, KCP, CXCP | FDMS | | | 10 ppm | | [35] |
| CP | HPLC–MS–MS | LLE | | 0.2 ng/ml (Q), 0.05 ng/ml (D) | Urine | [36] |
| CP, OHCP, 3-DCl, KCP, CXCP | HPLC–MS | SPE | MHA | 15 ng/ml CP, 3-DCl, KCP (D)<br>30 ng/ml CXCP, OHCP (D) | Plasma | [37] |
| IF, 2- and 3-DCl | GC–NPD and MS | LLE | | 50 ng/ml (Q) NPD | Plasma | [38] |
| IF, 2- and 3-DCl, 2,3-DDCl | NMR, MS | | | | Buffered solutions | [39] |
| IF, 2- and 3-DCl, OHIF | GC–NPD | LLE | PFBHA | 3.25, 0.8, 0.62, 0.08 μg/ml | Blood | [40] |
| IF | NMR | | | | Urine | [41] |
| IF | HPLC, NMR | | | | Urine | [42] |
| IPM | GC–MS<br>HPLC–UV | SPE | BSTFA, TMSI | | Plasma,<br>buffered solutions | [43] |
| IF, 2- and 3-DCl | GC–NPD | | 3-amph (OHIF) | 2- and 3-DCl 0.2 nmol/ml (D) | Blood, plasma | [44] |
| CAA | GC–ECD | | | 0.06 nmol/ml IF (D) | | |
| OHIF | HPLC–FLD | | | 0.05 nmol/ml OHIF (D) | | |

Table 1. Continued

| Compound | Method | Clean-up | Derivatization | Assay limits | Material | Ref. |
|---|---|---|---|---|---|---|
| 2- and 3-DCl | GC–NPD and MS | LLE | | 30 and 10 ng/ml plasma (D)<br>70 and 25 ng/ml urine (D) | Plasma, urine | [45] |
| IF | GC–NPD | LLE | | 2 ng/ml, 5 ng/ml | Plasma, urine | [46] |
| 2- and 3-DCl | HPLC–UV | LLE | | 2–5 μ*M* (various patients) | Urine | [47] |
| IF, IPM, 2- and 3-DCl, CXIF | TLC–PD | | NBP | | Urine | [48] |
| IF | GC–NPD | LLE | TFAA | 0.5 μg/ml | Plasma | [49] |
| IF | GC–NPD | LLE | | 100 ng/ml | Plasma | [50] |
| IF | HPLC–UV | LLE | | 100 ng/ml (D) | Serum | [51] |
| IF | GC–FID | SPE | | 0.5 μg/ml IF, 2-DCl<br>1.5 μg/ml 3-DCl | Plasma, urine | [52] |
| IF, CP, TR, Acr | HPLC–UV<br>HPLC–FLD | | <br>3-amph | 1 μg/ml (D) IF,CP,TR<br>18 ng/ml (D) Acr | Microsomal incubation | [53] |
| TR, IF, CP, 2-DCl, 3-DCl | GC–NPD | SPE | | | Serum, urine | [54] |
| IF, CP, TR, 2-DCl, 3-DCl | HPLC–UV | LLE | | 0.5, 0.7, 0.9 μg/ml IF, CP, TR | Microsomal incubation | [55] |
| ALCOCP | GC–MS | LLE | BSTFA | | Urine | [56] |
| CP, OHCP | HPLC–UV | SPE, LLE | DNPH | To 15 ng/ml | Plasma | [57] |
| OHIF | HPLC–UV | LLE | Semicarbazide | 100 ng/ml (Q), 10 ng/ml (D) | Plasma, erythrocytes | [58] |
| Acr | HPLC–FLD | | 3-amph | 5 ng/ml (D), 10 ng/ml (Q) | Microsomal incubation | [59] |
| OHCP | HPLC–FLD | | 3-amph | 50 ng/ml (Q) | Blood | [60] |
| OHCP | HPLC–UV | LLE | Semicarbazide | 25 ng/ml (D) | Serum | [61] |
| OHCP, ALDOCP | GC–MS | | PFBHA | 25 ng/ml | Blood | [62] |
| OHCP, ALDOCP | HPLC–UV | | DNPH | 22 ng/ml | Plasma | [63] |
| OHCP, CP, PM, ALCOCP NCOO | GC–MS, various columns | LLE | BSTFA | | Plasma | [64] |
| OHCP | GC–MS | LLE | KCN-$NaHSO_3$, BSTFA | 50 ng/ml (D) | Plasma | [65] |
| OHIF | Fluorometric assay | LLE | 3-amph | | Serum, urine | [66] |
| OHCP, ALDOCP | NMR | | MHA | | | [67] |
| OHIF, OHCP | Fluorometric assay | LLE | 3-amph | 0.5 nmol/ml urine | Urine, plasma | [68] |
| IFM | HPLC–UV | SPE | DDTC | 0.45 μ*M* (D) | Plasma | [69] |
| CEA | GC–MS | LLE | | 5 ng/ml (D) | Plasma | [70] |
| Chlorethylamine, NCOO | GC–MS | LLE | TFAA | | | [71] |
| IF, OHIF, 2-DCl, 3-DCl, IPM | GC–MS | LLE, SPE | KCN-$NaHSO_3$, MSTFA, BSTFA and TMSI | 0.1–0.5 μg/ml | Plasma | [72] |
| IPM | HPLC–UV<br>GC–MS | SPE | <br>BSTFA, TMSI | 50 ng/ml | Plasma | [73] |
| IPM | NMR | | | | Buffered solutions | [74] |
| Nitrogen mustards | LC–MS | | | | | [75] |
| NNM | GC–ECD | | HFBA | | | [76] |
| PM, NNM, CP | GC–MS | LLE | BSTFA | | Urine | [77] |
| IPM | GC–NPD | LLE | TFAA | 20 ng/ml (D) | Plasma | [78] |
| Nitrogen mustards | Colorimetric assay | | NBP | 0.5 μg/ml | Buffered solutions | [79] |
| CP (*R*), (*S*), 2-DCl, 3-DCl | Chiral GC–MS | LLE | | 100 ng/ml | Plasma, urine | [80] |
| IF (*R*), (*S*), 2-DCl, 3-DCl | Chiral GC–NPD | LLE | | | Plasma, urine, cerebrospinal fluid | [81] |

Table 1. Continued

| Compound | Method | Clean-up | Derivatization | Assay limits | Material | Ref. |
|---|---|---|---|---|---|---|
| CP (*R*), (*S*) | HPLC–UV, achiral/chiral | SPE | | 0.625 μg/ml | Serum | [82] |
| ALDOCP (*cis*), (*trans*) | NMR | | | | | [83] |
| IF (*R*), (*S*) | HPLC–UV, achiral/chiral | SPE | | 2.5 μg/ml | Serum | [84] |
| IF (*R*), (*S*), 2- and 3-DCl | Chiral GC–MS | LLE | | 250 ng/ml IF 500 ng/ml 2-and 3 DCl (D) in plasma 500 ng/ml IF, 2-and 3-DCl (urine) (D) | Plasma, urine | [85] |
| IF, CP, TR (*R*), (*S*) | HPLC–UV, achiral/chiral | | | 10 μg/ml | Serum | [86] |
| CP (*R*), (*S*) | HPLC–UV | | Amidoalkylation Acetylation | 1 μg/ml | Plasma | [87] |
| CAA | HPLC–FLD | | Adenosine | 10 ng/ml | Plasma | [88] |
| CAA | HPLC–UV | | Thiourea | 40 ng/ml | Plasma | [89] |
| CAA | TLC–radiometry | LLE | DNPH | | Urine | [90] |
| Acr | HPLC–FLD | | Luminarin | 100 pmol/ml (D) 300 pmol/ml (Q) | Plasma | [91] |
| Acr | Headspace GC–MS | | | 56–280 pg/ml (D) | Urine | [92] |
| Acr | HPLC–FLD | | 3-amph | | Urine | [93] |
| Acr | Fluorometric assay | | 3-amph | 2 ng/ml | | [94] |
| CP | NMR | | | | Urine | [95] |
| IF; its phosphorated metabolites | NMR | | | 2.5 μg/ml | Urine | [96] |
| IF and its phosphorated metabolites | NMR | | | 2.5 μg/ml | Plasma, urine | [97] |

used for the derivatization of oxazaphosphorines [99,100].

Investigations on the pharmacokinetics of oxazaphosphorines using GC–MS have been done both with [20,21,28] and without [15,25,33] derivatization. Momerency et al. [20] described a very sensitive method for the determination of the antitumour drug cyclophosphamide and six stable metabolites in plasma. The absolute detection limit for most metabolites was ~3 pg, with a signal-to-noise ratio of 3:1. The authors used 500 μl plasma for the clean-up procedure giving a detection limit of ~1.5 ng/ml. The liquid extraction procedures were adapted from previous work reported by Lartigue-Mattei et al. [28], Mehta and Calvert [49] as well as Jardine et al. [101], whereas the solid-phase extraction procedures were based on previous work from El-Yazigi and Martin [26], Burton and Jams [23] as well as Hardy et al. [27]. The clean-up procedure plays an important role in determination of drugs in various biological materials. In this case the removal of interfering substances from the oxazaphosphorines is the main problem. Solvents for the liquid–liquid extraction are usually chloroform [51,86], dichloromethane [99,102], ethyl acetate [32,46,49,50], ether [98] and mixtures of ether and isopropanol [30], as well as dichloromethane and isopropanol [103,104]. The solid-phase extraction is a further important clean-up procedure for the determination of oxazaphosphorine [17,20,22,23,26,27,37,52,54]. Goren [47] compared various clean-up procedures. The choice of clean-up procedure depends on the material

being extracted, e.g. plasma or urine, and the substances being determined. Here the pH-value plays an important role. Fig. 3 shows a typical chromatogram of a urine sample.

*3.1.2. Liquid chromatography*

High-performance liquid chromatography (HPLC) has been employed for the analysis of cyclophosphamide in pharmaceutical preparations [105,106]. Determination of oxazaphosphorines in serum, blood or plasma has been demonstrated using HPLC with detection at low UV wavelengths [23,24,27, 51,53,55]. For example Burton and Jams [23] described an HPLC–UV-method with a detection limit of 1 μg/ml using 250 μl plasma. This was sufficient for the carried out pharmacokinetic studies, although it is lower than that described for gas chromatographic methods. A typical chromatogram was shown from a patient who received 5 g/m$^2$ ifosfamide (Fig. 4). Rustum and Hoffman [24] demonstrated a rapid, simple, and sensitive HPLC–UV-method for the cytostatic drug cyclophosphamide in whole blood and plasma. The detection limit was 0.3 μg/ml. This method was used to determine the stability of cyclophosphamide in plasma at room temperature and at −10°C. Oxazaphosphorines do not have a strong chromophore and therefore it is necessary to monitor the drugs at very low wavelengths (190 nm). The baseline noise can be decreased if the grating monochromator of the detector is constantly bathed in helium gas and the mobile phase purged with helium gas.

A sensitive, specific and accurate high-performance liquid chromatography/ionspray–tandem mass spectrometry procedure (HPLC–MS–MS) has been developed to quantify cyclophosphamide in human urine from hospital personnel involved in drug preparation and administration of antineoplastic alkylating agents [36]. The assay was linear over the range from 0 to 3.2 ng/ml urine, with a lower limit of quantification of 0.2 ng/ml. HPLC–MS–MS seems to be the most appropriate analytical technique for analyses of compounds of low volatility and thermal instability. A sensitive HPLC–MS-method has also been developed for the simultaneous determination of cyclophosphamide and its metabolites 4-hydroxycyclophosphamide (aldophosphamide), 4-

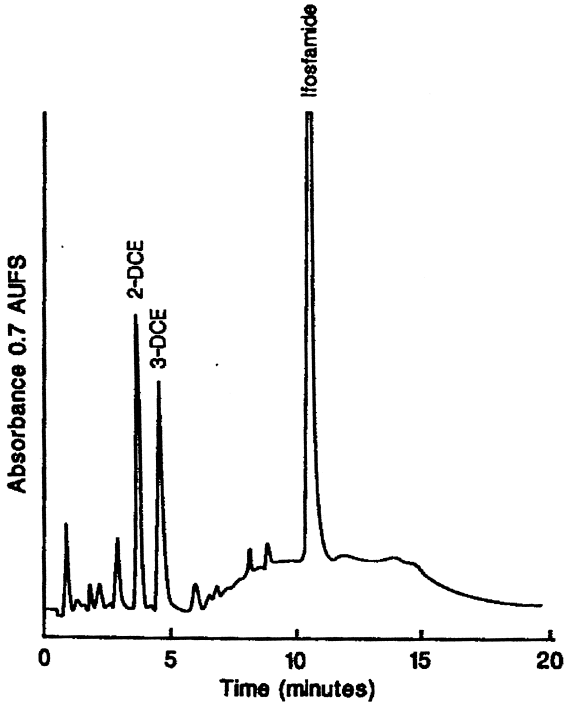



Fig. 3. Chromatogram of a urine sample obtained between 0 and 6 h after administration of 1.6 g/m$^2$ ifosfamide. The measured concentrations of 2-DCl, 3-DCl, and ifosfamide were 330, 805, and 615 μ*M*, respectively [47].

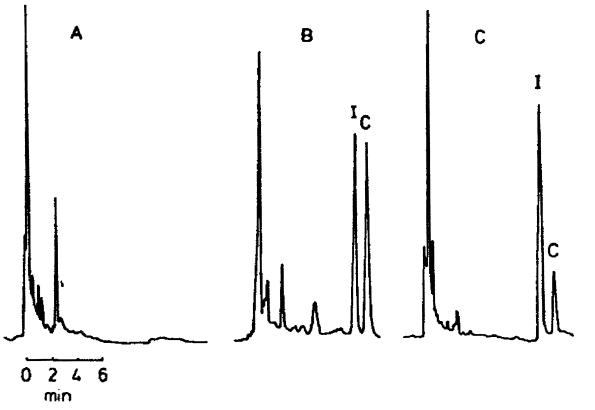



Fig. 4. Representative chromatograms from plasma. (A) Blank plasma; (B) plasma spiked with 30 μg/ml cyclophosphamide and 32 μg/ml ifosfamide; (C) plasma obtained from a patient who received 5 g/m$^2$ ifosfamide (sample contains 115 μg/ml ifosfamide). Peaks: C, cyclophosphamide; I, ifosfamide [23].

ketocyclophosphamide, carboxyphosphamide and 3-dechloroethylifosfamide in human plasma [37]. 4-Hydroxycyclophosphamide was converted with methylhydroxylamine to a stable methyloxime form. Other investigators have described the off-line coupling of HPLC and FDMS (field desorption mass spectrometry). The authors [31] indicated that highly sensitive, rapid and precise qualitative and quantitative investigations can be performed with this technique.

#### *3.1.3. Other methods*

An off-line method involving TLC (thin layer chromatography) and MS was described in the literature [33]. Other detection procedures using TLC are photography densitometry [22,48] and photometric determination [34]. In the photography densitometry method, the substances were treated with 4-(4′-nitrobenzyl) pyridine. The blue spots formed are unstable and therefore, for purposes of quantification, the plates have to be photographed within 10 s after the final treatment with alkali. In Ref. [22] the detection limit of the assay was 1 μg/ml for cyclophosphamide.

Polarographic and colorimetric methods have been described for the determination of cyclophosphamide and ifosfamide [18,29]. A colorimetric method based on measurement of nitroso derivative of cyclophosphamide or ifosfamide has been described. The procedures were applied to the determination of some pharmaceutical formulations of both substances. Also, the polarographic method is only suitable for determination of oxazaphosphorines in pharmaceutical formulations. It is more difficult to measure the wave height at less than 50 μg/ml. These methods are insensitive.

An important method for the investigations of metabolism and stability of oxazaphosphorines is NMR [14,19,39,42]. Misiura et al. [107] were the first to use $^{31}$P-NMR to study the urinary excretion of ifosfamide and its phosphorylated metabolites. Current 600-MHz HPLC–NMR technology allows detection of compounds at $<0.1$ μg/sample [42]. Given its rapid and continued growth, HPLC–NMR is likely to become one of the key technologies in pharmaceutic and/or biomedical analysis over the next decade.

### *3.2. Metabolites*

The oxazaphosphorines ifosfamide, cyclophosphamide and trofosfamide show a broad metabolite spectrum as demonstrated in Section 2.

#### *3.2.1. 4-Hydroxyoxazaphosphorines*

4-Hydroxyoxazaphosphorines are very unstable and therefore derivative formation is often necessary. A variety of substances are used for derivatization, e.g. *p*-nitrophenyl-hydrazine, semicarbazide, sodium cyanate, *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine and *o*-methyl-hydroxylamine [37,62,63,72, 108,109]. Indirect fluorometric methods involving the measurement of acrolein, a decomposition product of 4-hydroxy-oxazaphosphorines, have been used for quantitation of these metabolites [59,60,66,68].

4-Hydroxycyclophosphamide exists in equilibrium with aldophosphamide as does 4-hydroxyifosfamide with aldoifosfamide. These substances decompose to mustard and acrolein. Acrolein is a most active compound and reacts with many reagents. Based on the formation of fluorescent derivative 7-hydroxyquinoline by condensation of acrolein with 3-aminophenol, quantitation was performed without prior extraction or other sample clean-up procedures [59]. The method showed sufficient sensitivity with a limit of detection of 5 ng/ml and a limit of quantification of 10 ng/ml. 2-Butenal was used as internal standard. The resulting derivatives were measured by fluorescence detection with an excitation wavelength of 358 nm and an emission wavelength of 505 nm. Other than a centrifugation step, no other sample clean-up procedures were required for separation of microsomal proteins. Bohnenstengel et al. [59] concluded that protein binding of acrolein plays no important role in acrolein determination by this method. Various amounts of acrolein were added to samples containing denaturized rat liver microsomes. Denaturized proteins do not show the same characteristics as non-denaturized proteins. Proteins react with acrolein at 37°C, so that a predominant portion of acrolein is bound to proteins. Acrolein reacts with free sulfhydryl and/or free amino groups of proteins. Marinello et al. [10] reported that acrolein-induced destruction of cytochrome P-450 results from the alkylation of critical sulfhydryl groups located in cytochrome P-450. Uchida et al. [110] and Ester-

bauer et al. [111] reported that acrolein shows the highest reactivity with nucleophiles, such as the sulfhydryl groups of cysteine, the imidazole group of histidine, and the amino group of lysine. When mesna is added (300 μg/ml) to a protein solution (2 mg/ml), more than 90% of acrolein is bound to mesna at 37°C. Similar results are obtained in the determination of acrolein when using aqueous solutions and in experiments with proteins and mesna (own results, not published). This procedure is not practical for the determination of acrolein in plasma.

Wright et al. [60] described a method for the analysis of 4-hydroxycyclophosphamide in human blood. A aliquot of blood, collected for 4-hydroxycyclophosphamide assay, was immediately added (at the bedside in the case of clinical samples) to a solution containing perchloric acid, 3-aminophenol, hydroxylamine hydrochloride, sodium tungstate and methyl vinyl ketone as internal standard. The lower limit of quantitation is 0.2 μ*M*.

Various gas chromatographic methods with mass spectrometric detection have been used for determination of 4-hydroxyoxazaphosphorines [62,64,65]. The unstable metabolites 4-hydroxycyclophosphamide/aldophosphamide are derivatized with *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine to form a stable aldophosphamide oxime derivative (PBOX). [$^{2}H_{4}$]PBOX is used as an internal standard. PBOX is stable in whole blood for at least a week at room temperature. Previous GC–MS-methods [65,112] have used NaCN to ''trap'' 4-OH-CP/ALDOPH in human plasma followed by silylation of the resulting cyanohydrin derivative.

Measurement of 4-hydroxyoxazaphosphorines by HPLC–UV-detection has been described in the literature [58,63,109]. 4-OHIF [58] and 4-OHCP [109] are stabilized by derivatization with semicarbazide. The analyte is then determined by UV-detection at 230 nm. In Ref. [58] the lower limit of quantitation is 100 ng/ml using 1 ml of sample. Another type of derivatization step was used in an HPLC-method described by Johansson and Bielenstein [63]. Plasma proteins were precipitated with acetonitrile prior to derivatization with 2,4-dinitrophenylhydrazine at pH 2 using UV-detection at 357 nm. Concentration levels down to 22 ng/ml could be determined.

At this point we want to mention the determination of alcophosphamide [56]. Alcophosphamide is thought to be a metabolite following administration of cyclophosphamide formed via aldophosphamide. This metabolite has been quantified in rat plasma using gas chromatography–mass spectrometry with ammonia chemical ionization. The sensitivity limit for rat plasma was 20 ng/ml. A further GC–MS-method [20] and an HPLC–MS-assay [13] have been reported.

### *3.2.2. Dechloroethyloxazaphosphorines*

Several methods for determination of dechloroethyloxazaphosphorines 2-DCl and 3-DCl have already been listed in Section 3.1. For completeness, there are additional methods, for example GC–NPD- [13,17,38,40,44,45,54], GC–MS- [15,20,38,45,72], HPLC–MS- [37], HPLC–UV- [47,55] and TLC–PD-assays [48] which can be added to this list. The GC–NPD-method is usually used for direct analysis of ifosfamide or cyclophosphamide and their metabolites 2- and 3-DCl. A small amount of blood can be used since the NP-detector is highly sensitive. These assays may be applied in conjunction with routine clinical pharmacokinetic studies. Kaijser et al. [45] described a method for gas chromatographic determination of 2- and 3-DCl in plasma and urine using NPD. The major advantages of this procedure are that it is simple, fast and sensitive, and only 50-μl samples are required, which is especially useful in pediatric oncology. The extraction recoveries of 2- and 3-DCl are smaller than those of ifosfamide and cyclophosphamide. The liquid–liquid extraction (LLE) described by Goren [47] and the solid-phase extraction (SPE)-method of Momerency et al. [113] for example, appear to have the highest extraction recovery for 2- and 3-DCl (75 and 80%, respectively), whereas all LLE- and SPE-methods have comparable extraction recoveries of ifosfamide and cyclophosphamide (>95%). The SPE-method developed by Hardy et al. [27] showed a recovery of 91% for ifosfamide, but only 40% recovery for both 2- and 3-DCl. In Ref. [37] the recovery rate of 3-DCl was ~75% using an SPE-method. Not only the extraction recoveries of 2- and 3-DCl are important, but also the separation of these substances.

### *3.2.3. Keto- and carboxy-compounds*

Carboxyifosfamide (CXIF) and carboxyphosphamide (CXCP) are unstable. Ludeman et al. [114]

demonstrated in 1992 that the hydrolysis of CXCP was pH-dependent and was facilitated under acidic conditions. They reported $t_{1/2}$ values of 77 min at 37°C and pH 4, of 7 days at pH 7.4, and of 23 days at pH 9. In Ref. [37] $t_{1/2}$ for carboxyphosphamide was reported to be 14.5 h at room temperature and pH 7. The first study on the stability of CXIF was that reported by Hartley et al. [115] in 1994. Their study on the temporal stability showed that CXIF was stable at room temperature and pH 6–7, for at least 4 h, but that it was degraded by one-third at 24 h. A similar value has been observed in the case of CXCP at room temperature and pH 7 [37], although Joqueviel et al. [14] showed that CXCP is more stable than CXIF in urine at various pH values (7.0 or 5.5), indicating that the nature of the sample plays an important role in the stability. Ketoifosfamide and ketocyclophosphamide are stable at room temperature and at pH 7, but at low pH values ($<3$) both substances degraded.

Various methods have been used for the determination of these compounds: GC–NPD [13,16], GC–MS [20,25], TLC–PD [22], HPLC–MS [37] and FDMS [31,35]. HPLC–UV-methods are not suitable on account of the low concentrations in plasma and weak chromophore groups in the molecules. A sensitive HPLC–MS-method [37] was developed for simultaneous determination of cyclophosphamide and its metabolites 4-hydroxycyclophosphamide (aldophosphamide), 4-ketocyclophosphamide, carboxyphosphamide and 3-DCl in human plasma. A solid-phase extraction was used with $C_{18}$ cartridges followed by HPLC–MS with a single mass spectrometer (SSQ 7000 Finnigan). 3-DCl has a similar retention time to carboxyphosphamide, but this substance shows no interferences at mass 293 (CXCP), indicating that the separation is sufficient. The substances were diluted in 30% methanol/70% 20 m*M* ammonium acetate. Apart from carboxyphosphamide, all other metabolites of cyclophosphamide were stable. Carboxyphosphamide can be determined up to 2 h by storage in an autosampler. Kalhorn et al. [13] reported an HPLC–MS-method for the determination of carboxyphosphamide using a autoinjector equipped with a cooled (4°C) sample tray. Ketocyclophosphamide was determined by GC–NPD-assay with derivatization. The limit of quantification was 1.5 μ*M* for carboxyphosphamide.

de Bruijn et al. [25] investigated the possible formation of cyclization products of 4-ketocyclophosphamide similar to cyclophosphamide and this process can be monitored by mass spectrometry. Both cyclization products could be determined selectively and it appeared that cyclization of 4-ketocyclophosphamide was negligible in situ.

### *3.2.4. Mustard and related compounds*

The bioanalysis of ifosfamide mustard (IFM) and phosphoramide mustard (PM) is hampered by their high reactivity, polarity and lack of a UV chromophore. Their high solubility makes it difficult to purify the analyte effectively from biofluids. Various methods have been described in the literature: GC–NPD [16,78], GC–MS [20,43,64,72,73,76,77], GC–ECD [76], HPLC–MS [13,75], TLC–PD [22,48], colorimetric estimation [79], NMR [74] and HPLC–UV [69]. Kaijser et al. [69] reported a sensitive and selective HPLC–UV-method for the analysis of IFM in plasma. The assay involves direct derivatization of ifosforamide mustard in plasma with diethyldithiocarbamate and subsequent solid-phase extraction of the resulting derivative. The samples were stabilized by addition of semicarbazide and sodium chloride. 4-Hydroxyifosfamide reacts with semicarbazide to form semicarbazone and does not decompose into IFM and acrolein. After derivatization a product is obtained that is stable and can be detected at 280 nm. The derivative decomposes at pH$<$6.5. Therefore, all solutions used in the derivatization procedure were made up in 0.5 *M* phosphate buffer solution at pH 8. The high semicarbazide hydrochloride concentration necessitated a solution with a high buffer capacity in order to maintain a pH$>$8. The detection limit was 0.45 μ*M* (100 ng/ml).

Wang and Chang [72] published a GC–MS-assay in which 4-hydroxyifosfamide was first converted to more stable cyanohydrin adducts. IPM was extracted by $C_{18}$ reversed-phase resin. All analytes were converted to their silyl derivatives before GC–MS analysis. 2-DCl, 3-DCl, 4-hydroxyifosfamide, and ifosfamide were also determined in addition to IPM. The sensitivity limits ranged from 0.1 to 0.5 μg/ml using 100 μl of plasma.

Kalhorn et al. [13] described an HPLC–MS-method for the determination of carboxyphosphamide, alcocyclophosphamide and phosporamide mustard

(PM). Cyclophosphamide, 4-ketocyclophosphamide and 3-DCl were determined with a GC–NPD-assay. Non-polar compounds were extracted into methylene chloride, concentrated and analyzed by GC–NPD after derivatization, and the remaining aqueous fraction was deproteinated with acetonitrile–methanol prior to separation via reversed-phase HPLC and detection using atmospheric pressure ionization (API)-MS. The limit of quantification for PM was 3.0 $\mu M$.

Zheng et al. [73] developed a sensitive and specific GC–MS-assay for IPM in plasma. This method was used to study pharmacokinetics of IPM in rats, and results indicated rapid disposition of IPM. The routine detection limit for the assay was 50 ng/ml. Zheng et al. [73] also described an HPLC-method for the analysis of IPM in buffers. This method is rapid and does not require derivatization, but it cannot be used for IPM in biological materials. Though more stable than PM, using this method, IPM was shown to be unstable in buffer. The stability was influenced by the pH of the medium, higher stability being seen at acidic and at basic pH values. IPM is apparently more stable in plasma and tissue culture medium than in buffer. A further investigation [74], deals with NMR studies on the kinetics of bisalkylation of ifosfamide and phosphoramide mustard.

Phosphoramide mustard is an active alkylating species, which reacts via an aziridinium intermediate. PM undergoes cleavage of a phosphoramide residue to form nor-nitrogen mustard (NOR), a metabolite of cyclophosphamide which has been detected in human serum and urine [101,116,117]. Various methods have been described for the determination of nor-nitrogen mustard and the aziridinium intermediate [16,76,77].

IPM decomposes into chloroethylamine and phosphoric acid. Chloroethylamine can react with bicarbonate ion to form 1,3-oxazolidine-2-one. Highley et al. [71] published a method for determination of chloroethylamine and 1,3-oxazolidine-2-one following ifosfamide administration in humans. Chloroethylamine and 1,3-oxazolidine-2-one were isolated by liquid extraction with ethyl acetate at pH 10. One-step derivatization using trifluoroacetic anhydride was used and GC–MS then carried out in the electron impact ionization mode. Significant quantities were detected in vivo following high-dose ifosfamide administration.

A GC–MS-assay for the determination of *N*-2-chloroethylaziridine (CEA), a volatile cytotoxic metabolite of cyclophosphamide, has been developed by Lu and Chan [70]. The high volatility problem of CEA during isolation procedure was overcome by the combined use of a deuterium-labeled analog as the internal standard and a Snyder column-concentrator assembly. The routine detection limit was 5 ng/ml. The dichloromethane extraction recoveries were in the range from 91 to 101% for concentrations between 33 and 1333 ng/ml. The analytical method was used to evaluate formation of CEA from its precursors in sodium phosphate buffer, in cell culture media, and degradation of CEA in these media. The CEA formed was very stable in culture media with a degradation half-life of 265 h.

### *3.2.5. Acrolein*

As shown in Section 2, acrolein is formed during the metabolism of oxazaphosporines. Phosphoramide or isophosphoramide mustards are formed spontaneously and concomitantly with release of acrolein. Acrolein is responsible for the urotoxic side effects. Mesna is administered to prevent urothelial toxicity. Acrolein forms a stable thio-ether by addition of mesna to a double bound of acrolein. Since oxazaphosphorine metabolism is sensitive to auto-induction, as well as to inter- and intraindividual variations, an accurate measurement of acrolein levels in plasma should facilitate individual pharmacokinetic and metabolism studies of these drugs and thus lead to a better understanding of the variation in metabolism.

Different methods have been described for quantitative determination of acrolein in water [118,119], in air and exhaust gas [120–125] and in biological samples [91–94].

A rapid, sensitive and specific high-performance liquid chromatographic method for the quantification of acrolein has been developed by Paci et al. [91]. Condensation of acrolein with Luminarin® resulted in a fluorescent derivative that could be specifically detected and quantified. The method using fluorimetric detection showed high sensitivity with a limit of detection of 100 pmol/ml and a limit of quantification of 300 pmol/ml (16.8 ng/ml). This assay does

not require heating of the sample and consequently the risks of modified kinetics, as well as those of denaturation or artifact production, are virtually eliminated.

Most of the assays relate to the work of Alarcon [94]. Indirect measurement of 4-hydroxyoxazaphosphorines is an estimation of acrolein content. Acrolein reacts with 3-aminophenol to form 7-hydroxyquinoline. This compound can be measured fluorometrically as described in Section 3.2.1. Al-Rawithi et al. [93] developed a method for determination of acrolein in urine by liquid chromatography and fluorescence detection of its quinoline derivative. Derivatization with 3-aminophenol was carried out in the presence of ferrous sulfate solution in sulfuric acid. The effluent was monitored fluorometrically at excitation and emission wavelengths of 360 and 495 nm, respectively.

Sakura et al. [92] described a rapid and sensitive gas chromatographic and mass spectrometric (GC–MS) method based on the headspace technique. The calibration curve demonstrated good linearity for concentrations ranging from 1 to 1000 n*M*. However, due to the wide variation in acrolein evaporation from human urine, a calibration curve must be established for each urine specimen by preparation of standard solutions in urine. The detection limit varied from 1 to 5 n*M* (56–280 pg/ml).

#### *3.2.6. Chloroacetaldehyde*

Chloroacetaldehyde is a nephrotoxic and neurotoxic metabolite of oxazaphosphorines. It is produced as a byproduct by an alternative P450-catalyzed *N*-dechloroethylation reaction during the formation of the therapeutically inactive metabolites 2- and 3-DCl. About 45% of ifosfamide is usually metabolized to chloroacetaldehyde via the *N*-dechloroethylation pathway, while in the case of cyclophosphamide only 10% of the parental drug is *N*-dechloroethylated to yield chloroacetaldehyde [126]. Consequently, neurotoxicity occurs in ~20% of patients treated with ifosfamide, but is rare in cyclophosphamide treated patients.

Huang and Waxman [88] developed a simple and sensitive HPLC-method with fluorescence detection and applied this to the measurement of chloroacetaldehyde (CA) formation by liver cytochrome P450 enzymes in vivo in ifosfamide-injected rats and in vitro in liver microsomal incubations. This method is based on the formation of a highly fluorescent adduct, 1-$N^6$-ethenoadenosine, from the reaction of CA with adenosine at pH 4.5 upon heating at 80°C for 2 h. Heating at 80°C for 1–2 h was suggested as the preferred incubation condition since derivatization can be obtained within a relatively short time while minimizing sample degradation [127]. The limit of detection of CA in plasma using this method is $<0.1$ μ*M* and only 50 μl of plasma is required for the assay.

Kaijser et al. [89] published an HPLC–UV-method for determination of CA. Before analysis CA in plasma is derivatized with thiourea and the UV absorbance of 2-aminothiazole measured at the wavelength of maximum absorbance, 254 nm. The limit of detection (signal-to-noise ratio of 3:1) is 0.5 nmol of CA per ml of plasma (~40 ng/ml) with an injection volume of 20 μl. Formaldehyde solution is added directly to the blood samples in order to stabilise the substances to be measured.

Derivatization of CA with 2,4-dinitrophenylhydrazine in combination with thin-layer chromatography of the resulting hydrazone has been utilized to quantify radiolabelled CA [90]. After derivatization of CA with 2-hydrazinobenzothiazole [128] or 3-methyl-2-benzothiazolonehydrazone [129], the reaction product can be analyzed colorimetrically. Underivatized CA has been analyzed by means of gas chromatography [12,130–132] with flame ionization [130] or electron-capture detection [12,131].

### *3.3. Special aspects of oxazaphosphorine analysis*

#### *3.3.1. Stereochemistry*

Oxazaphosphorines are chiral molecules which are administered as a racemic mixture of their two enantiomeric forms. The molecules contain an asymmetrically substituted phosphorous atom. Previous investigations have revealed a considerable variability in enantiomer-specific oxazaphosphorine metabolism and cytotoxicity among different animal species and tumor models [107,133–139]. Various assays have been described for investigations of stereochemical effects: chiral GC–MS [80,85], chiral GC–NPD [81], achiral/chiral coupled HPLC–UV-methods [82,84,86] and a two-step derivatization

followed by assay using a standard HPLC–UV-method [87]. The main difference between these methods is in the chiral separation of oxazaphosphorine, i.e. the preliminary derivatization of the enantiomers. Gas chromatographic methods have employed various columns, including a chiral stationary phase composed of heptakis (6-*O*-hexyldimethyl-silyl-2-3-di-*O*-methyl-β-cyclodextrin) [80,85] and a Chirasil-L-Val column [81].

Corlett and Chrystyn [82] used an HPLC achiral–chiral coupled assay to measure the serum concentration of the enantiomers of cyclophosphamide. 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 $\alpha_1$ acid glycoprotein column. The limit of determination was 1.25 μg/ml for each enantiomer. UV detection was employed at 195 nm. Corlett and Chrystyn [84] used the same arrangement (Fig. 5) for determination of ifosfamide. Ifosfamide is separated from its metabolites and interfering serum components on an achiral $C_1$ column as Fig. 6 shows. The eluent containing racemic ifosfamide is then selectively transferred onto an $\alpha_1$ acid glycoprotein column for quantification of the enantiomers. Masurel and Wainer [86] described chromatographic separation of the enantiomers of ifosfamide, cyclophosphamide and trofosfamide. An achiral–chiral coupled column approach was used for analytical determinations. In this system, the OD-CSP (based upon cellulose-tris (3,5-dimethylphenylcarbamate)) was coupled to an achiral precolumn based on D,L-napthylalanine.

Reid et al. [87] developed a two-step chiral derivatization sequence for cyclophosphamide. The sequence involves amidoalkylation of cyclophosphamide with anhydrous chloral containing 1% dimethylformamide followed by acylation of the resulting secondary alcohol with a chiral carboxylic acid chloride, (+)-6-methoxy-α-methyl-2-naphthaleneacetyl chloride, to form a diastereomeric pair. Investigations at lower concentrations indicated that the enantiomers could be detected in plasma at the 100-ng/ml level. Thus, the two-step chiral amidoalkylation/acylation method is applicable to the routine bioanalytical determination of cyclophosphamide enantiomers in plasma at therapeutically relevant concentrations.

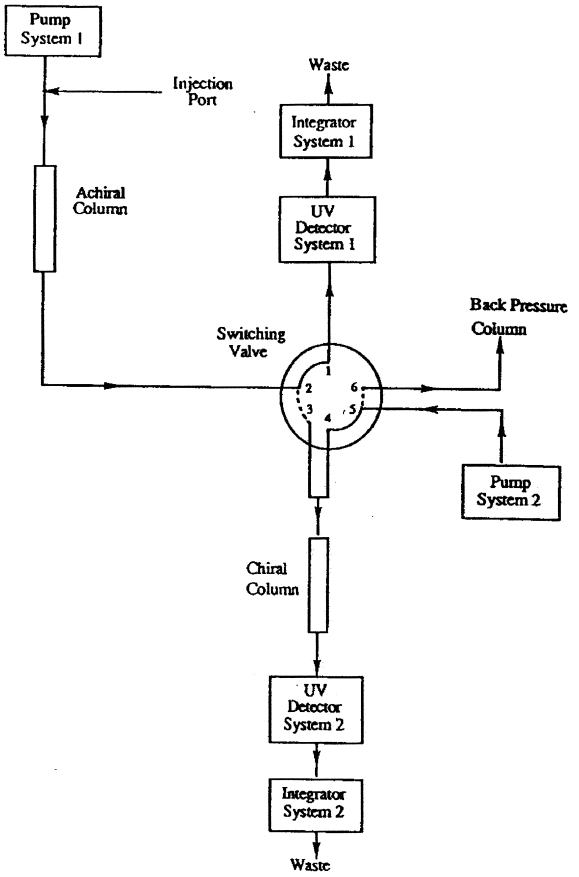



Fig. 5. Schematic design of the coupled system [84].

### *3.3.2. DNA-adducts*

Various metabolites of oxazaphosphorines form various adducts with glutathione, and nucleic acids in DNA and RNA. Development of drug resistance against alkylating cytostatic drugs has been associated with higher intracellular concentrations of glutathione and increased expression of glutathione S-transferase enzymes [140]. The mechanisms underlying the cytotoxic and teratogenic properties of oxazaphosporines are not completely understood, therefore it is necessary to investigate the relationships between the various metabolites and DNA, RNA

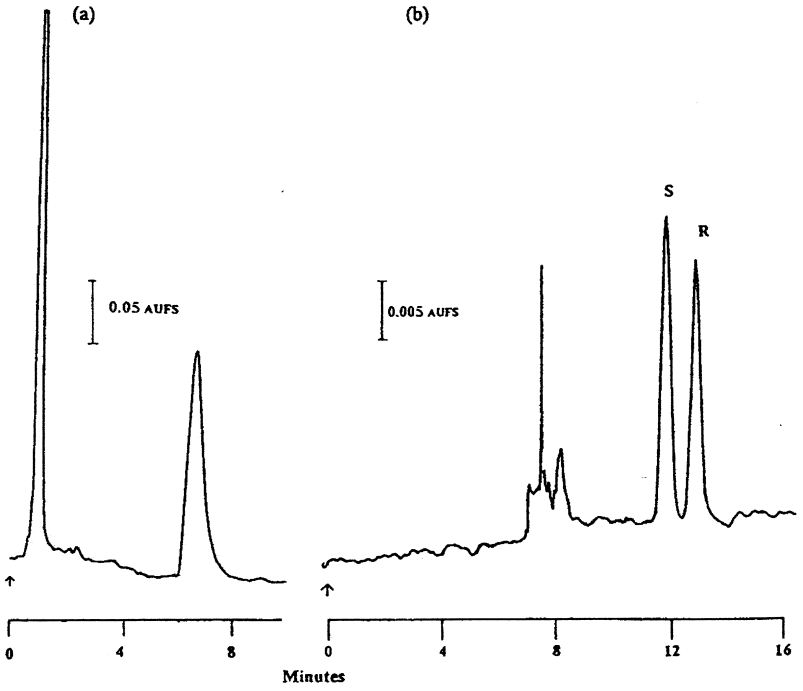



Fig. 6. Chromatogram of (a) recemic ifosfamide (50 mg/ml), and (b) *R*- and *S*-ifosfamide (25 μg/l of each) from a serum sample using the coupled achiral–chiral system [84].

substances such as glutathione. Any of the methods described could be used for investigation of these properties but the methods of choice for determination of these adducts are those based on NMR [140,141,145,147,149,150], MS [140,143–147], HPLC–UV [141,142,145,149,150], or HPLC–fluorescence detection [148].

Mirkes et al. [143] using tandem mass spectrometry, showed that the monofunctional adduct *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)ethyl] amine (NOR-G) and the bifunctional adduct *N*,*N*-bis[2-(7-guaninyl)ethyl] amine (G-NOR-G) can be detected in the DNA in rat embryos during organogenesis after an in vitro exposure to 4-hydroperoxycyclophosphamide. The high sensitivity of this approach should enable the induction and removal of specific cyclophosphamide-DNA adducts during the initial stages of teratogenesis to be followed in future studies.

Acrolein is a metabolite of all oxazaphosphorines described. It reacts readily with DNA bases to form various cyclic propano adducts [151–154].The identities of the adducts have been confirmed using HPLC co-migration, NMR and UV spectra, and by comparing the chemical properties with those of synthetic standards [141].

### *3.3.3. Investigations on stability and metabolism of oxazaphosphorines by means of NMR*

NMR plays an important role in investigations on stability and metabolism of oxazaphosphorines. $^{31}$P nuclear magnetic resonance (NMR) spectroscopy has been used in conjunction with cell perfusion techniques to monitor the intracellular chemistry of the cyclophophamide metabolites, 4-hydroxycyclophosphamide and aldophosphamide, in human histiocytic and erythroleukemia cells [155]. The investigation of Sonawat et al. [156] deals with intracellular transformation of *cis*-mafosfamide in P388 mice leukemia cells using $^{31}$P-NMR spec-

troscopy. The authors established that the cell membrane was permeable to 4-hydroxycyclophosphamide and aldophosphamide and less permeable to phosphoramide mustard.

Investigations on the stability and decomposition of oxazaphosphorines and metabolites using NMR have been described in the literature [14,19, 39,74,157–161]. Iminocyclophosphamide has been identified using $^{1}$H-NMR as a product resulting from the treatment of 4-alkylthio-substituted cyclophosphamide derivatives. Although iminocyclophosphamide is unlikely to be important in the direct metabolism of cyclophosphamide, it is probably the critical intermediate in the activation of mafosfamide and the further metabolism of other 4-thio-substituted cyclophosphamide intermediates [158].

Gilard et al. [96] published a NMR-method for determination of the urinary excretion of ifosfamide and its phosphorylated metabolites. The accuracy of the quantification was acceptable at concentrations ranging from $2\times10^{-5}$ to $10^{-3}$ *M*. However, the analytical error was high at the lowest concentration used ($\approx10^{-5}$ *M*), the detection limit of the spectrometer employed. Moreover, since $^{31}$P-NMR enables intact body fluids to be studied directly, the problems encountered in extraction, recovery, and chemical derivatization, as well as those stemming from the pH sensitivity of many metabolites, are avoided. The main limitation of $^{31}$P-NMR is however, its low sensitivity compared with chromatographic methods.

## 4. Conclusion

The metabolism of oxazaphosphorines is complex and leads to a large variety of metabolites. This explains the high number of analytical assays employed and the large number of analytical methods for ifosfamide and cyclophosphamide as well as their metabolites in particular. Trofosfamide and mafosfamide are substances whose clinical use is limited and so fewer methods are available for the determination of these compounds. Since oxazaphosphorines resemble each other closely many of the methods described can be used for all oxazaphosphorines. Many of the assays for cyclophosphamide can also be used for ifosfamide. On the other hand trofosfamide is a relatively hydrophobic compound and chromatographic conditions must be changed for this substance.

The most widely used assay for the determination of oxazaphosphorines is the GC–NPD-method, with or without derivatization. The advantage of nitrogen–phosphorus detection is the high selectivity and sensitivity. A highly sensitive (quantification limit 0.2 ng/ml), specific and accurate HPLC–MS-method has been developed to quantify cyclophosphamide in human urine from hospital personnel involved in the preparation of drug formulations. HPLC–MS- and GC–MS-methods were often used for the quantitation of metabolites.

Since many metabolites are not stable, these substances have to be either derivatized or the solutions containing them cooled. The fluorimetric assay is a highly sensitive method for the determination of 4-hydroxyoxazaphosphorines. Studies describing the detection of 4-hydroxy-trofosfamide and trofosfamide mustard in biological samples have not yet been reported.

## 5. Nomenclature

*Compounds*

| | |
|---|---|
| Acr | Acrolein |
| ALCOCP | Alcocyclophosphamide |
| ALDOCP | Aldocyclophosphamide |
| ALDOIF | Aldoifosfamide |
| CA | Chlorethylamine |
| CAA | Chloroacetaldehyde |
| CEA | *N*-2-Chloroethylaziridine |
| CP | Cyclophosphamide |
| CXCP | Carboxyphosphamide |
| CXIF | Carboxyifosfamide |
| 2-DCl | 2-Dechloroethylifosfamide |
| 3-DCl | 3-Dechlorethylifosfamide |
| 2,3-DDCl | 2,3-Didechloroethylifosfamide |
| IF | Ifosfamide |
| IPM | Isophosphoramide mustard |
| KCP | Ketocyclophosphamide |
| KIF | Ketoifosfamide |
| NCOO | *N*-Chloroethyl-1,3-oxazolidine-2-one |
| NNM | Nor-nitrogen mustard |
| OHCP | 4-Hydroxycyclophosphamide |
| OHIF | 4-Hydroxyifosfamide |

| | |
|---|---|
| PM | Phosphoramide mustard |
| TR | Trofosfamide |

*Methods and detectors*

| | |
|---|---|
| ECD | Electron capture detector |
| FDMS | Field desorption mass spectrometry |
| FLD | Fluorescence detection |
| GC | Gas chromatography |
| HPLC | High performance liquid chromatography |
| LC | Liquid chromatography |
| MS | Mass spectrometry |
| NMR | Nuclear magnetic resonance spectrometry |
| NPD | Nitrogen–phosporus detection |
| PD | Photographic densitometry |
| TLC | Thin-layer chromatography |
| UV | Ultra violet chromatography |

*Clean-up*

| | |
|---|---|
| CF | Chloroform |
| DCM | Dichloromethane |
| Etac | Ethyl acetate |
| LLE | Liquid–liquid extraction |
| SPE | Solid-phase extraction |

*Derivatization*

| | |
|---|---|
| 3-amph | 3-Aminophenol |
| BMC | Benzylmercaptan |
| BSC | Benzene sulphonyl chloride |
| BSTFA | *N,O*-bis (Trimethylsilyl)trifluoroacetamide |
| DDTC | Diethyldithiocarbamate |
| DFDA | to *N,N*-dimethylformamide dimethyl acetal |
| DNPH | 2,4-Dinitrophenylhydrazine |
| HFBA | Heptafluorobutyric anhydride |
| MHA | Methylhydroxylamine |
| MSTFA | *N*-Methyl-*N*-methylsilyltrifluoroacetamide |
| NBP | 4-(4′-Nitrobenzyl)pyridine |
| PBH | 4-Pyridinaldehyde-2-benzothiazolylhydrazine |
| PFBHA | Pentafluorobenzylhydroxylamine |
| TCA | Trichloracetic acid |
| TFAA | Trifluoroacetic anhydride |
| TMSI | *N*-Trimethylsilylimidazole |

*Limit*

| | |
|---|---|
| D | Detection limit |
| Q | Quantification limit |

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