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Trace Analysis of the Oxazaphosphorines Cyclophosphamide and Ifosfamide in Body Fluids

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Cyclophosphamide and Ifosfamide
Oxazaphosphorine metabolites

1 Introduction

The oxazaphosphorines cyclophosphamide CY, 2-(bis-[2-chloroethyl]-amino)tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide and ifosfamide IF, 3-([2-chloroethyl]-2-C-[2-chloroethyl]amino)-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide are amongst the most widely used drugs in cancer chemotherapy [1]. The drugs are inactive *per se*: activation by liver enzymes results in formation of alkylating products with structures related to mustard [1]. The mutagenic mustard-related structures induce DNA damage whereas other metabolites such as acrolein are believed to be responsible for the urotoxic properties and/or neurotoxic symptoms. In both routine cancer therapy and experimental cancer research, many people might be exposed to CY and IF during pharmaceutical handling of the agents.

No methods are yet available for the routine screening of uptake of IF, CY, and mustard-related compounds during exposure to oxazaphosphorines: as a result, the risks to nurses and physicians working in clinical oncology, and pharmacists and to researchers working in cancer research, cannot yet be estimated.

The hazards of exposure to oxazaphosphorines may be estimated by use of any one of several methods for the determination of the mutagenic activity of biological fluids containing mustards; the methods, however, either lack a link with uptake of unchanged, inactive oxazaphosphorines or are laborious and require extensive sampling of biological fluids [2].

An analytical method for routine determination of CY, IF, and their toxic products should be (a) highly sensitive, since these compounds are present at low nanogram levels per mL of biological fluid and 10–100 µL of blood (fingertip) or urine should be sufficient for 2 determinations, and (b) highly selective since a large number of mustard-related metabolites might be present [1]. In addition, the analytical system must be highly deactivated, since some mustard compounds are notoriously reactive and readily adsorbed by active surfaces.

Existing methods for the determination of oxazaphosphorines in biological fluids have recently been reviewed [3]: bioanalysis of CY and IF has been performed by both gas and high performance liquid chromatographic methods, some of which are also suitable for determination of active metabolites. The methods enable adequate monitoring of patients treated with CY and IF but lack the sensitivity required for trace analysis.

If oxazaphosphorines are analyzed by capillary GC-MS, derivatization may not be essential [4]; preliminary experiments in our laboratory have shown that the compounds could be determined in water without any sample pretreatment simply by dilution of only 1–5 µL of the sample to 1 mL with ethyl acetate [4]. We extended these observations to biological fluids and it appeared that both CY and IF could be easily determined in blood below the ng/ml level.

This report presents, for the first time, an analytical method for the determination of trace quantities of CY and IF, and some of their metabolites, in biological fluids. The capacity of the method for estimating the risks of handling oxazaphosphorines by persons involved in health care and cancer research is described in detail.

2 Experimental

2.1 Instrumentation and Reagents

Gas chromatographic determination was performed by both electron capture and flame ionization detection.

GC-ECD was used for determination of the compounds of interest at pg/mL levels, and also traces of metabolites of the oxazaphosphorines, such as *nor*-N-mustard formed from CY. The analysis was accomplished with a Hewlett-Packard 5890 gas chromatograph equipped with ECD (^{63}Ni, 56.1×10^{-7} Bq) and FID, and fitted with a 25 m × 0.32 mm i.d. capillary column coated with 0.12 µm film of CP-Sil 8 CB (Chrompack).

Samples were introduced by on-column injection: helium was used as carrier gas at a flow rate of 5 mL/min and the oven temperature was programmed from 85 to 100 °C at 15°/min, then to 160 °C at 50°/min, and finally at 5°/min to 220 °C, which was maintained for 5 min (the temperature program was slightly

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modified for analysis of the oxazaphosphorine metabolites). The detector temperature was 250 °C and data were acquired by a combination of a Hewlett-Packard 3390A integrator and a Spectra-Physics SP4290 integrator.

GC-FID was used for determination of CY and IF in pharmaceutical solutions without sample pretreatment [4] and for routine monitoring of IF and CY in body fluids of patients and rats for a period of 48 h after treatment with the drugs of interest, since concentrations were much higher than those trace levels requiring analysis by ECD. These analyses were performed with a Carlo Erba HRGC 5160 Mega series chromatograph fitted with a 5 m × 0.53 mm i.d. column coated with a 2.65 μm film of polydimethylsiloxane HP-1 (Hewlett-Packard): the system was not essentially different from that described elsewhere [4].

Samples were introduced by on-column injection and the oven temperature was programmed from 85 to 100 °C at 15°/min, then to 160 °C at 50°/min, then to 180 °C at 1°/min, and finally to 220 °C at 5°/min. Data were acquired by a Spectra-Physics 4290 integrator.

The mustard analogs of interest were obtained from ASTA Pharma AG (Frankfurt, FRG) and were used without further purification. Compressed gases were UHP grade, additionally purified with gas filter traps. AR grade sodium hydroxide and ethyl acetate were purchased from E. Merck (Darmstadt, FRG), ethyl acetate was distilled twice before use.

2.2 Adsorption Traps: Solid Phase Extraction (SPE)

A rapid and simple method for the isolation of IF and CY from biological fluids has recently been described [5]. Adsorption sample traps were prepared from cyclohexyl-bonded silica and were used for IF and CY concentrations between 10 ng/mL and 100 μg/mL: linear calibration curves were obtained ($r > 0.9994$). Once packed, each trap was eluted with p.a. grade methanol (50 mL; Merck) and dried with helium (50 mL/min) for 1 h at ambient temperature. Traps could be stored in the freezer at −30 °C for several weeks without degradation or loss of IF and CY. When concentrations were below 10 ng/mL, recovery was nonlinear and liquid–liquid extraction (LLE) was preferred.

2.3 Procedure

2.3.1 *Sampling*

Blood (10 μL–1 mL) was obtained from volunteers (blank samples), patients treated with the oxazaphosphorines, and nurses handling cytostatic agents in clinical oncology. After centrifugation, plasma (5–500 μL) was isolated and 0.05–5 mL ethyl acetate was added. Urine was obtained from the same individuals upon spontaneous voiding and the fluid was treated in the same way as plasma.

In addition, 50 μL of blood was taken from the tail vein of a rat at different times following oral administration of CY in micro doses of 0.07 mg/kg. The method of administration was not essentially different from that reported earlier, but the dose was only 1/100 and cannulation of the carotid artery was not performed [6]. The blood was treated as described above.

2.3.2 *Analysis*

Trofosfamide (TF), an experimental oxazaphosphorine, was added to the ethyl acetate–plasma mixture as an internal standard. The solution was vortex mixed for 15 s followed by centrifugation at 5000 rpm for 1 min. The organic layer was isolated, evaporated to dryness using an evaporative concentrator, re-dissolved in ethyl acetate (10 μL), and analyzed. Working standards containing CY, IF, and the metabolites of interest, i.e. dechloroethyloxazaphosphorine, 4-ketooxazaphosphorines, and CY- and IF-related mustards, were prepared at the following concentrations: 0.11, 0.22, 0.44, and 0.88 μg/mL, and 0.11, 0.22, 0.44, and 0.88 mg/mL.

Two different working standards were injected before and after each sample analysis such that the working standard concentrations bracketed the sample concentrations. Sample aliquots and working standards were both injected in triplicate, and concentration calculations were based on the means of the three determinations. Urine was treated in the same way as plasma. Plasma and urine blanks were submitted to the same procedures as the samples from volunteers, patients, and animals included in the study.

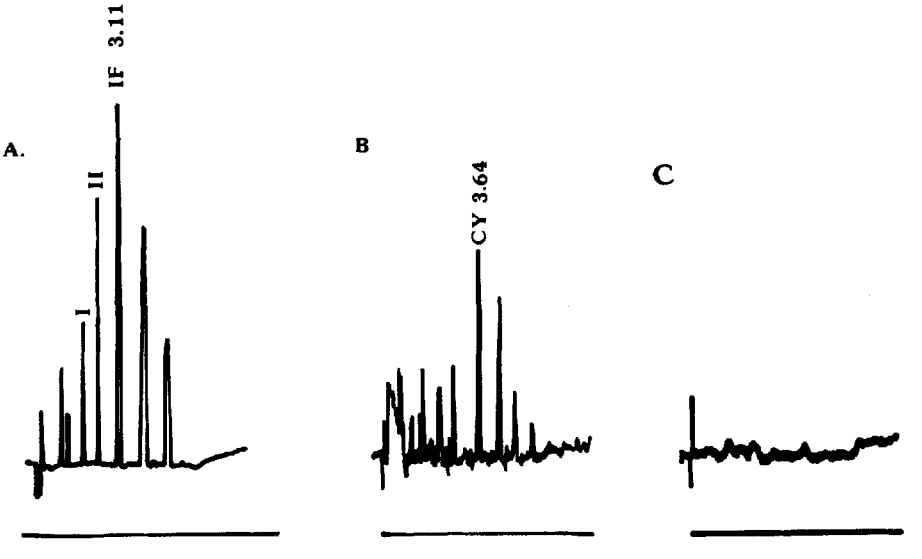

Figure 1

Chromatograms of 1 μL injections of (A) plasma extract from patient blood (20 μL) containing 12.7 μg/mL of ifosfamide (IF), (B) plasma extract of rat blood (20 μL) containing 2 ng/mL cyclophosphamide (CY), and (C) blank plasma obtained from a patient just before administration of IF. I and II were identified as 2-dechloroethylifosamide and 3-dechloroethylifosfamide, respectively, metabolites of IF, by GC-MS [4].

3 Results and Discussion

Chromatograms of IF and several of its metabolites from samples obtained from (A) a patient treated with IF and (B) a rat treated with orally administered CY are shown in **Figure 1**. Ethyl acetate (100 µL) was added to the patient plasma sample (10 µL), the sample was mixed and centrifuged, and 1 µL of the organic layer was subsequently analyzed by GC-ECD. Evaporation and concentration was necessary with rat plasma owing to the low concentration of CY in the sample: the patient sample contained 12.7 µg/mL IF; that from the rat contained only 2 ng/mL.

The patient plasma was also analyzed by SPE. The chromatogram then obtained was not essentially different from that obtained following LLE: the concentration then measured was 13.4 µg/ml. Several metabolites of IF appeared in the chromatogram obtained from the sample taken from the patient: dechlorinated oxazaphosphorines (2-dechloroethylifosfamide and 3-dechloroethylifosfamide) and keto- and carboxyoxazaphosphorine [4, 7, 8] were identified by GC-MS.

By use of a different temperature program starting with an injection temperature of 70 °C, a reaction product was identified which originated from IF-mustard. Its detection might be useful for estimation of the metabolic activation of IF. The presence of similar compounds was demonstrated following administration of CY to the rat; capillary GC of CY demonstrated, however, the presence of two peaks [4, 7, 8].

3.1 GC-MS

GC-MS confirmation of the identities of IF, CY and several of their metabolites was performed on a Hewlett-Packard 5970A (MSD) instrument fitted with a 25 m × 0.20 mm i.d. column coated with a 0.32 µm film of polydimethylsiloxane HP-UP (Hewlett-Packard) and interfaced with a Hewlett-Packard 9825B data system. Electron ionization mass spectra at 70 eV were obtained at a rate of 2/s. Samples were injected splitless (split valve closed for 30 s) at 60 °C; the oven was then ballistically heated to 150 °C which was maintained for 2 min and then programmed from 150 °C to 300 °C at 10°/min. The column head pressure was 20.0 psi and the interface temperature 290 °C.

GC-MS was also used for random control of patient samples.

3.2 Recoveries

The efficiency of the analytical procedure was determined by loading plasma and urine samples with 10 µL aliquots of IF and CY standards in water and extracting the samples by LLE as described above. The recoveries and relative standard deviations for spiked plasma samples over a wide range of concentrations are presented in **Table 1**, data obtained from spiked urine samples were similar.

The recovery of IF and CY from biological fluids was examined during the early stages of this work and shown always to be above 85 %. Linear calibration curves ($r^2 > 0.9992$) were obtained in the range depicted in Table 1. Recovery studies also showed that extracts of IF and CY could be stored for 8 weeks at −20 °C and 24 hours at room temperature. Slight reductions in the recovery figures were noted when extracts were stored for 8 weeks or more at 4 °C (loss *ca.* 10 %).

When IF and CY concentrations were above 44 ng/mL, *e.g.* for experimental drug monitoring [6], we preferred to use GC-FID because the calibration curves obtained for plasma and urine were linear over a wider range than those obtained by GC-ECD.

Table 1

Recovery of IF and CY from plasma (pg/mL–ng/mL range).

Concentration [ng/mL]	% CY recovered	% *RSD*	% IF recovered	% *RSD*
0.11 (n = 6)	94.9	2.1	95.2	3.1
0.22 (n = 6)	86.3	4.9	91.8	5.2
0.44 (n = 4)	91.1	3.3	93.7	4.2
0.88 (n = 4)	92.5	1.9	92.2	1.9
1.10 (n = 6)	88.5	3.9	89.6	3.8
2.20 (n = 6)	90.0	2.7	92.3	2.7
4.40 (n = 6)	87.8	4.0	88.9	2.6

3.3 Analysis of Biological Samples

This method has been applied to the analysis of (a) micro volumes (10–50 µL) of blood samples taken from patients and animals treated with IF and CY during the first 48 hours after starting treatment and (b) traces of IF and CY in 100 µL–1 mL volumes of blood of different origin. Concentrations of IF and CY in samples collected during the first two days were so high that GC-FID could be used for routine monitoring of the drug; the GC-ECD system, however, required only micro volumes of blood. This is of particular interest for routine monitoring of oxazaphosphorines in pediatric sessions (blood from childrens' fingertips) and in small laboratory animals.

The method seems to be particularly useful for monitoring patients subjected to long term oral CY treatment for breast cancer (CMF) [9, 10]. Patient compliance can be determined bi-weekly by analysis of fingertip blood samples and dose adjustments can then be made on the basis of systemic measurements of CY levels and the development of toxic effects [9, 10]. When 1000 mg doses of CY were administered *i.v.* to such patients, application of the method showed blood concentrations to be in the range 170 ng/mL–40 µg/mL during the first 48 hours (n = 19). Six days after administration the CY concentration (12–256 pg/mL) had dropped to well below the ng/ml level: 1 mL of blood was required for determination of the latter concentration.

As far as we know, this is the first time that the latter concentrations have been determined routinely. The data clearly demonstrate the applicability of the GC-ECD method to the determination of trace levels of oxazaphosphorines: the limits of determination, using 1 mL samples of blood plasma, are 10 pg/mL and 6 pg/mL for CY and IF respectively (signal to noise ratio: 3 : 1).

The method has also been applied to the monitoring of trace levels of IF and CY in rats to which the oxazaphosphorines were administered in micro doses (0.07 mg/kg). The range of concentrations encountered with IF and CY was 20 ng/mL (C_{max} at t = 5 min post-administration [6]) down to 16 pg/mL (t = 48 h post-administration). 1 mL of blood was required for determination of the latter concentration.

The method has also been used to determine trace levels of IF and CY in samples collected from nurses working with IF or CY for two months in oncological environments. Thus far no detectable levels of IF or CY have been demonstrated in 1 mL samples of the body fluids of interest (n = 6). This study is continuing in order to

increase the number of individuals included ($n > 100$). A detailed report of these data, and the implications with regard to mutagenicity, will be published elsewhere.

This method will be useful in other areas of cancer research for determining the risks associated with the handling of cytotoxic drugs. Most importantly, the presence of trace levels of IF, CY, and their metabolites can now be related to mutagenic effects by comparing data of sister chromatid exchanges (SCE) induced by exposure of CHO cells to biological fluids containing the compounds of interest [11]. This will finally result in a definition of the "mutagenic levels" of CY, IF, and/or their metabolites in humans.

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References

[1] *N. Brock*, Cancer Res. **49** (1989) 1–7.

[2] *E. Bingham*, N. Engl. J. Med. **313** (1985) 1173–1178.

[3] *U.R. Tjaden* and *E.A. De Bruijn*, J. Chromatogr. **531** (1990) 235–294.

[4] *H. Lambrechts, K.A. Van Cauwenberghe, G. Pattyn, A.T. Van Oosterom, E. Gheuens, P.A. Leclercq*, and *E.A. De Bruijn*, HRC **13** (1990) 567–569.

[5] *L.C. Burton* and *C.A. James*, J. Chromatogr. **431** (1988) 450–454.

[6] *E.A. De Bruijn, Y. Geng, J. Hermans*, and *O. Driessen*, Int. J. Cancer **45** (1990) 935–939.

[7] *E.A. De Bruijn, P.A. Leclercq*, and *U.R. Tjaden*, HRC & CC **9** (1986) 89–94.

[8] *E.A. De Bruijn, A.T. Van Oosterom, P.A. Leclercq, U.R. Tjaden, J.W. De Haan*, and *L.Y.M. Van de Ven*, Biomed. Environ. Mass. Spectr. **14** (1987) 643–647.

[9] *E.A. De Bruijn, P.H.Th.J. Slee, A.T. Van Oosterom, D.N. Lameijer, K.J. Roozendaal*, and *U.R. Tjaden*, Pharm. Weekbl. Sci. Ed. **10** (1988) 200–206 .

[10] *E. Gheuens, P.H.Th.J. Slee*, and *E.A. De Bruijn*, Onkologie **13** (1990) 203–206.

[11] *A.T. Natarajan, E.A. De Bruijn, P. Leeflang, P.H.Th.J. Slee, G.R. Mohn*, and *O. Driessen* Mutation Res. **121** (1983) 39–45.

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Capillary GC-MS Investigation of the Metabolism of Formyldienolone in Man

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1 Introduction

Formyldienolone (androsta-1,4-diene-2-carboxaldehyde-11,17-dihydroxy-17-methyl-3-oxo-(11α,17β); $C_{21}H_{28}O_4$; RN 2454-11-7), an anabolic steroid with therapeutic uses and low androgenic properties, is an ingredient of the Italian pharmaceutical product "Esiclene". It can, however, also be used illegally in sport with the aim of enhancing athletic performance. Studies of its chemical and pharmacological properties have been reported [1–3] and toxicological investigation has also been performed [4].

The detection of anabolic steroids and their metabolites in human urine is generally performed by GC-MS, usually after formation of a suitable derivative [5–11]. This paper reports a specific and sensitive method for the detection of formyldienolone (FD) metabolites in man. The method is based on extraction of urine, derivatization of the steroids, and capillary GC-MS. The urinary excretion of the drug's metabolites has been investigated by using capillary GC-MS with SIM to monitor the metabolites' characteristic ions: ingestion of the drug can be easily proved 2–3 days after the administration of a single therapeutic dose.

This method is useful for doping control in sport and for studying the excretion and metabolism of anabolic steroids.

2 Experimental

2.1 Chemicals and Reagents

Esiclene tablets containing 5 mg of formyldienolone were obtained from LPB, Pharmaceutical Institute SpA, Cinisello Balsamo, Milan, Italy.

N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), *N*-methyl-*N*-trimethylsilylheptafluorobutyramide (MSHFB), trimethylchlorosilane (TMSCl), and trimethylsilylimidazole (TSIM)

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