Monitoring the behaviour of 4-ketocyclophosphamide during capillary gas chromatography by mass spectrometry

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Monitoring the Behaviour of 4-Ketocyclophosphamide *versus* Cyclophosphamide during Capillary Gas Chromatography by Mass Spectrometry

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Capillary Gas Chromatography (CGC) is capable of determining underivatized cyclophosphamide (CPA) using SCOT OV 275 columns. Then CPA is subjected to *in situ* degradation resulting in formation of a cyclization product which can be determined selectively in biological fluids. In routine bioanalysis however cyclization products of CPA metabolites might interfere, e.g. 4-keto CPA. In the present study possible formation of cyclization products of 4-keto CPA similar to CPA was monitored by Mass Spectrometry. Cyclization of 4-keto CPA *in situ* was demonstrated to occur, resulting in a product similar to that of CPA. Both cyclization products could be determined selectively and it appeared that *in situ* cyclization of 4-keto CPA was negligible (<5%), probably owing to extra stabilization of the CPA metabolite by keto-enol tautomerism as has been demonstrated by NMR.

INTRODUCTION

Cyclophosphamide (CPA) (Fig. 1; 2-(bis-2-chloroethyl-amino)-tetrahydro-2H-1,3,2-oxazophosphorin-2-oxide) is a drug frequently used in cancer chemotherapy and as an immunosuppressant.[1,2] CPA is metabolically activated *in vivo* by the cytochrome P_{450} system to form 4-hydroxycyclophosphamide (4-OHCPA), iminocyclophosphamide (i-CPA), aldophosphamide (A-CPA), alcophosphamide (a-CPA) and N-dechloroethylcyclophosphamide (N-dClEt-CPA). Subsequent metabolism results in 4-ketocyclophosphamide (4-kCPA), chloroacetaldehyde, carboxyphosphamide (CPE), phosphoramide mustard (PM), acrolein, hydracrylic acid, nor-HN_2, 3-(2-chloroethyl)-1,3-oxazolidin-2-one and N-2-chloroethylaziridine (Fig. 1).

4-OHCPA is believed by most investigators of the mechanisms of action of CPA to be the primary extracellular mediator of antitumour activity.[3-5] Recently an important role for i-CPA has been demonstrated.[6] Pharmacokinetics and metabolism of CPA have been found to vary widely;[7,8] measurements of CPA and metabolites might be important in optimization of treatments with the alkylating agent.[9,10] Sensitive and selective assays are required then; to date methods of analysis include radioactivity measurements of ^{14}C-labelled CPA,[11] mass spectrometry,[12] gas chromatography/mass spectrometry (GC/MS),[13,14] gas–liquid chromatography with and without derivatization of CPA,[15-19] high performance liquid chromatography (HPLC)[20] and HPLC/field

desorption mass spectrometry.[21] GC is the most widely used technique in clinical pharmacological studies, both with and without derivatization. Introduction of unchanged CPA into GC results in a derivative as depicted in Fig. 2; conditions for the reaction can be optimized in such a way that CPA can be determined in body fluids of CPA-treated patients properly without derivatization.[13,18,19]

The selectivity of the assays with respect to other drugs regularly combined with CPA has been demonstrated. Taking metabolities of CPA into account, however, products like the 'on-column' product of CPA, the fundamental of the assay, might be formed and hinder selective analysis of CPA. Introduction of metabolities of CPA in capillary GC assays as described earlier by us[19] revealed that only 4-kCPA might interfere. Injection of 4-kCPA in a gas chromatographic system consisting of SCOT OV-275 capillary columns and electron capture detection resulted in two peaks if 4-kCPA solutions were prepared just before introduction into the analytical system (Fig. 3). The peaks were thought to be related to unchanged 4-kCPA and an 'on-column' product as presented in Fig. 4.

Besides formation of 'on-column' products, another point needs further investigation: the peak shape of peak 2, which was considerably aberrant of peaks with comparable retention times in SCOT OV 275 capillary columns under similar conditions.[19]

The data of the present investigations place particular emphasis on the behaviour of 4-kCPA during elution on SCOT OV-275 columns and implications for the selectivity of CPA analysis by capillary GC without derivatization.

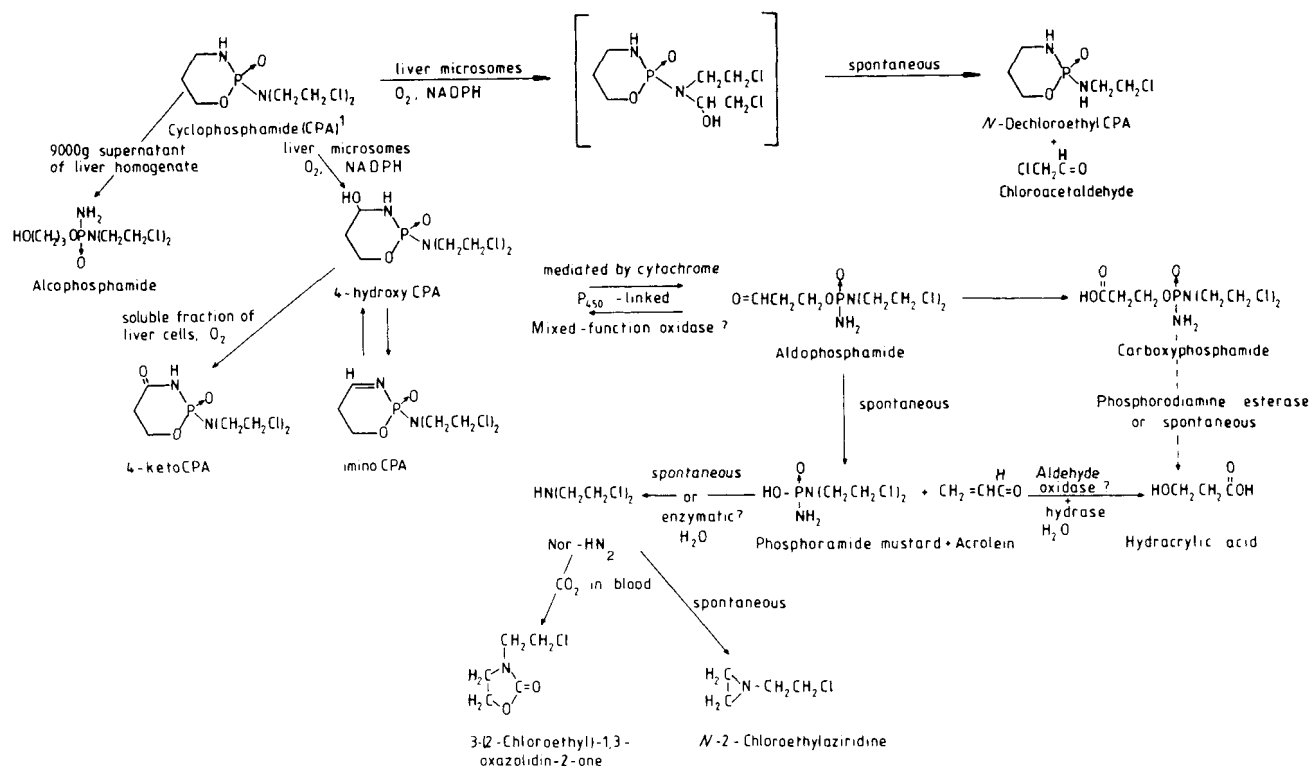† Author to whom correspondence should be addressed.

Figure 1. Metabolism of cyclophosphamide.

EXPERIMENTAL

Chemicals

CPA and metabolites of interest were kindly donated by ASTA-Werke (Bielefeld, FRG). Solvents used were of analytical grade and obtained from Baker (Deventer, The Netherlands) and Merck (Darmstadt, FRG). Freshly prepared ethyl acetate samples were introduced into the column at 250 °C (falling needle) and 83 °C (on column).

Columns

A fused silica WCOT CP SIL 5CB column (25 × 0.21 mm i.d.) was used for elution of 4-kCPA.

Mass Spectrometry

A Finnigan model 4000 quadrupole mass spectrometer (Finnigan, Sunnyvale, California, USA) was used both in the electron impact (EI) and chemical ionization (CI) mode. Mass spectral data were obtained under the following conditions: ionizing electron energy, 70 eV;

electron current, 0.30 mA; scan time, 1 s; source temperature, 250 °C (EI) and 200 °C (CI). For the CI mode, NH_3 reactant gas was introduced via the make-up gas line. The ion source was maintained at 0.15 Torr gauge
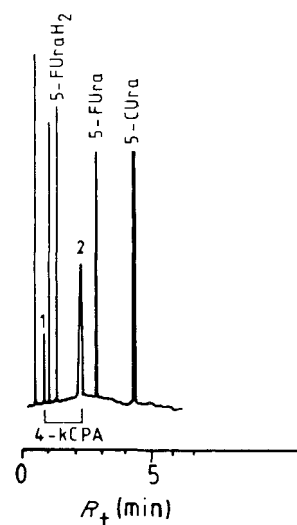

Figure 3. A chromatogram of 4-kCPA (1) and an 'on-column' product (2) and compounds not related to CPA and/or 4-kCPA. 5-FUraH₂: 5,6 dihydro-5-fluorouracil; 5-FUra: 5-fluorouracil; 5-CUra: 5-chlorouracil.
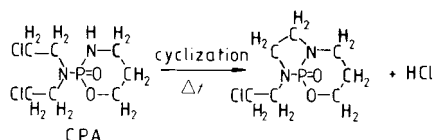

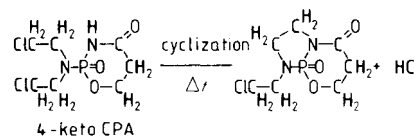**Figure 2.** Cyclization process of CPA following injection on capillary GC.
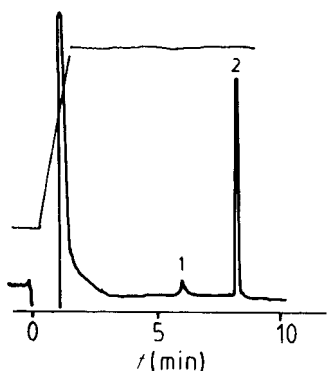

Figure 4. Possible 'on-column' product of 4-kCPA.

Figure 5. Chromatographic profile of 4-kCPA following on-column injection at 83 °C. Temperature programme: 83 °C to 225 °C (1.5 deg. C/s).
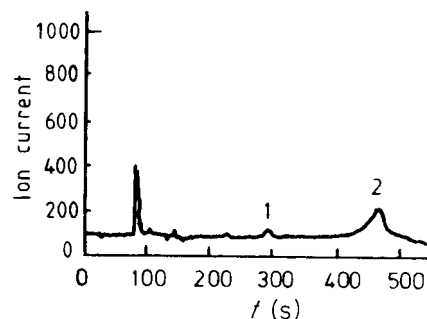
Figure 6. Total ion current chromatogram of 4-kCPA using solid injection at 250 °C. A fused silica WCOT CP Sil-5 column (25 × 0.21 mm i.d.) at 230 °C was used.

reading. The WCOT fused silica column was operated isothermally in MS experiments.

RESULTS AND DISCUSSION

A chromatogram obtained upon on-column injection at 83 °C using a fused silica WCOT CP SIL-5 column followed by programmed temperature increase from 83 °C to 225 °C at 1.5 deg./s is presented in Fig. 5. Two peaks can be noted, with retention times (t_R) of 6.1 and 8.3 min, respectively. The peak area of peak 1 was less than 5% of peak 2. A slightly raised baseline between peaks 1 and 2 could be distinguished, indicating that on-column reactions as well as reactions in the injection system of 4-kCPA take place.

When a total ion current chromatogram of 4-kCPA was recorded using split injection at 250 °C under otherwise similar conditions, again two peaks were observed, with $t_R = 4.5$ min and $t_R = 7.0$ min (Fig. 6). The peak area of peak 1 was 8% of peak 2. Furthermore, asymmetric peaks were observed in contrast to peaks of Fig. 5.

EI mass spectral fragmentation

The EI mass spectrum of peak 1 is given in Fig. 7(a). Fragment ions m/z 189, 161 and 117 were produced. The ions are degradation products of the M^+ ion at

m/z 238, which was not observed owing to rapid degradation to the ion at m/z 189 by loss of CH_2Cl. The EI mass spectrum of peak 2 is given in Fig. 7(b). Ions at m/z 225, 189, 163, 161, 134, 117 and 92 were formed. The fragmentation processes, together with their percentages as obtained from EI mass spectrometry, of peaks 1 and 2 are presented in Fig. 8.

CI mass spectral fragmentation

The CI mass spectrum of peak 1 is given in Fig. 9(a). Two ions were formed: MH^+ (m/z 239) and MNH_4^+ (m/z 256). The CI mass spectrum of peak 2 is presented in Fig. 9(b). MH^+ (m/z 275) and MNH_4^+ (m/z 292) were formed, as well as the fragment ion $MH^+ - HCl$ (m/z 239). Pathways of degradation during capillary GC and CI mass spectrometry with percentages of products formed are depicted in Fig. 10.

As Figs 8 and 10 demonstrate, peak 1 is associated with a cyclization product of 4-kCPA produced during capillary chromatography, while selectivity towards unchanged 4-kCPA is sufficient. The comparable cyclization product of CPA and unchanged CPA could be well separated from 4-kCPA and its 'on-column' product. Moreover, the amount of the 'on-column' product of CPA is negligible when capillary GC with a temperature programme is used. Keto-enol tautomerism of 4-kCPA was thought to be related to the relatively low amounts of 'on-column' products of 4-kCPA as well as some forms of peak asymmetry. This possibility was
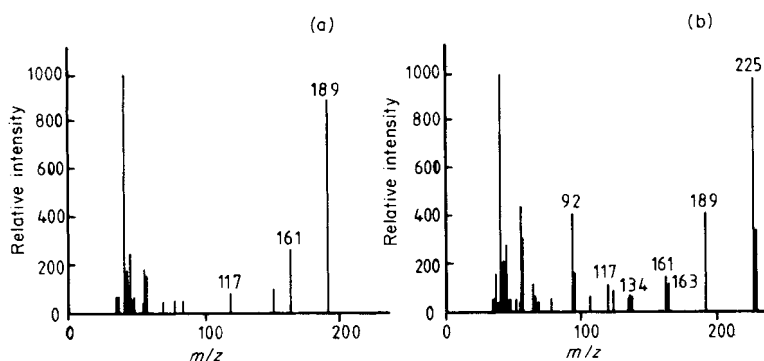
(a)

(b)

Figure 7. EI mass spectra of (a) peak 1 and (b) peak 2, as presented in Fig. 6.

GC mol wt 238.GC peak 1   −HCl   4-ketoCPA,mol wt 274.GC peak 2
                         Thermal degrada-
                              tion

t_{inj} 250 °C

MS |e⁻ (EI) |e⁻ (EI)

[M⁺⁺,m/z 238, 0 %]   −HCl   [M⁺⁺, m/z 274, 0 %]
                          ?

                   −CH₂Cl   −CH₂Cl   −(ClCH₂CH₂)N⁺

m/z 189, 100%   −HCl   m/z 225, 100%   ClCH₂CH₂N=CH₂   m/z 134, 10%
                 (40%)

−28   −72
CO?

m/z 161, 20%   m/z 163, 15%   m/z 92, 40%

m/z 117, 10%

Figure 8. Fragmentation processes of EI mass spectrometry of 4-kCPA.

Figure 9. CI mass spectra of (a) peak 1 and (b) peak 2, as presented in Fig. 6.

GC mol wt 238, GC peak 1   −HCl   4-ketoCPA, mol wt 274, GC peak 2
                          Thermal degrada-
                               tion

t_{inj} 250 °C

MS |NH₄⁺ (CI) |NH₄⁺(CI)

MH⁺, m/z 239, 100%   −HCl   MH⁺, m/z 275, 100 %
                      (40%)

and   and

MNH₄⁺, m/z 256, 10 %   [Adduct ions [M + NH₄]⁺ also formed]   MNH₄⁺, m/z 292, 10%

Figure 10. Fragmentation processes of CI mass spectrometry of 4-kCPA.

Figure 11. Thermal desorption of 4-kCPA.

Figure 12. Thermal desorption mass spectra of (a) peak A and (b) peak B, as presented in Fig. 11.

No thermal degradation   4-kCPA, m/z 274, ECP
                                TD peak 1(or 2)   TD peak 2(or 1)

|e⁻ (EI)

M⁺⁺ m/z 274, 1%

m/z 239, 2%   −HCl (2%)   −CH₂Cl   −(ClCH₂CH₂)N

m/z 189, 5%   −HCl (5%)   m/z 225, 100%   −ClCH₂CH₂N=CH₂   O=P m/z 134, 5%

Minor fragment ions

m/z 163, 8%   m/z 92, 25%

Figure 13. Thermal desorption fragmentation processes of 4-kCPA.

further investigated by thermal desorption mass spectrometry and nuclear magnetic resonance (NMR). Measurements by thermal desorption (EI) mass spectrometry with a fast temperature programme (0.15 A/s) and NMR confirmed the existence of keto-enol tautomerism at temperatures below 150 °C, as can be seen in Figs 11, 12, 13 and 14. Both peaks (A and B), as depicted in Fig. 11, show identical mass spectra (Fig. 12(a) and (b)).

Figure 13 shows pathways of degradation during thermal desorption mass spectrometry, together with percentages of formed products. NMR data of 4-kCPA solutions in $CDCl_3$ finally give confirmation of the existence of keto-enol tautomerism as an OH signal at 1.65 ppm.

Tautomerism might also be responsible for peak asymmetry. However, peak broadening can increase to such an extent[19] that absorption of one or both of the compounds of the tautomeric misture cannot be excluded. Implications of the keto-enol tautomerism of 4-kCPA with respect to *in vivo* metabolism of 4-kCPA are currently being investigated; indications for reversible CPA metabolism have now been found in which the tautomerism might play a key role.[22]
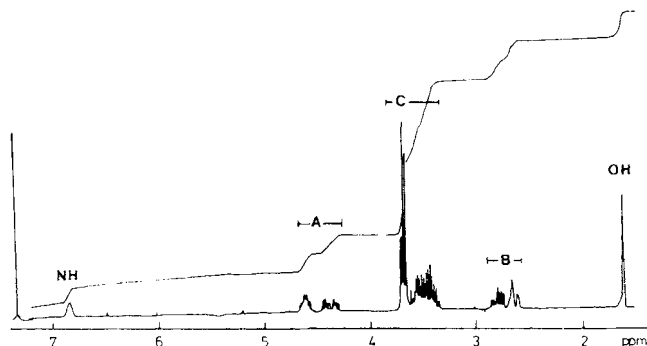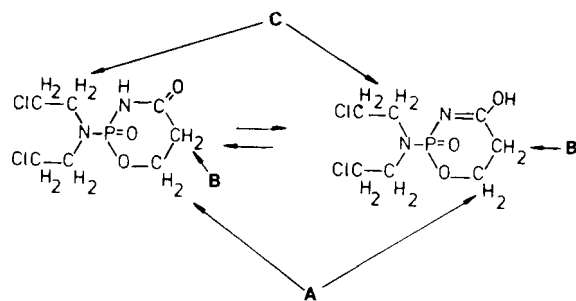

Figure 14. NMR spectrum of 4-kCPA in $CDCl_3$.

CONCLUSIONS

The experimental evidence presented here strongly supports the selectivity of capillary GC of underivatized CPA and 4-kCPA with respect to their 'on-column' products as well as unchanged compounds. A keto-enol tautomerism of 4-kCPA has been demonstrated at temperatures below 150 °C; the tautomerism is assumed to be a determining factor for the behaviour of 4-kCPA during capillary GC.

Acknowledgements

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