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Citation for published version (APA):

Bruijn, de, E. A., Leclercq, P. A., & Tjaden, U. R. (1986). The autoderivatization of 4-ketocyclophosphamide during capillary gas chromatography. *HRC & CC, Journal of High Resolution Chromatography and Chromatography Communications*, 9(2), 89-94. <https://doi.org/10.1002/jhrc.1240090207>

DOI:

[10.1002/jhrc.1240090207](https://doi.org/10.1002/jhrc.1240090207)

Document status and date:

Published: 01/01/1986

Document Version:

Publisher's PDF, also known as Version of Record (includes final page, issue and volume numbers)

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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The Autoderivatization of 4-Ketocyclophosphamide during Capillary Gas Chromatography

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Key Words:

Gas chromatography, GC
Capillary columns
Mass spectrometry
Autoderivatization
Cyclophosphamide
4-Ketocyclophosphamide

Summary

The selectivity of a capillary gas chromatographic assay for the anti-neoplastic and immunosuppressive agent cyclophosphamide (CPA) towards one of its naturally occurring metabolites, *i.e.* 4-ketocyclophosphamide (4-keto CPA), has been studied. Mass spectrometry studies showed that a cyclization product of 4-keto-CPA can form using the same chromatographic conditions as those under which CPA was determined. However, the amount produced is relatively small (less than 10%) compared with the percentage produced from CPA. Furthermore, both cyclization products, formed by loss of HCl and intra-alkylation of the parent compounds, can be separated well under suitable chromatographic conditions.

1 Introduction

Cyclophosphamide [N,N-bis(2-chlorethyl)-tetrahydro-2H-1,3,2-oxazaphosphorin-2-amine-2-oxide] (**Figure 1**) is an alkylating agent with a broad spectrum of activity against tumors in humans, and is probably the best overall agent for immunosuppression [1,2]. The biological properties of CPA are mediated by the compounds generated from the metabolism of this drug. CPA is activated by hepatic mixed function oxidases to a wide variety of active metabolites (**Figure 3**) [3]. Metabolites of main interest are 4-hydroxy CPA, 4-keto-CPA, CPE and PM. 4-Hydroxy-CPA is believed by most investigators of the mechanisms of action of CPA to be the primary extracellular mediator of antitumor activity [4]. PM is thought to be both an extra- and an intracellular acting mediator with antitumor activity [5], while recently, an important role for imino-CPA was described [6].

Based on a paper presented at the Sixth International Symposium on Capillary Chromatography.

Figure 1

Metabolic conversion of CPA.

Metabolism by hepatic microsomal enzymes depends on numerous factors including age, co-administration of drugs, heredity, chronobiological effects, and disease state (cancer patients) [8,9]. The pharmacokinetics and metabolism of CPA have been found to vary widely [10,11]; individual adjustment of dosage based on monitoring unchanged CPA and metabolites could provide an optimized therapeutic efficacy and minimized toxicity. This requires sensitive and selective assays for CPA and metabolites. To date, methods of analysis include radioactivity measurements of ^{14}C -labeled CPA [12], mass

Abbreviations used are: CPA: cyclophosphamide, 4-hydroxy CPA: 4-hydroxycyclophosphamide, 4-keto-CPA: 4-ketocyclophosphamide, CPE: carboxyphosphamide, PM: phosphoramidate mustard, imino CPA: imino cyclophosphamide, Rt: retention time.

Figure 2

Cyclization processes of CPA and 4-keto-CPA following injection on capillary gas chromatography.

spectrometry [13], gas chromatography-mass spectrometry [14,15], gas-liquid chromatography, both with and without derivatization [16-20], high performance liquid chromatography [21], and high performance liquid chromatography-field desorption mass spectrometry [22]. Since radiolabeling is clinically inconvenient and techniques using mass spectrometry hamper routine application in extensive pharmacological studies, gas chromatography with nitrogen phosphorus specific detection and high performance liquid chromatography seem attractive methods [16-21]. Furthermore, derivatization is time-consuming and then assays not requiring derivatization techniques seem to be preferable [17-21]. The derivatization of CPA during capillary gas chromatography is based on autoderivatization of CPA by intramolecular alkylation, depending on injection and column temperatures and injected amounts of CPA [14,18] (Figure 2). The selectivity of these assays with respect to other drugs regularly combined with CPA has been established. The gas chromatographic assay described by our group enables a good separation of 4-keto-CPA in presence of CPA itself, CPE, and other compounds of interest [20]. However, taking metabolites into account (Figure 1), products such as cyclization product of CPA on which the determination of CPA is based (Figure 2) could be formed and therefore interfere with the CPA assay.

Since 4-hydroxy-CPA and imino-CPA are assumed to be unstable and rapidly changed into 4-keto-CPA and/or CPE, a cyclization product of 4-keto-CPA only could result in a serious hindrance for the determination of CPA by measuring CPA's cyclization product. Besides possible interferences of 4-keto-CPA with the determination of CPA, another point needs further attention: the peak of 4-keto-CPA was quite different from neighboring peaks ([20], see also Figure 3).

The present investigations are the first in a series intended to gain more insight into the behavior of the CPA metabolite 4-keto-CPA during capillary gas chromatography and its

Figure 3

A chromatogram of 4-keto-CPA (2), the cyclization product of 4-keto-CPA (1), CPA (a), the cyclization product of CPA (3), and of compounds not related with CPA, (b, c, and d). ASCOT OV-275 capillary column was used (10 m $\times$ 0.31 mm i.d.). Temperature: programmed from 205 to 220°C at 2°C/min. Temperature program started 9 s after injection.

possible interference in the gas chromatographic determination of CPA itself.

2 Experimental

Chemicals. CPA and metabolites including 4-keto-CPA were kindly supplied by ASTA-Werke (Bielefeld, FRG). All solvents were of analytical grade and obtained from Baker (Deventer, The Netherlands) and Merck (Darmstadt, FRG).

Gas Chromatography. For routine measurements, two gas chromatographs were used: a Packard Becker Model 420 (Packard Becker, Delft, The Netherlands), equipped with a Model 18-789 nitrogen-phosphorus selective detector (Hewlett-Packard, Avondale, PA, USA), and a Hewlett-Packard Model 5713 A, equipped with a ^{63}Ni pulse-modified electron-capture detector. The samples were introduced onto the column by means of a solid-sample injection system [18-20].

Columns. Support-coated open-tubular (SCOT) OV-275 columns (7 m $\times$ 0.31 mm i.d.), made of Duran 50 glass, were used when routine analyses were carried out for clinical pharmacological studies. A more detailed description was

reported recently by us [20], including temperatures of injection, columns, and detection systems. For mass spectrometry a fused silica WCOT CP SIL-5CB column (25 m $\times$ 0.21 mm i.d.) was used.

Mass Spectrometry. A Finnigan Model 4000 quadrupole mass spectrometer (Finnigan, Sunnyvale, CA, USA) was used both in the electron impact (EI) and chemical ionization (CI) mode. The mass spectral data presented were acquired under the following conditions: ionizing electron energy, 70 eV; electron current, 0.30 mA; scan time, 1 s per scan; 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 reading. The WCOT fused silica column was directly inserted into the ion source. The column was operated isothermally. Samples were dissolved in ethyl acetate and injected onto the column at 250°C (falling needle) and 83°C (on-column).

3 Results and Discussion

A chromatogram of 4-keto-CPA and CPA using a capillary SCOT OV-275 column and ECD detection is presented in Figure 3. A good separation between the cyclization product of CPA and noncyclized 4-keto-CPA was obtained. A broad peak of 4-keto-CPA was observed compared with neighboring peaks.

3.1 GC/(EI) Mass Spectrometry

A total ion current chromatogram of 4-keto-CPA using split injection at 250°C onto a fused silica WCOT column coated with a nonpolar phase is shown in Figure 4. Two peaks can be distinguished with $R_t = 4.5$ min and $R_t = 7.0$ min. The peak area ratio peak 1: peak 2 under the described conditions, as determined by peak integration, was 0.08. Furthermore, asymmetric peaks were encountered in contrast to the peaks obtained with OV-275 capillary columns (Figure 4 versus Figure 3).

Figure 4

Total ion current chromatogram of 4-keto-CPA using solid injection at 250°C. Fused silica WCOT CP Sil-5 column (25 m $\times$ 0.21 mm i.d.) at 230°C.

Figure 5

A chromatogram of 4-keto CPA after on-column injection at 83°C. Temperature program from 83°C to 225°C at 1.5°/s. Otherwise similar conditions as described for Figure 4.

A chromatogram obtained after on-column injection at 83°C under otherwise similar conditions is shown in Figure 5. Two peaks with $R_t = 6.1$ min and $R_t = 8.3$ min were observed. The peak area ratio peak 1: peak 2 was less than 0.05. Much less asymmetry was observed for the second peak. These results suggest that degradative cyclization occurs in the injector as well as on the column. The latter effect, on-column reaction, could be distinguished by a slightly raised baseline between peaks 1 and 2 in Figure 5.

3.2 EI Mass Spectral Fragmentation

The EI mass spectrum of peak 1 is given in Figure 6a. Fragment ions at m/z 189, 161, and 117 were formed. These ions are degradation products of the M^+ ion at m/z 238, which was not observed due to rapid conversion to the molecular ion at m/z 189 by loss of CH_2Cl under the EI conditions described.

The EI mass spectrum of peak 2 is given in Figure 6b. Ions at m/z 225, 189, 163, 161, 134, 117, and 92 are formed. The fragmentation processes, together with their percentages, as obtained from EI mass spectrometry of both peaks 1 and 2, are presented in scheme 1.

3.3 CI Mass Spectral Fragmentation

The CI mass spectrum of peak 1 is given in Figure 7a. Apart from the protonated molecular ion MH^+ at m/z 239, the MNH_4^+ ion at m/z 256 is formed. The CI mass spectrum of

Figure 6

EI mass spectra of a) peak 1 and b) peak 2 as presented in Figure 4.

peak 2 is given in **Figure 7b**. MH^+ , m/z 275 and MNH_4^+ , m/z 292 were formed; in addition, the fragment ion $\text{MH}^+ - \text{HCl}$ at m/z 239 is found.

The pathways of degradation during both capillary GC and CI mass spectrometry together with their percentages are presented in scheme 2.

As schemes 1 and 2 show, peak 1 is related to a cyclization product of 4-keto-CPA, formed under the conditions described for capillary gas chromatography. This product, which is produced in relatively low abundance, could be easily separated from the unchanged 4-keto-CPA. The comparable cyclization product of CPA could be well

Figure 7

CI mass spectra of a) peak 1 and b) peak 2 as presented in Figure 4.

separated from the cyclization product of CPA [14,18-20] (Figure 2). In contrast to CPA cyclization, the product of 4-keto-CPA cyclization is formed in relatively small amounts. Nevertheless, it can be questioned whether the GC assay for CPA, based on cyclization of CPA by a loss of HCl under suitable chromatographic conditions, is hindered by incomplete separation of the 4-keto-CPA cyclization product. Since 4-keto-CPA has been demonstrated to be a naturally occurring metabolite of CPA using capillary GC without derivatization [20], CPA concentrations as measured by its cyclization product could be too high. On temperature programmed SCOT OV-275 columns, the cyclization product of 4-keto CPA is eluted just after the injection peak or is present in plasma matrix front peaks (Figure 3, [18], [20]). In our opinion, the relatively

low abundance of cyclization product of 4-keto-CPA formed during capillary GC is due to extra stabilization of 4-keto-CPA by keto-enol tautomerism, as depicted in schemes 1 and 2.

3.4 Thermal Desorption Mass Spectrometry

Measurements by thermal desorption (EI) mass spectrometry with a fast temperature program (0.15 A/s) and by NMR confirmed the existence of keto-enol tautomerism at temperatures below 150°C; these data will be published separately [24]. The tautomerism could be responsible for the peak asymmetry, using the columns described for mass spectrometry, or peak broadening, as observed when SCOT OV-275 columns were applied [20]. However, the

4-keto-CPA peak is twice as broad as the neighboring peaks presented in Figure 3: this indicates that absorption of one or both of the compounds of the tautomeric mixture together with the very existence of the tautomerism is responsible for the remarkable peak shape.

4 Conclusions

The data obtained from this study show that processes occur during injection capillary gas chromatography of 4-keto-CPA comparable with those on which the assay in blood plasma of CPA is based. A cyclization product of 4-keto-CPA, produced by loss of HCl, was demonstrated by capillary gas chromatography with EI and CI mass spectrometry. The amount of the cyclization product of 4-keto CPA produced appeared to be relatively low (less than 10%) as opposed to the amount of cyclised CPA produced under identical circumstances.

Probably, keto-enol tautomerism prevents cyclization of 4-keto-CPA in large amounts during capillary gas chromatography; the peak broadening and asymmetry can not be explained by this phenomenon alone.

Both capillary SCOT OV-275 and nonpolar WCOT columns enable sufficient separation of 4-keto-CPA, CPA, and their cyclization products.

Acknowledgment:

Supported in part by a grant of "Saal van Zwanenbergstichting".

References

- [1] H. Arnold and F. Boerseaux, *Angew. Chem.* **70** (1958) 539.
- [2] J. Holland and E. Frei, III, (Eds.) "Cancer Medicine", Lea & Febiger, Philadelphia (1973).
- [3] B. E. Domeyer and N. E. Sladek, *Cancer Res.* **40** (1980) 174.
- [4] N. Brock and H. J. Hohorst, *Z. Krebsforsch.* **88** (1977) 185.
- [5] P. J. Cox, B. J. Philips, and P. Thomas, *Cancer Res.* **35** (1975) 3755.
- [6] C. Fenselau, M.-N. Kan, and S. S. Rau, *Cancer Res.* **37** (1977) 3738.
- [7] R. F. Struck, M. C. Krik, M. H. Witt et al., *Biomed. Mass. Spectrom.* **2** (1975) 46.
- [8] F. Vessell, *Clin. Pharmacol. Ther.* **22** (1977) 659.
- [9] D. D. Breimer, *Clin. Pharmacokin.* **8** (1983) 371.
- [10] P. H. T. J. Slee, E. A. de Bruijn, O. M. J. Driessen, J. Hermans, and A. T. van Oosterom, *Anticancer Res.* **3** (1983) 269.
- [11] H. Mouridsen, O. Farmer, and L. Skovsted, *Acta Pharmacol. Toxicol.* **35** (1974) 98.
- [12] J. Cohen, J. Jao, and W. Jusko, *Br. J. Pharmacol.* **43** (1971) 677.
- [13] U. Bahr, H. R. Schulten, O. R. Hammes, and F. Aerts, *Clin. Chim. Acta* **103** (1980) 183.
- [14] C. Pantarotto, A. Martini, G. Belvedere, M. G. Donelli, A. Frigerid, *Cancer Treat. Rep.* **60** (1976) 493.
- [15] C. Lartigue-Mattei, J. L. Chabard, C. Touzet, H. Bargnoux, J. Petit, and J. A. Berger, *J. Chromatogr.* **310** (1984) 407.
- [16] F. D. Juma, H. J. Rogers, J. R. Trounce, and I. D. Bradbrook, *Cancer Chem. Pharmacol.* **1** (1978) 229.
- [17] T. Fachinetti, M. D. d'Incalci, G. Martinelli, L. Cantoni, G. Belvedere, and M. Salmona, *J. Chromatogr.* **145** (1978) 315.
- [17a] B. Whiting, S. H. K. Miller, and B. Caddy, *Brit. J. Clin. Pharmacol.* **6** (1978) 373.
- [18] N. Van den Bosch and D. de Vos, *J. Chromatogr.* **183** (1980) 49.
- [19] N. Van den Bosch, O. Driessen, A. Emonds et al., *Meth. and Exptl. Clin. Pharmacol.* **3** (6) (1981) 377.
- [20] E. A. de Bruijn, U. R. Tjaden, A. T. van Oosterom, P. Leeflang, and P. A. Leclercq, *J. Chromatogr.* **279** (1983) 603.
- [21] R. W. Hardy, C. Erlichman, and S. J. Soldin, *Therapeutic Drug Monitoring* **6** (3) (1984) 313.
- [22] M. Przybylski, *Arzneim.-Forsch/Drug Res.* **32** (II) (9) (1982) 995.
- [23] P. M. J. van der Berg and T. Cox, *Chromatographia* **5** (1972) 301.
- [24] E. A. de Bruijn, P. A. Leclercq, J. W. de Haan, L. J. M. van de Ven, and U. R. Tjaden (to be submitted to *Biom. Mass Spectrom.*).