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Introduction

Recent technological advances in capillary gas chromatography (CGC) during the last decade have generated bioassays which can be applied routinely in biochemical and pharmacological studies. CGC is widely applied now in different areas and the number of applications is still growing.[1] In bioanalysis the use of GC was limited to compounds which can be introduced into the gas phase easily with or without derivatization.[2,3] In cancer research the use of CGC is limited since compounds of interest, either endogenous or exogenous, frequently have large molecular weight and/or are polar and thermally labile. Nevertheless, impressive results have been obtained for the determination of several compounds of interest in cancer research such as polyamines[4] and antitumour agents like 5-fluorouracil (5-FUra).[5] Compounds of interest generally need derivatization; however, during the past five years we have demonstrated that some thermally labile antitumour agents can be monitored in body fluids of cancer patients routinely by CGC without derivatization at all.[6,7] Owing to the sensitive and selective determination the antitumour agents cyclophosphamide (CPA) and 5-FUra could be monitored without derivatization in tumour bearing rats at the low nanogram level while only 0.1 ml of blood plasma per time point was needed.[8,9] The use of short capillary columns with a polar phase (OV 275) enabled sufficient resolution in the determination of CPA and 5-FUra in the presence of several metabolites.[10]

The article provides data on the capacity of capillary columns used for bioassays of antitumour agents including sample pretreatment. Determination of CPA and 5-FUra in body fluids — two frequently administered antitumour agents — is described as well as pitfalls which can be encountered in routine analysis.

Sample Pretreatment

Antitumour agents generally are reactive compounds which react with targets responsible for cell proliferation and are subjected to inactivation as well as activation processes. Administration of CPA and 5-FUra results in production of several metabolites responsible for antitumour effects as well as undesired side-effects (Figs 1 and 2).

Data on the metabolism of CPA and 5-FUra reveal problems which can be encountered when unchanged drug and/or metabolites need to be determined by CGC. During sample pretreatment items as depicted in Table I need attention.

The stability of the compounds to be determined during an analysis is rarely considered when developing bioassays. In routine analysis of CPA and 5-FUra the stability of some of their metabolites during sample pretreatment

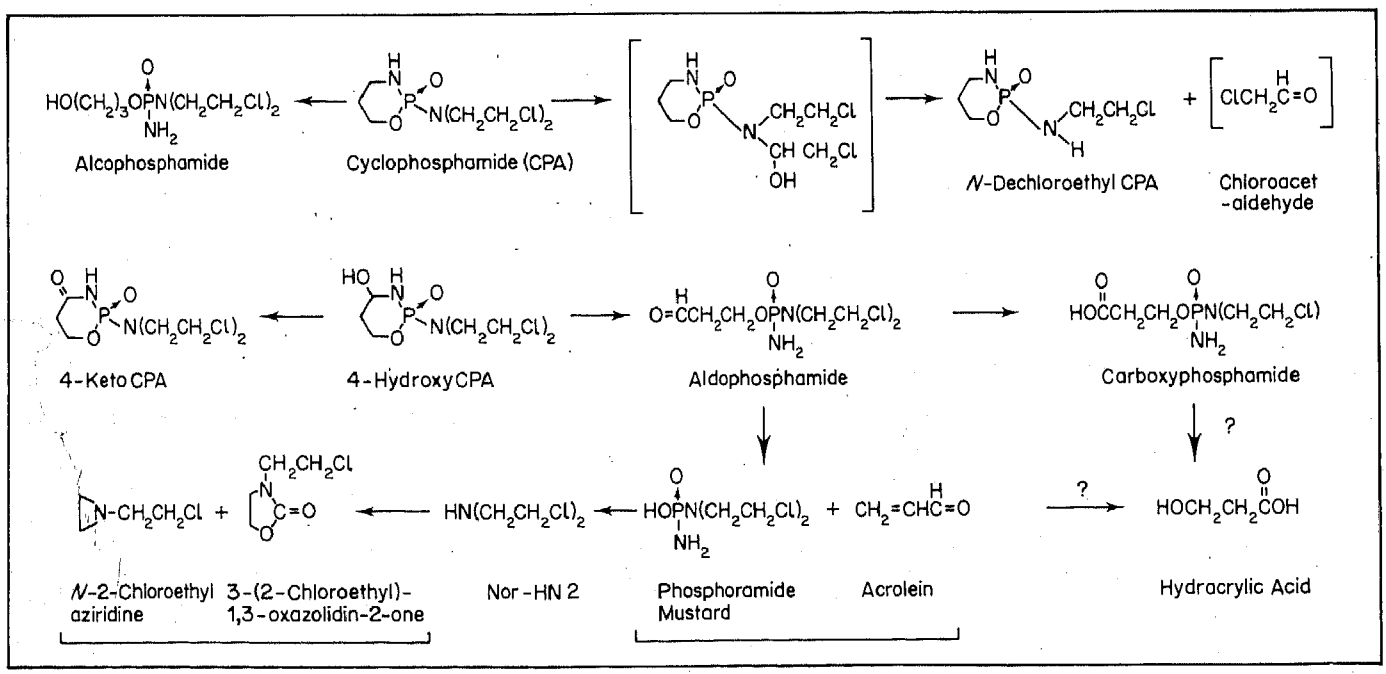

Figure 1. Structures of cyclophosphamide and metabolites. 4-OH cyclophosphamide and phosphoramide mustard are assumed to be mediators of CPA antitumour activity.

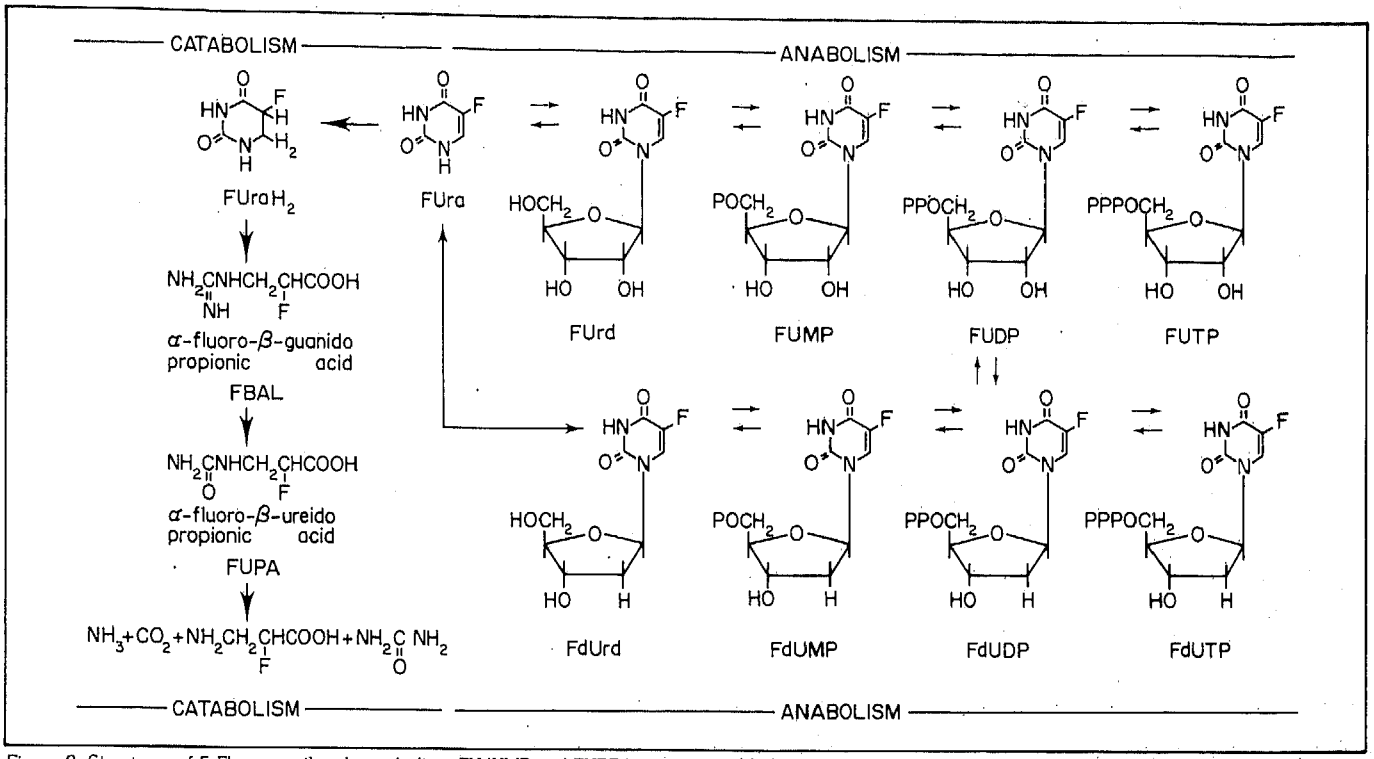

Figure 2. Structures of 5-Fluorouracil and metabolites. FUdUMP and FURTP are responsible for antitumour activity as they inhibit DNA and RNA synthesis respectively.

Stability
Selectivity/Specificity
Speed
Simplicity
Sensitivity
$

Table I. Important items in routine determination of thermally labile compounds of interest in cancer research.

is of importance. CPA itself is stable but instability of metabolites such as 4-OHCPA has been reported extensively while instability of 5-FUra in blood has recently been published.[11,12] Furthermore 5-FUraH_2 appeared to be highly unstable in blood and blood plasma owing to the presence of NADPH/H^+ dependent degradative enzyme systems.[13] This implies that isolation of the compounds of interest until introduced into a capillary chromatograph has to be carried out under special conditions from the moment that body fluids are collected from treated patients and laboratory animals.[11-13] Determination of 4-OHCPA, for instance, requires trapping techniques,[11] that of 5-FUra and 5-FUraH_2 should include collection of blood on ice since both compounds can be degraded then.[12,13] Isolation of 5-FUraH_2 should include handling procedures which prevent rapid degradation of this metabolite such as temperature and/or pH adjustments during liquid–liquid extraction.[13] Besides stability several other 'S's (Table I) can be taken into account: proper choice of adsorbtion materials for liquid–solid extraction and fluids for liquid–liquid and liquid–solid–liquid extraction increase selectivity of the bioassay and determine the speed of analysis, costs and possibilities for automation ($). Furthermore selective extraction methods can increase sensitivity when compounds originating from the biological matrix can be removed optimally before introduction into capillary columns.

Sample Introduction

Injection of the samples to be analysed by capillary chromatography is probably the most important part of the bioassay. A large number of reports dealing with injection in capillary chromatography are available now and development of new techniques and optimization is still going on. In the analysis of CPA and 5-FUra solid sample injection proved to be stable in routine analysis.[14,15,16] Only 4-ketoCPA might be subjected to intramolecular changes during injection[17,18] which still enables determination of this metabolite in body fluids by CGC. Solid sample injection of CPA, 5-FUra and metabolites did not conflict with the items in Table I: the method allows a selective, simple and sensitive determination of compounds of interest with a sample throughput enabling drug monitoring in two patients/day/technician/apparatus.

Elution on Capillary Columns

Open tubular CGC still offers the highest separating efficiency in comparison to HPLC since the diffusion coefficients are highest in the gaseous mobile phase. This, together with its compatibility with sensitive detection systems, such as mass spectrometry and nitrogen–phosphorus specific detection, makes it the method of choice, providing that the sample is volatile and thermally stable. Most compounds of interest in antitumour drug monitoring, however, are thermally labile and/or, involatile which favours application of HPLC or requires derivatization. Until recently such compounds have been analysed by HPLC methods which do not require derivatization. HPLC is generally used for routine determination of 5-FUra and the products of both the anabolic and catabolic pathway as depicted in Fig. 2. Then radiolabelling is required in order to obtain sufficient sensitivity.[19] In order to exploit the separation capabilities of CGC we used short open tubular capillary columns with a polar phase (SCOT OV 275, 7-10 m, 0.3-0.4 mm id) for the determination of CPA and 5-FUra without any derivatization at all.[7,10] A mixture of CPA, 5-FUra and several metabolites could be separated easily without destruction of

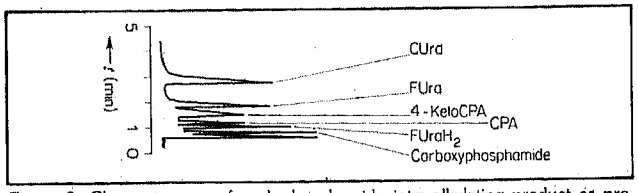

Figure 3. Chromatogram of cyclophosphamide intra-alkylating product as presented in Fig. 4, 4-ketocyclophosphamide, carboxyphosphamide, 5-Fluorouracil, 5,6 dihydro-5-fluorouracil (5-FUraH$_2$) and 5-Chlorouracil (5-CUra). A capillary support open tubular column coated with OV-275 was used (7 m, 0.31 mm id).

compounds during elution at temperatures above 200°C owing to short residence times in the short capillaries (Fig. 3).

Using this technique, simultaneous determination of CPA, 5-FUra and metabolites could be performed at the low nanogram level in blood plasma of patients and laboratory animals treated with the antitumour agents. Using CGC without derivatization in bioassaying compounds such as CPA and 5-FUra one has to study the behaviour during elution in order to exclude artefacts originating from the biological matrix including products of metabolism (Figs 1 and 2). For instance, CPA undergoes intra-alkylation during CGC resulting in a peak of unchanged CPA and one of the intra-alkylating products (Fig.4).

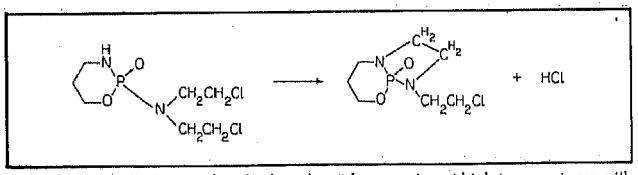

Figure 4. Intra-alkylation of cyclophosphamide occurring at high temperatures with loss of HCl.

The reproducibility of CPA intra-alkylation over a wide concentration range allows the use of peak heights of the intra-alkylating product for calculation of CPA concentrations in biological fluids. As far as selectivity is concerned, products of metabolism can also form intra-alkylating products. Therefore metabolites such as 4-ketoCPA might interfere with the determination of CPA as the assay is based on intra-alkylation of CPA. Indeed, data from mass spectrometry studies showed that an intra-alkylating product of 4-ketoCPA can be formed during injection and/or elution (Fig. 5).

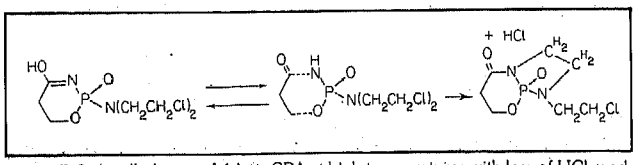

Figure 5. Intra-alkylation of 4-ketoCPA at high temperatures with loss of HCl moderated by keto–enol tautomerism.[17,18]

However, resolution of the intra-alkylating product of CPA is sufficient, especially when a temperature programme is used.[17,18] Studies on CGC of 4-ketoCPA gave indications for keto–enol tautomerism which is thought to be responsible for peak broadening and moderates intra-alkylation of 4-ketoCPA.[17,18]

For determination of unchanged 5-FUra by CGC, two points need further attention. First, metabolites and analogues of the anabolic pathway form 5-FUra upon introduction into CGC. For determination of 5-FUra in the blood plasma of patients treated with 5-FUra the problem is negligible since 5-FUra anabolite concentrations are below the limit of determination. However, when intracellular concentrations of anabolites have to be determined or blood concentrations of 5-FUra analogues such as 5′-deoxy-5-fluorouridine,[5] derivatization is required in order to prevent degradation of the compounds to be

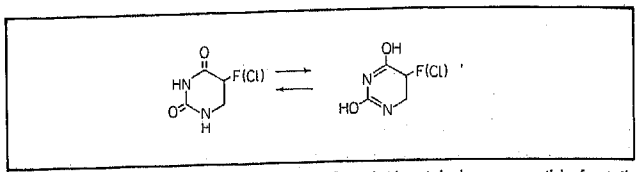

Figure 6. Tautomerism of 5-FUra and 5-CUra which might be responsible for tailing during CGC and capillary SFC.[21]

determined to 5-FUra during capillary elution at elevated temperatures. Second, some tailing of 5-FUra and analogues like 5-CUra — used as internal standard —can be observed possibly related to tautomerism according to the mechanism presented in Fig. 6. Tailing can be diminished by the application of a temperature programme.

Detection

The major advantage of CGC in the determination of compounds of interest in cancer research is its compatibility with highly sensitive detection systems. If necessary this can be exploited optimally by derivatization with halogens, e.g. pentafluorobenzylation.[5] When 5-FUra and CPA are assayed by CGC without derivatization it is essential to know about the structure of compounds to be detected. For instance, simultaneous introduction of CPA and 4-ketoCPA in CGC results in four peaks: unchanged CPA and 4-ketoCPA and two peaks related to products of intra-alkyation (Figs 4 and 5). Further analysis by mass spectrometry is required then in order to distinguish unchanged products from intra-alkylating products.

Fourier transform infrared spectrometry is capable of determining shift of keto–enol tautomerism of 4-ketoCPA as a function of residence time and temperature during capillary elution. With respect to the items described in Table I, detection systems used in CGC fulfil the demands of sensitivity and selectivity such as electron capture detection.

Future Developments

In practice polar capillary columns prove to be useful in the determination of thermally labile compounds such as CPA. The use of capillary columns in cancer research presumably will be extended in the next five years when SFC is exploited in this research area. SFC seems useful since it combines the advantage of HPLC in analysing involatile thermally labile compounds, i.e. antitumour antibiotics and DNA-adducts of Pt complexes, and its potential to be coupled with the sensitive detection systems of CGC.[20] Preliminary experiments showed that SFC enables determination of products both of the anabolic and catabolic pathway in one run without labelling techniques (Fig. 1).[21] Therefore future developments in capillary chromatography will be directed into the field of SFC.

Conclusion

Bioanalysis of compounds of interest in cancer research by CGC without derivatization is possible for thermally labile compounds, providing that short open tubular capillary columns are used and products formed during elution are known. Stability of compounds of interest during all steps of the bioassay has to be determined including sample pretreatment. The use of short capillaries enables selective and sensitive determination at low costs with sufficient sample throughput for routine pharmacological/oncological studies.

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Ubbo R. Tjaden studied analytical chemistry. After his Ph.D. in 1976 at the University of Amsterdam he got a position at the University of Leiden. Nowadays he is senior staff member of the Division of Analytical Chemistry of the Center for Bio-Pharmaceutical Sciences. The main research themes of the division include chromatographic techniques (HPLC, SFC) in combination with mass spectrometry and laser induced fluorescence in the field of bioanalysis.

Piet Leclercq is senior staff member at Eindhoven University of Technolgy and head of mass spectroscopy facilities; he is responsible for laboratory automations. He obtained his Ph.D. with a thesis entitled: Biomedical Applications of Mass Spectrometry in 1975 at the Eindhoven University of Technology.