Journal of Chromatography, 531 (1990) 235–294
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5406

Review

Chromatographic analysis of anticancer drugs

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(First received April 29th, 1990; revised manuscript received May 27th, 1990)

CONTENTS

LIST OF ABBREVIATIONS

Methods

AAS	Atomic absorption spectrometry
AUC	Area under the (plasma) concentration–time curve
CE	Capillary electrophoresis
CGC	Capillary gas chromatography
CI	Chemical ionization
ECD	Electron capture detection
ED	Electrochemical detection
EI	Electron impact
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-multiplied immunoassay technique
FAB	Fast atom bombardement
FIA	Flow injection analysis
FID	Flame ionization detection
FPIA	Fluorescence polarization immunoassay
FTIR	Fourier transform infrared
GC	Gas chromatography
HMDE	Hanging mercury drop electrode
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
LLE	Liquid–liquid extraction
MDR	Multi drug resistance
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NPD	Nitrogen–phosphorus detection
PDA	Photodiode-array detection
RIA	Radioimmunoassay
SCOT	Support coated open tubular
SFC	Supercritical fluid chromatography
SIM	Selected-ion monitoring
SPI	Solid-phase isolation
TLC	Thin-layer chromatography

UV	Ultraviolet
WCOT	Wall-coated open tubular

Compounds

AMSA	4′-[(9-Acridinyl)amino]methanesulfon-m-aniside, amsacrine
APD	1-Hydroxy-3-aminopropylidene-1,1-bisphosphonate
Ara-C	Cytosine arabinoside, cytarabine
Ara-CTP	Cytosine arabinoside triphosphate
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosurea, carmustine
Bleo	Bleomycin
CBDCA	*cis*-Diammine(1,1-cyclobutanedicarboxylato) platinum(II), JM-8, carboplatin
CCNU	Lomustine
CDDP	*cis*-Diamminedichloroplatinum(II), cisplatin
DHFR	Dihydrofolate reductase
CHIP	*cis*-Dichloro-*trans*-dihydroxo-*cis*-bis(isopropylamino)platinum(IV), JM-9, iproplatin
CLB	Chlorambucil
CY	Cyclophosphamide
DAMPA	2,4-Diamino-N-10-methylpteroic acid
Dauno	Daunorubicin
DDTC	Diethyldithiocarbamate
dFUrd	5′-Deoxy-5-fluorouridine, doxifluridine
DX	Doxorubicin
FdUMP	5′-Fluoro-2′-deoxy-5′-uridine monophosphate
FT	Ftorafur
FUra	5-Fluorouracil
$FUraH_2$	5,6-Dihydro-5-fluorouracil
HU	Hydroxyurea, hydroxycarbamide
IF	Ifosfamide
IFN	Interferon
IL-2	Interleukin-2
JM-8	Carboplatin
JM-9	Iproplatin
MeCCNU	Methyl-CCNU, semustine
MMC	Mitomycin C
6-MP	6-Mercaptopurine
MTX	Methotrexate
7-OHMTX	7-Hydroxymethotrexate
L-PAM	Melphalan
PCA	Perchloric acid
TCA	Trichloroacetic acid
TEPA	Triethylenephosphoramide

6-TG	6-Thioguanine
Thio-TEPA	1,1,1″-Phosphinothioylidine trisaziridine
VCR	Vincristine
VDS	Vindesine
VLB	Vinblastine
VM-26	Teniposide
VP-16	Etoposide

1 INTRODUCTION

Pharmacotherapy of cancer, including chemotherapy and immunotherapy, has during recent decades become increasingly important in the treatment of neoplastic diseases. It can be expected that this trend will continue for at least another 15–20 years because new treatment modalities need a decade for establishment. A real breakthrough is lacking at the moment, despite impressive progress in the insight into molecular and immunological backgrounds of cancer.

There are now *ca.* 50 drugs commercially available for the treatment of cancer. Cancer pharmacotherapy started with the introduction of nitrogen mustard in the late 1940s, and most recently interleukin-2 was introduced and suramin was discovered to have anticancer activity. It can be expected that new agents will be selected from a large number of substances either synthesized or isolated from fermentations, plant extracts from earth surface, the depths of the ocean or even from procedures performed in space. Before a drug is used clinically, analytical assays will have been developed for structure elucidation after isolation, for studies of stability control in pharmaceutical preparations and for monitoring behaviour in laboratory animals.

Until the 1980s, it was generally stated that the routine use of therapeutic drug monitoring in antineoplastic drug therapy was limited to one drug *i.e.* methotrexate. During the past ten years however, routine drug monitoring in oncology has been extended to other drugs. Especially in regional therapy, drug monitoring has enabled the fraction of unchanged drug reaching the systemic circulation, and the fraction extracted by the organ infiltrated with malignant tissue, to be determined. Furthermore, studies of anticancer drug plasma pharmacokinetics proved to be useful to optimize infusion time to regulate maximum plasma concentrations without affecting the area under the curve.

The limiting factors in the application of therapeutic drug monitoring in oncology have been clarified recently [1–4] and are listed in Table 1. In contrast to clinical oncology there are many other clinical situations in which drug monitoring has proved to be of value, such as anticonvulsant and antiarrhythmic therapy, antiepileptic therapy and lidocaine administration. The high success rate of extraoncological therapeutic drug monitoring is based on one clear-cut relationship, *i.e.* plasma concentration–(side-)effect. In considering all the factors listed in Table 1, it is clear that in pharmacological oncology they all hinder the definition

TABLE 1

FACTORS LIMITING ROUTINE APPLICATION OF THERAPEUTIC DRUG MONITORING IN ONCOLOGY

1	Heterogeneity of malignant neoplasms
2	Complexity of drug–tumour interaction
3	Application of combination pharmacotherapy inducing drug–drug interactions
4	Continuous treatment resulting in cumulative (side-)effects
5	*In vivo* instability of anticancer drugs and metabolites
6	Genetic instability of malignant cells during continuous therapy associated with changes in sensitivity to a certain drug (receptor down-regulation; selection of MDR cells)
7	Bio-activation at the extratumoural (hepatic) and intratumoural site, products not trapped in the systemic circulation
8	Large variation of blood supply of tumours from tumour type to tumour type but also within tumour (intratumoural and intertumoural)
9	Late onset of toxicity

of a concentration–effect relationship. In contrast and most intriguingly, clinical oncology includes almost all the factors that favour therapeutic drug monitoring (Table 2).

Large variations of anticancer agent pharmacokinetics between patients have been described frequently, and it might be expected that the wide variations observed in plasma levels would show an effect on the outcome of (side-)effects. Remarkably, in most cases it has not been clearly demonstrated that patient-to-patient variations in pharmacokinetics are associated with comparable variations observed in patients in the therapeutic efficacy of the drugs applied. Therapeutic drug monitoring in oncology aiming at identification of optimal efficacy is limited to a few drugs only, with the most striking examples being methotrexate and very recently suramin. Nonetheless, therapeutic drug monitoring has proved to be very useful in unravelling factors other than that associated with definition of therapeutic outcome, such as determination of hepatic extraction.

TABLE 2

FACTORS INDICATING THERAPEUTIC MONITORING IN CLINICAL ONCOLOGICAL SETTINGS

1.	Heterogenous population of patients differences between tumour types, sex-related diseases, wide range of ages, genetics influencing biotransformation of drugs, variation in compliance owing to disease status, diet, concomitant drugs
2	Assessment of systemic availability upon non-systemic administration
3	Low therapeutic index of agents used
4	Complexity of measurement of drug effects
5	Identification of therapeutic failure
6	Problems with organs involved in drug clearance (*e g.* the liver)
7	Drug–drug interactions at the pharmacokinetic level.

The present review concentrates on the chromatographic quantification of unchanged agents and some of their metabolites in blood plasma and other body fluids of interest in regional cancer therapy, such as peritoneal fluids (intraperitoneal instillations) and bladder content (intravesical instillation). Many of these drugs are applied in such doses that the resulting plasma levels are extremely low. As a consequence the frequent problems encountered in trace level analysis, *i.e.* losses due to instability, adsorption and similar aspects, demand careful sample handling and analysis. A separate section is devoted to some of the anticancer agents suspected of or known to show severe problems with respect to stability, adsorption and other aspects affecting the analysis.

2. ALKYLATING AGENTS

The cytotoxic mechanism of the alkylating agents is based on the transfer of alkyl groups to essential components of the cellular matrix. The alkylating agents are well known for their reactivity and instability. Decomposition during storage of the biological samples, during sample pretreatment and during chromatography needs to be taken in account. Recently an isomer of cyclophosphamide, *i.e.* ifosfamide, was added to the list of clinically established alkylating agents.

During the 1970s bioassays were generally based on radiolabelling, and included the monitoring of plasma radioactivity. The introduction of chromatographic techniques in the late 1970s and early 1980s enabled the selective determination of the unchanged drug, its metabolites and possible degradation products. In the second half of the 1980s high-performance liquid chromatography (HPLC) appeared also to be useful for routine monitoring with sufficient selectivity, and even proved to be capable of separating chiral oxazaphosphorines.

2.1. Chlorambucil

Chlorambucil (CLB), *p*-(di-2-chloroethyl)amino-γ-phenylbutyric acid (Fig. 1), is a bifunctional nitrogen mustard alkylating agent, which has been used in the treatment of malignant lymphomas, chronic lymphocytic leukaemia, ovarian

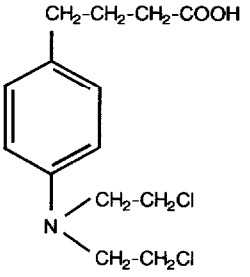


Fig. 1. Structure of chlorambucil

cancer and head and neck carcinomas The agent, along with several others in its therapeutic class, has been associated with the development of acquired drug resistance, which can lead to decreased drug uptake into target cells, increased DNA repair mechanisms and increased reaction of the alkylating agent with intracellular nucleophiles. Consequently its cytotoxic activity is decreased [5]. An extensive metabolism yields phenylacetic mustard, which compound shows a comparable cytotoxicity in *in vitro* studies with human cell lines [6–8].

The stability of CLB has been studied extensively [9–11]; it is found to be dependent on pH, temperature and chloride ion concentration. Binding to albumin also contributes to improved CLB stability in plasma. In general, HPLC and gas chromatographic (GC) methods have been described for CLB monitoring.

High-performance liquid chromatography

The first CLB assays based on HPLC with ultraviolet (UV) detection were generally capable of monitoring the parent compound and its metabolite phenylacetic acid [12,13]. In order to perform pharmacokinetic studies after oral administration of therapeutic doses of CLB, improved HPLC assays have been introduced during the past five years [14–17]

Adair *et al.* [14] solved some problems of earlier HPLC methods, such as liquid–liquid extraction (LLE), the need of gradient elution and relatively long retention times. The sample clean-up involved protein precipitation with concentrated perchloric acid (PCA), followed by solid-phase isolation (SPI). The analysis time was less than 5 min, and UV detection at 260 nm resulted in a determination limit close to that of gas chromatography–mass spectrometry (GC–MS) (10 ng/ml). Besides CLB, melphalan could also be determined by this system. The investigators demonstrated that plasma samples may be stored on ice (2°C) over a period of 6 h without hydrolysis of CLB. Furthermore, the methanolic extracts of the alkylating agents obtained after SPI proved to be stable under storage.

Workman *et al.* [15] developed an isocratic, ion-pair reversed-phase system suitable for the rapid analysis of CLB and several analogues. Tetrabutylammonium hydroxide was the ion-pairing agent and detection was performed with a twin-channel fixed-wavelength UV detector recording at 254 and 280 nm. Plasma samples were deproteinated by methanol, and CLB and analogues were interchanged as internal standards. Calibration curves were linear over the pharmacologically relevant range (0.14–100 μg/ml); the recovery at the μg/ml level was 76%. The limits of determination for CLB and analogues ranged from 0.03 to 0.13 μg/ml.

Bank *et al.* [17] demonstrated the potential of HPLC in a study of CLB pharmacokinetics and DNA binding in chronic lymphocytic leukaemia lymphocytes. In this study, isotope measurements were performed after HPLC discrimination between parent drug and metabolites.

Dulik *et al.* [18] recently reported on the characterization of glutathione conjugates of CLB. The formation of glutathione conjugates may play a role in the

development of acquired drug resistance, and therefore measurements of the conjugates are of importance The mono- and diglutathionyl conjugates of CLB were purified by reversed-phase HPLC and identified by fast atom bombardment (FAB) MS. The mono- and dihydroxy hydrolysis products of CLB were described by positive-ion thermospray liquid chromatography (LC)–MS.

Gas chromatography

GC–MS with selected-ion monitoring (SIM) offers the most selective and sensitive method for determination of CLB [19]. Derivatization is by alkylation or by silylation of the carboxylic group. Conversion of the nitrogen mustard group into a thiazane by reaction with sodium sulphide gave higher chemical stability. Both CLB and phenylacetic acid could be determined at the 2 ng/ml level.

2.2. Melphalan

Melphalan (L-PAM) (Fig. 2) is used in the treatment of multiple myeloma, malignant melanoma and ovarian cancer. L-PAM shows limited *in vitro* stability, which is affected by the pH and chloride ion concentration. Bile acids and albumin concentrations co-determine the hydrolysis rate [20–23]. Methods applied to L-PAM analysis include HPLC, GC and MS (GC–MS).

High-performance liquid chromatography

HPLC with UV detection is the most widely used analytical tool for L-PAM [24–29] Fluorescence detection with HPLC enables monitoring of L-PAM levels at the low ng/ml [25,26,28]. Several LC separations have been performed at elevated temperatures (40–60°C), which might adversely affect the chromatographic yield of L-PAM. Ehrsson *et al.* [28] exploited conversion of L-PAM into a stable N-acetylcysteine derivative by reaction with N-acetylcysteine prior to HPLC separation. Fluorometric detection (260/360 nm) allowed determination of L-PAM concentrations exceeding 5 ng/ml of plasma Deproteination was based on addi-

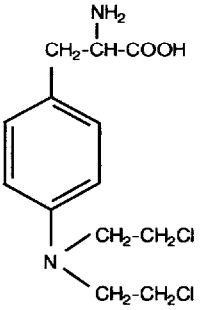


Fig. 2 Structure of melphalan

tion of trichloroacetic acid (TCA). Chromatography was performed on a C_{18} column with a citrate buffer containing octanesulphonic acid as mobile phase.

Bean *et al.* [30] recently reported on solvent–sample interactions in thermospray MS of antineoplastic nitrogen mustards, including L-PAM. The lower limit for detection of L-PAM using direct injection and summing the ion current in the m/z range between 120 and 870 was *ca.* 150 ng. Successful thermospray LC–MS was concluded to be possible using either high percentages of methanol in the mobile phase or increasing the organic content by post-column solvent modification. For chromatography, C_{18} columns with ODS guard columns were used; for direct injection analysis the guard column alone was used. Applications in biological studies of this technique must be awaited.

Gas chromatograhpy

L-PAM analysis by GC with SIM was already developed at the end of the 1970s by Pallante *et al.* [31]. Derivatization of L-PAM included trifluoroacetylation followed by esterification with diazomethane. The assay is still amongst the most sensitive: the limit of determination in plasma amounts to 2 ng/ml.

2.3. Busulfan

Busulfan (1,4-butanediol dimethanesulphonate, Fig. 3) is a bifunctional alkylating agent that is administered at high doses (16 mg/kg) in combination therapy prior to bone marrow transplantation . The high-dose busulfan regimens induce a dose-related antitumour effect in acute leukaemias and solid tumours. It is also used as a myelosuppressive agent before allogenic or syngeneic bone marrow engraftment in immunodeficiencies or inborn metabolism errors. Busulfan exhibits a different stability in comparison with the nitrogen mustards chlorambucil and melphalan: it is more stable in aqueous solution [32] but less stable in plasma [33].

Methods available for busulfan monitoring include radiolabelling, HPLC with UV detection and GC.

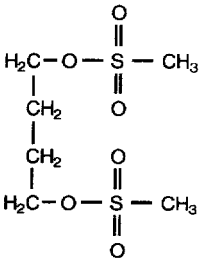


Fig 3. Structure of busulfan

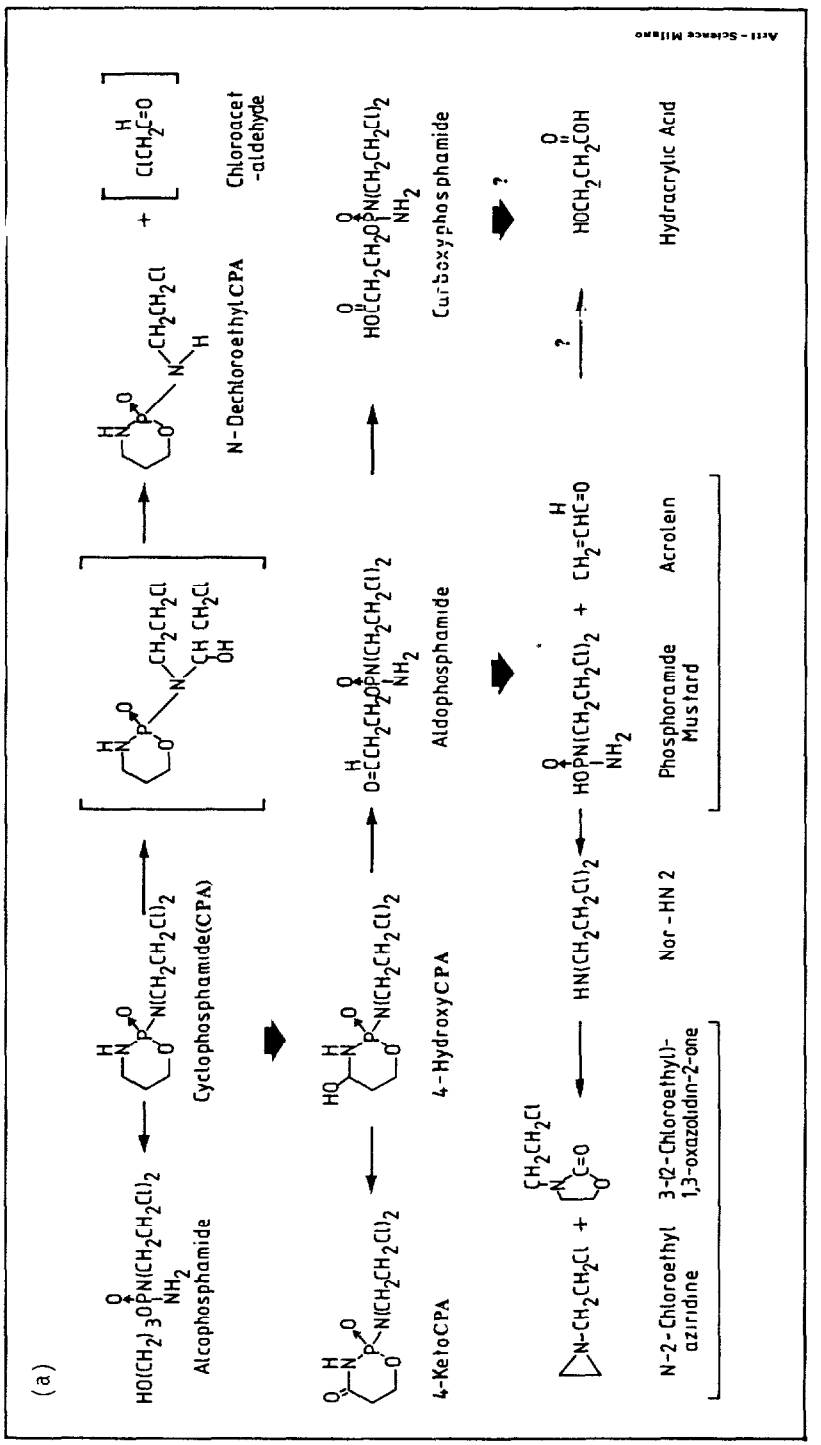


Fig 4 (a) Structures of cyclophosphamide and its metabolites, (b) ifosfamide is a structural analogue of cyclophosphamide and follows a similar pattern of metabolism

High-performance liquid chromatography

Henner *et al.* [34] used HPLC and derivatization of busulfan to 1,4-bis(diethyldithiocarbamoyl)butane; the method seems to be easier than GC but is less sensitive, so its application is limited.

Gas chromatography

In GC analysis [35,36] busulfan is converted into 1,4-diiodobutane by reaction with sodium iodide. Detection is performed by SIM and electron-capture detection (ECD). The latter is more sensitive but less selective, owing to interfering peaks in the chromatograms. Both methods use 1,5-pentanediol dimethylsulphonate as internal standard Chen *et al* [37] also reported on GC–ECD of busulfan, and it might be expected that data obtained with this assay correlate well with those obtained by the methods described by Hassan and Ehrsson [38].

Vassal *et al.* [39] adapted the method of Ehrsson and Hassan [35], but used a deuterated analogue, [$^2H_4$]busulfan as an ideal internal standard. Split injection was applied and chromatography was performed on a wall-coated open tubular (WCOT) capillary column. Mass spectra were recorded in the electron-impact (EI) mode. The method allows detection of 10 pg injected, corresponding to a limit of determination of 0.5 ng/ml.

2.4. Oxazaphosphorines

2.4.1. Cyclophosphamide

Cyclophosphamide (CY) (Fig. 4a) is still amongst the most frequently administered anticancer agents owing to its activity against a vast number of malignancies, its therapeutic index and its characteristics in the pharmaceutical phase (oral and intramuscular administration possible). Since CY is a prodrug that needs activation by hepatic metabolism, assays capable of determining CY as well as its metabolites are preferable. The metabolism of CY and ifosfamide is outlined in Fig. 4a. The initial step includes formation of the 4-hydroxyoxazaphosphorine and subsequent ring-opening to the "aldol" form. Spontaneous degradation (acrolein and phosphoramide mustard) and metabolism yields inactive compounds (keto and carboxy analogues) and structures thought to be specifically related to antitumour activity as well as to toxicity (acrolein)

CY exhibits optical isomerism owing to the presence of an asymmetrically substituted phosphorus atom. Since its introduction into clinical usage *ca.* 30 years ago, CY has been used as the racemic mixture. Since oxazaphosphorines require enzymatic activation, the possibility exists for stereoselective metabolism, and therefore it is not surprising that several studies were undertaken to reveal a distinct therapeutic advantage for one enantiomer relative to its optical antipode. In a limited patient population, CY recovered from the urine revealed a statistically significant stereoselectivity for the formation of 4-keto-CY when (+)-CY was administered compared with racemic CY [40]

High-performance liquid chromatography

Reversed-phase HPLC for the analysis of CY in the presence of its hydrolysis products was reported over a decade ago [41,42]. UV detection at 200 nm was applied, the technique was judged to lack the sensitivity required for drug monitoring [41]. More recently, however, determination of CY in serum of plasma by HPLC with detection at low UV wavelengths has been reported [43,44]. The relatively complex LLE of CY can be substituted by an SPI [44]. Ifosfamide can be applied as an internal standard [45].

Examples in which HPLC has been used to achieve enantiomeric separation can be found in the literature and have been the subject of several recent reviews and monographs [46,47] Enantiomeric separations are generally performed by utilizing stationary phases and chiral mobile phase additives or via precolumn derivatization with chiral reagents to form diastereomeric products. Determination of CY enantiomers in a biological sample is still an analytical challenge. An interesting approach using a two-step precolumn chiral derivatization has been described recently [48]. The analysis includes amidoalkylation of CY with anhydrous chloral containing 1% dimethylformamide followed by acetylation of the resulting secondary alcohol with a chiral carboxylic acid chloride, (+)-6-methoxy-α-methyl-2-naphthaleneacetyl chloride, to form a diastereomeric pair. Reversed-phase HPLC results in separation of derivatized (−)-CY and (+)-CY. The method is claimed to the applicable to clinical sessions including CY, and data on possible stereoselective *in vivo* metabolism of CY enantiomers are pending.

Gas chromatography

GC techniques for the determination of CY have been described extensively. Most reports deal with prior derivatization of the oxazaphosphorine. Trifluoroacetic anhydride or heptafluorobutyric anhydride are the most frequently used derivatization agents Underivatized CY partly decomposes during injection and elution, resulting in two peaks in the chromatogram: one of CY and one originating from an intramolecular alkylating process. Optimization of injection and column temperatures results in a quantitative conversion into the dehydrohalogenated compound resulting from intra-alkylation [49,50] Underivatized metabolites that may be subjected to the same procedures can be determined selectively using polar capillary columns, and for quantification several detection techniques can be used: flame-ionization detection (FID), nitrogen–phosphorus detection (NPD), ECD and SIM [49–51]. Both ECD and SIM enable monitoring of CY concentrations at the lower ng/ml level. Identification of the metabolites of CY and their monitoring is of great importance since CY itself is not active at all. The number of chromatographic methods available for the monitoring of metabolites to date is limited. Two important metabolites with cytolytic activity, phosphoramide mustard and nornitrogen mustard, can be determined by SIM, ECD or NPD [49,52].

Special attention has to be paid to the (incompleteness of) derivatization [52]. Although a proper choice of temperature programming enables the elution of some metabolites without derivatization [49], trifluoroacetylation is to be recommended. Methylation prior to trifluoroacetylation of phosphoramide mustard increases the derivatization yield in comparison with diazomethylation [52]. Artefacts are easily generated during injection and chromatography [51]. Carboxyphosphamide and 4-ketocyclophosphamide can be determined by capillary GC (CGC) without derivatization, but decomposition to nornitrogen mustard during prechromatographic procedure has to be prevented or co-determined.

Thin-layer chromatography

Separation and purification of CY and its metabolites from biological samples by thin-layer chromatography (TLC) has been reported as a technique prior to derivatization for determination of compounds of interest by MS. Hadidi and Idle [53] elegantly exploited TLC for the quantification of CY and its four principal metabolites in urine. The assay combines adsorption of drug-related material onto Amberlite XAD-2 and TLC with spot visualization using 4-(4-nitrobenzyl) pyridine, rapid photography and densitometry. An intra-assay coefficient of variation for each compound of less than 6% and limits of detection at the low μg/ml level for CY, phosphoramide mustard, bis(2-chloroethyl)amine, 4-ketocyclophosphamide and carboxyphosphamide allow determination of the full metabolic spectrum of CY in patients without administration of radioisotopically labelled drug at low costs [54].

2.4.2. Ifosfamide

Ifosfamide (IF), 3-(2-chloroethyl)-2-(chloroethylamine)tetrahydro-2*H*-1,3,2,-oxazaphosphorineoxide (Fig. 4b), differs chemically from CY in the shift of a 2-chloroethyl group from the nitrogen mustard to the heterocyclic nitrogen. The formation of alkylating metabolites follows a pattern similar to that of CY, implying a comparable demand of selective and sensitive assays for unchanged IF and its metabolites as described for CY. The usual methods for the determination of IF in biological fluids are HPLC [55–63] and GC [64]. Several assays described for CY can also be used for IF [60–64], and one assay developed for the determination of phosphoramide mustard can possibly be used for the related metabolite of IF [59].

High-performance liquid chromatography

HPLC for the analysis of IF with detection at short UV wavelengths has been introduced to perform pharmacokinetic studies [57]. An elegant improvement of HPLC in the analysis of IF has been described by Burton and James [63] A rapid and simple method for the extraction of IF from plasma completed the assay using SPI cartridges containing a cyclohexyl bonded-silica sorbent. Since the method also enables determination of CY, this oxazaphosphorine can be used as

internal standard Careful choice of the column packing material was required to ensure separation Satisfactory peak shapes and baseline separations were found with a special C_{18} column. The method has a good linear range up to levels generally monitored in clinical sessions, implying that dilution is not needed. Thus, together with a limit of determination of 1 μg/ml using 250 μl of plasma, and absence of interferences from co-administered drugs commonly used in clinical oncology, puts this assay amongst the preferred assays for IF.

Gas chromatography

GC generally requires derivatization with trifluoroacetic or heptafluorobutyric anhydride, which is time-consuming but increases sensitivity when ECD is used [55,56,58,60,61]. The assays based on GC frequently include a dilution step owing to deviations from linearity at higher IF concentrations GC determination of underivatized IF appeared to be possible at concentrations down to 1 ng/ml [64]. The reports describing GC determination of underivatized CY, can generally be adapted for determination of IF A low limit of determination is easily obtained by using ECD, which allows the routine monitoring of IF *in vivo* behaviour in small laboratory animals when only small volumes of biological fluids (50 μl) are available.

Blaschke *et al.* [65,66] applied GC, HPLC and ^{3}H-labelling, and ^{31}P nuclear magnetic resonance (NMR) in order to determine the importance of enantiomer metabolism in the pharmacotherapeutic efficacy of IF. It was concluded that in mice both enantiomers of IF are eliminated at almost equal rates, and that 4-ketoifosfamide is formed stereoselectively from *S*(−)-IF. In mice this was not related to difference in biological activity.

Nevertheless, determination of oxazaphosphorine enantiomers probably will be of interest in the 1990s, and therefore demands for stereoselective methods of determination will increase [67].

2.5. 1,1,1″-Phosphinothioylidine trisaziridine

The alkylating agent 1,1,1′-phosphinothioylidine trisaziridine (thio-TEPA, N,N′,N″-triethylenethiophosphoramide), which is also a well known mutagen, has been used in the clinic and in experimental genetics for many years. Thio-TEPA is metabolized to triethylenephosphoramide (TEPA, Fig. 5), which is also an alkylating agent. The main application for thio-TEPA is intravesical instillation in the (local) treatment of superficial bladder cancer; however, this role is decreasing owing to the efficacy of intravesical mitomycin C. Because of the high cytotoxicity of both thio-TEPA and TEPA, simultaneous determination of both compounds is of interest. At the moment various chromatographic techniques [68–72] are available.

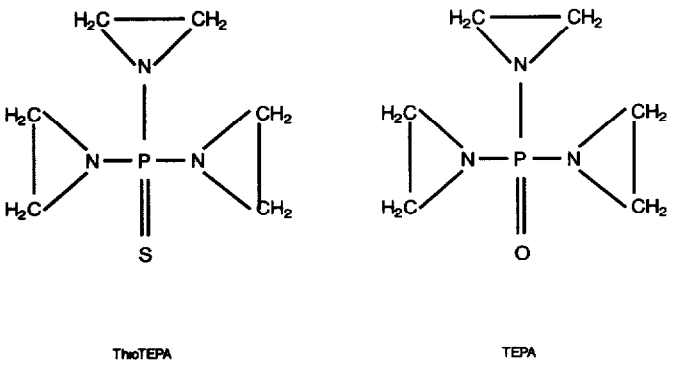


Fig 5 Structures of thio-TEPA and its metabolite TEPA

High-performance liquid chromatography

The assay developed by Sano *et al.* [68,69] might be selected for the analysis of thio-TEPA and TEPA. After derivatization, the products were separated on a C_{18} column. Derivatization was performed with sodium sulphide, taurine and *o*-phthalaldehyde to give fluorescent products. Sample pretreatment was performed by clean-up with an Extrelut 3 column. Fluorescence detection of the eluates (340/440 nm) enabled determination of thio-TEPA and TEPA in plasma as low as 10 and 20 ng/ml estimated, respectively. The recoveries of thio-TEPA and TEPA from plasma were 66.1 and 80.3%, respectively.

Gas chromatography

GC methods were reported in the second half of the past decade; the methods are capable of very sensitive determination of both thio-TEPA en TEPA owing to the use of NPD and proper choice of column materials.

However, GC requires a high operating temperature and this might be responsible for problems in the bioanalysis of the thermally unstable thio-TEPA and TEPA [71,72].

2.6. Nitrosureas

The nitrosureas 1,3-bis-(2-chloroethyl)-1-nitrosurea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea (CCNU) and 1-(2-chloroethyl-3-(4-*trans*-methylcyclohexyl)-1-nitrosurea (MeCCNU) (Fig. 6) are drugs used in the treatment of brain tumours, lymphomas (including Hodgkin's disease), myeloma, lung cancer and melanoma [73]. The high lipid solubility and low molecular mass permit these nitrosureas to cross the blood–brain barrier. The pharmacological effect is based on the decomposition of the agents to unstable intermediates capable of DNA alkylation.

The chemical stability is limited: the nitrosureas rapidly decompose at physio-

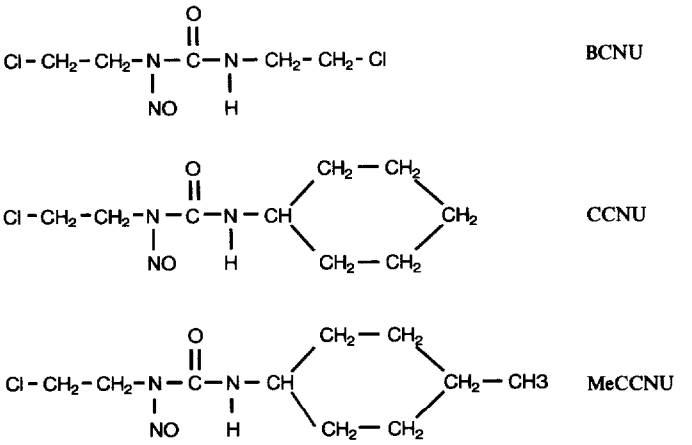


Fig. 6. Structures of the nitrosureas BCNU, CCNU and MeCCNU.

logical pH. Adjustment of pH to 2–4 before isolating BCNU by solvent extraction is recommended, since the stability of nitrosureas was found to be maximal at this pH [74].

Several techniques have been utilized for the analysis of nitrosureas in biological fluids, including HPLC and GC.

High-performance liquid chromatography

BCNU and MeCCNU were determined by reversed-phase HPLC applying UV detection (230–237 nm) [75], and BCNU could also be measured with electrochemical detection (ED) [76]. Concentrations down to 30 ng/ml could be determined by HPLC.

Gas chromatography

GC seems the most interesting analytical tool for the determination of nitrosureas, with its high efficiency and low limits of determination achievable (1 ng/ml) [77,78]. GC with SIM for the determination of BCNU, CCNU and MeCCNU in plasma required a time-consuming sample pretreatment. This included TLC prior to derivatization in order to separate denitrosated metabolites. Subsequently, conversion of the nitrosureas into 1,3-diacyl-1,3-diethylureas by reaction with trifluoroacetic anhydride was performed.

El-Yazigi and Sawchuk [79] used GC with NPD for simultaneous determination of BCNU and CCNU. Plasma was extracted with benzene with a yield of at least 87%. Chromatography was performed on a 25 m × 0.33 mm I.D. fused-silica SE-30 capillary column. To ensure complete separation, a temperature programme was needed. The limits of determination were comparable with those reported for GC–MS.

3 PLATINUM COMPOUNDS

The discovery of the cytotoxic activity of *cis*-diamminedichloroplatinum(II) (CDDP) (Fig. 7) turned out to be a major breakthrough in anticancer chemotherapy [80] Already known as Peyrone's chloride in 1845, the square planar platinum complex proved to be the most active platinum complex out of a series of complexes tested in preclinical sessions. With its broad spectrum of antitumour activity, CDDP is one of the most important weapons in the armoury of clinical oncology developed in the second half of this century. CDDP increased cure rates dramatically in testicular cancer and greatly enhanced the therapy of ovarium cancer, bladder cancer and small cell lung cancer [81,82].

Dose-limiting nephrotoxicity, as well as nausea and vomiting, neurotoxicity and ototoxicity, are amongst the most important observed side-effects. Synthesis of CDDP complexes with a better therapeutic index and enlarged applicability was a second important step in the platinum story. Aqua[1,1-bis(aminomethyl) cyclohexane]sulphatoplatinum(II) (spiroplatin, TNO-6), ethylenediamminemalonatoplatinum(II) (JM-40), diammine(1,1-cyclobutanedicarboxylato)platinum (II), CBDCA, carboplatinum are amongst platinum analogues with enhanced therapeutic index that have been tested clinically. This increased the demand for bioanalytical methods to perform studies on the pharmaceutical and pharmacokinetic phase behaviour of CDDP and related compounds.

High-performance liquid chromatography

CDDP determinations by HPLC were described in the late 1970s and early 1980s [83–85]. Diethyldithiocarbamate (DDTC) proved to be a useful reagent for

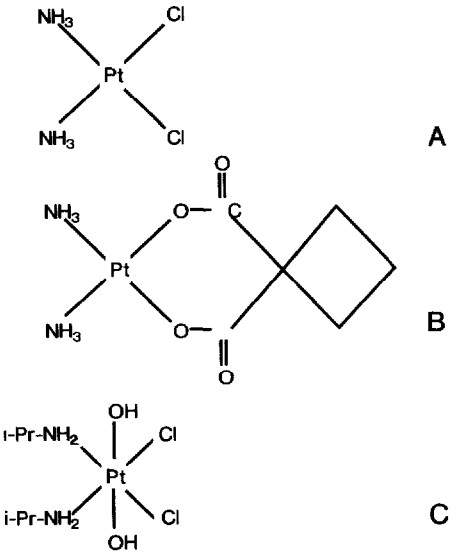


Fig 7 Structures of cisplatinum (A), carboplatin (B) and iproplatin (C).

precolumn derivatization in the non-specific determination of CDDP. DDTC forms a product with CDDP (DDTC/Pt), which can be separated on cyano or C_{18} bonded silica columns. The detection limit reported is 13 n*M* in plasma ultrafiltrate [84]. Addition of nickel chloride as an internal standard improved the accuracy [83–85], and the method provides an attractive alternative to graphite furnace atomic absorption spectrophotometry (AAS) of ultrafiltrate platinum.

Pharmacokinetic studies, however, generally require assays that quantify the parent drug, possible degradation products and metabolites. For this demand HPLC without precolumn derivatization was preferred. Application of strong anion-exchange columns resulted in broad peaks, which were detected by off-line AAS. Further work by Riley *et al.* [86–88] resulted in improved peak shape; a solvent-generated ion-exchange system was used with the hexadecyltrimethylammonium ion as the counter-ion. Application of column switching and off-line AAS column switching in this assay resulted in a detection limit of 6.7 μM for CDDP in urine. Daley-Yates and McBrian [89] studied renal clearance of CDDP and metabolites *in vivo* by off-line detection of [^{191}Pt]CDDP.

The next step in the specific determination of CDDP and metabolites was the development of on-line procedures. Direct UV detection of CDDP is possible at 301 or at 203 nm with molar absorptivities of 130 and 5200 mol l^{-1} cm^{-1}, respectively. A detection limit of 0.5 μM in plasma and urine was reported by Kizu *et al.* [90] using direct UV detection of CDDP at 203 nm Interesting contributions in the specific determination of CDDP were the more specific UV detection of CDDP following post-column derivatization with sodium bisulphate [91] and by quenching of biacetyl phosphorescence as reported by Gooyer *et al.* [92]. However, HPLC with UV detection is still the method of choice in routine monitoring of CDDP because of its straightforward approach.

On-line ED, however, proved to be superior to UV with respect to sensitivity and selectivity. CDDP could be assayed by both reductive and oxidative ED: mercury-drop electrodes in the reductive mode are to be preferred since higher sensitivity can be obtained in comparison to a solid Au/Hg electrode. Moreover, Au/Hg electrodes were found to be more likely to become contaminated [93–96]. Bannister *et al.* [93] reported a detection limit of 0.005 μM at a hanging mercury-drop electrode (HMDE) by amperometric detection at 0 V *versus* an Ag/AgCl reference electrode at a temperature of 60°C. At room temperature the limits of detection at an Au/Hg electrode and at an HMDE were 0.3 μM and 0.2 μM, respectively [95,96]. Selectivity could be further increased by combination of an Au/Hg electrode with oxidative detection at a glassy carbon electrode in a dual-electrode cell [96]. Oxidative amperometric detection appeared to be catalysed by the presence of chloride ions in the eluent [97]. O'Dea *et al.* [98] recently optimized assays as developed by Parsons and Leroy [94] with respect to the mobile phase employed, and compared the use of mercury and glassy carbon electrodes as ED systems. Separation was achieved using ion-pair chromatography with octanesulphonic acid as ion-pairing agent. Limits of detection were *ca.* 5 μg/ml

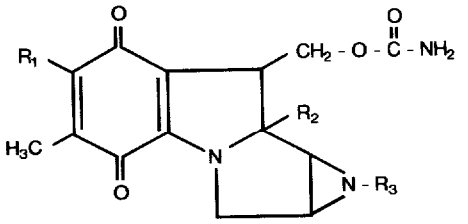
(HMDE) and 370 ng/ml (single glassy carbon). Operation of a dual glassy carbon electrode in a parallel orientation even reduced the detection limit to 25 ng/ml CDDP. Unfortunately, electrode fouling is a major disadvantage in the use of glassy carbon necessitating the polishing of the electrode once a day.

The introduction of a new generation of platinum compounds, including spiroplatin (TNO-6), iproplatin, and carboplatin, led to a new wave of assay development. Some of the assays mentioned before proved to be capable of determination of iproplatin and carboplatin. An important contribution to the analysis of platinum analogues was the work of Van der Vijgh and co-workers [99–102]. ED in the differential pulse mode was used for detection of antitumour Pt(II) complexes, enabling detection at a minimum level of 0.1 μM For aqua[1,1-bis(aminomethyl)cyclohexane]sulphatoplatinum(II) (Spiroplatin, TNO-6) and its hydrolysis and oligomerization products, Elferink *et al* [101] developed a reversed-phase HPLC analysis with HMDE detection at -540 mV *versus* Ag/AgCl. The limit of detection was 0.05 μM and the analysis also enabled detection of diaqua, monoaquamonosulphate, monoaquamonochloro, dichloro and hydroxy-bridged dimer complexes of [1,1-bis(aminomethyl)cyclohexane]sulphatoplatinum(II) in mutual equilibrium. On-line HPLC differential pulse polarographic detection was used for the determination of carboplatin (reduction at -1.77 V *versus* Ag/AgCl). With an injection volume of 50 μl, the detection limits for carboplatin in plasma ultrafiltrate and in urine were 0.1 and 1 μM, respectively [102]. In comparison with CDDP, the analogues are more prone to UV detection owing to their organic ligands enabling a more sufficient separation from biological components that also have a maximum UV absorption at 200 nm. HPLC–UV methods were developed for monitoring carboplatin in plasma. With silica-bonded diol columns, detection limits at the low μM level could be obtained [103,104]. HPLC–UV was also successful in the selective and convenient determination of another platinum analogue, ethylenediamminemalonatoplatinum(II) (JM-40), in plasma ultrafiltrate and urine A linear dynamic range of at least three decades (3–3000 μM) was achieved with detection at 214 nm; the limit of determination was 1 μM [105]

4 ANTITUMOUR ANTIBIOTICS

4.1. Mitomycins

The mitomycins are a group of potent antitumour antibiotics (Fig. 8), which are activated *in vivo*. After activation, cytotoxic activity can be observed owing to covalent binding and cross-linking of DNA, DNA alkylation and enzyme inhibition. Mitomycin C (MMC) has proven clinical efficacy in the treatment of several malignancies and is now amongst the drugs of first choice in the intravesical instillation for the treatment of superficial bladder cancer. New findings in the pharmacokinetics, metabolic, and drug-resistance aspects of MMC have recently



	R_1	R_2	R_3
Mitomycin A	H_3CO	OCH_3	H
Mitomycin B	H_3CO	OH	CH_3
Mitomycin C	H_2N	OCH_3	H
Porfiromycin	H_2N	OCH_3	CH_3

Fig. 8 Structures of the mitomycins

been highlighted by Dorr [106]. Hoey *et al.* [107] described reductive activation of MMC, whereas Tomasz and co-workers [108,109] provided direct proof for bifunctional alkylation of DNA by MMC. In this study several tools were used: HPLC, Fourier transform infrared (FTIR), UV spectrophotometry, circular dichroism spectroscopy and FAB-MS

Several analytical procedures have been described for drug monitoring: microbiological assays [110–116], immunological assays [117,118], polarography [119] and assays based on HPLC [120–133] Interesting recent developments in MMC monitoring were the introduction of a completely automated system [134] and the application of phase system switching as on-line sample pretreatment in the bioanalysis of MMC using supercritical fluid chromatography (SFC) [135].

High-performance liquid chromatography

Reversed-phase HPLC is prefered in the bioanalysis of MMC. The pH of the mobile phase should be between 4 and 8 in order to prevent degradation [136]. Gradient elution showed limited advantage over isocratic systems [124], but might be useful in the analysis of mitomycin, mitosane and mitosene derivatives [137,138]. All reversed-phase systems are largely similar, in that either C_{18} or C_8 alkyl bonded silica stationary phases are used. Most HPLC assays utilize UV detection at 360–365 nm. Limits of determination with UV detection are at the lower ng/ml level owing to the high molar absorptivity (21380 mol l^{-1} cm^{-1}).

Furthermore, detection at the wavelength mentioned offered sufficient selectivity with respect to biological matrix substances As an alternative to UV detection, ED can be applied [123,132,136], which exploits the favourable reduction potential of the 7-aminoquinoid chromophore at $E = -368$ mV. Tjaden *et al* [123] compared ED (with an HMDE) and UV detection and found a limit of detection at 250 pg of MMC (*versus* 150 pg for UV) which resulted in a somewhat higher limit of determination than with UV detection.

A survey of MMC studies [120–134,136] reveals that HPLC–UV at 360–365 nm is the method of choice. During the past five years attention has been paid to the stability of MMC and its behaviour, either in the pharmaceutical or in the pharmacokinetic phase (*e.g.* intravesical instillation therapy) [138–145]. Systemic concentrations were found to be extremely low following intravesical instillations, resulting in a demand for lowering of detection limits [145].

For studies of MMC metabolism thermospray LC–MS and FAB–MS offer interesting possibilities but applications in clinical sessions have so far been limited [108,120,137,146].

Stability

Stability needs special attention in routine monitoring of MMC. In order to avoid misinterpretations in MMC bioanalysis, samples must be handled with care. Biological fluids can be stored below −20°C for at least one month without any loss of MMC, provided that the pH range is kept to 5–7 [119,124,145].

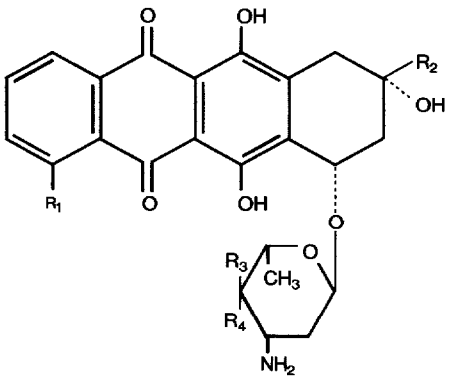
MMC proved to be stable under normal *in vitro* assay conditions such as a clonogenic assay [140,147]. For sample clean-up, several procedures have been introduced: LLE [122] and SPI [123], the latter instilled either off-line or on-line [131,135], on-line dialysis [134] and a protein precipitation step with methanol, which is suitable only for the serum of small laboratory animals [148] In order to prevent MMC degradation, the evaporation temperature of the vehiculum should not exceed 40°C [122].

4.2. Anthraquinone glycosides

4.2.1. Doxorubicin (adriamycin)

The anthracycline antitumour antibiotics doxorubicin (adriamycin, Adriblastina®) and daunorubicin (Cerubidine®), originating from *Streptomyces peuciticus var. caesius* and *S. peuceticus*, respectively, as well as related compounds, are the most frequently used anticancer drugs in current cancer chemotherapy. This is reflected in the extensive number of papers selected for this review [149–257]. The therapeutic index of the first anthracyclines was mainly determined by drug-induced cardiotoxicity, caused by effects on cardiac mitochondria [149]. The toxicity prompted a search for new, natural or semi-synthetic derivatives with possibly improved therapeutic index [150,151] (Fig. 9)

In general, anthracyclines are extensively metabolized, but only the 14-hydroxy derivatives are of clinical importance. These metabolites have the same cytotoxic activity as the intact drugs. The (clinical) importance of the anthracyclines has led to many reviews [149,152,153] dealing with fundamental and clinical aspects. From the clinical point of view, it was already evident in the early 1970s that both intact drugs and their reduced metabolites should be monitored, since a high therapeutic response has been suggested to be associated with a high degree of formation of reduced metabolites. Therefore, in this review attention



	R1	R2	R3	R4
doxorubicin	OCH_3	$COCH_2OH$	H	OH
4'-deoxydoxorubicin	OCH_3	$COCH_2OH$	H	H
4'-epidoxorubicin	OCH_3	$COCH_2OH$	OH	H
daunorubicin	OCH_3	$COCH_3$	H	OH
idarubicin	H	$COCH_3$	H	OH
doxorubicinol	OCH_3	$CHOHCH_2OH$	H	OH
carminomycin	OH	$COCH_3$	H	OH

Fig. 9 Structures of the anthraquinone glycosides

has been focused on methods that include co-determination of drug metabolites present in plasma and (of significant importance) also in tumour cells [154].

Aspects that are of great importance for sample pretreatment, such as adsorption and stability, will be discussed separately. An interesting alternative separation technique for anthracyclines is isotachophoresis, as described by Akedo and Shinkai [155]. Doxorubicin concentrations could be determined at a level of 5 ng/ml after extraction with *n*-butanol.

Adsorption and stability

Tomlinson and Malspeis [156] reported the adsorption onto container walls of doxorubicin and analogues, which can be decreased by the use of co-solvents and the selective use of container materials (*e.g.* polypropylene and silanized glass). Desipramine is capable of preventing adsorption, and therefore it was frequently added to HPLC mobile phases [157,189] and extraction media [159,253]. Schutz *et al.* [175] reported the adsorption of daunorubicin onto silanized glassware, whereas Andrews *et al.* [160] demonstrated that silylation of glassware had no effect on extraction efficiencies; Benvenuto *et al.* [176] recommended poly(vinyl chloride) containers after a comparative study with glass containers. In conclusion, the adsorption of anthracyclines onto glassware and laboratory materials should always be taken into account, especially when low concentrations (below $2 \cdot 10^{-7}$ *M*) have to be determined.

In the development of an analytical method for anthracyclines care should be

taken to prevent degradation during sample pretreatment and analysis. Several precautions concerning the handling of the samples have been detailed [161]. In clinical drug monitoring generally, whole blood is collected in heparinized tubes on the assumption that this will not interfere with the bioanalyses. However, interaction cannot be excluded completely [162]. Hydrolysis by blood esterases in the collected blood samples must be avoided, and immediate separation of the plasma fraction from the blood cells is a necessity since cells are also prone to anthracycline accumulation [163,177]. Cytoplasmic aldo-keto reductase activity subsequently results in significant anthracycline clearance [161,164,178], whereas in cell-free plasma this activity was not observed [154]. Anthraquinone glycosides are stable in plasma for longer periods when stored at −80°C [161]. Data on the stability of doxorubicin in plasma samples stored at −20°C are inconclusive [165,178], and prompted further investigation; at 4°C the compound of interest remained stable for 24 h [178]. Degradation of carminomycin/carminomycinol [166], 4′-epidoxorubicin [167] and menogarol [168] was not observed at all when these substances were stored at −20°C for several weeks.

Determination of anthracyclines in plasma has frequently been associated with extraction of the unchanged drug and its metabolites with a non-polar organic solvent [157,160,162,164–166,226,253]. It is assumed that the compounds remain stable during this extraction. Subsequent re-extraction of the glycosides into an acidic aqueous phase and storage in the dark for a short period was proven to be free of degradation [161,169].

Tissue samples containing daunorubicin can be preserved at −20°C or −4°C for prolonged periods [179]; urine samples can be stored intact at −20°C [167] but need acidification when stored at 4°C [180].

The stability of anthracyclines in solution and in pharmaceutical preparations has been studied extensively. The stability of doxorubicin is limited after dissolution, and degradation is pH-dependent. Hydrolysis is dependent on pH, temperature and ionic strength [169,171], whereas optimum drug stability was found in the acid region at pH 3–4 [181,182].

The anthracyclines have been demonstrated to be photolabile and therefore samples should be protected from light [183–185]. Besides pH, the nature of the solvent also determines the photolysis rate. For the preparation of stock solutions several solvents have been tested Doxorubicin can be stored in frozen (−20°C) sterile water for injection (U.S.P.) for one month [186], but storage in water cannot be recommended [166,167,172,173]. The degradation rate also appears to depend on the initial drug concentration [181] and the presence of metal ions [187]. However, differences between the stability behaviour of several anthracyclines have been reported: carminomycin was found to be stable in methanol for one day, in contrast to doxorubicin [170,173]. Furthermore, glycosides are more subject to degradation than aglycones [162,172].

Commercially doxorubicin and daunorubicin (Adriblastina and Cerubidine, respectively), delivered as hydrochloride salts in a lyophilized state containing five

parts by weight of lactose (Adriblastina) or D-mannitol (Cerubidine) as excipient are assumed to be stable for years if kept dry and in well closed containers at room temperature in the dark [174,187].

In conclusion, standard solutions of anthracyclines should be stored in the refrigerator or frozen, protected from light. Stock solutions ought to contain 10^{-1} to 10^{-2} *M* phosphoric acid [161]

High-performance liquid chromatography

HPLC is generally accepted for anthracycline pharmacokinetic analysis [188] since it permits sufficient selectivity and sensitivity for the parent compounds and their metabolites. Both normal-phase and reversed-phase HPLC have been reported; reversed-phase HPLC seems to be preferred owing to higher reproducibility, better peak symmetry and better resolution of the aglycones.

In the normal-phase mode cyano-aminoalkyl bonded silica and microporous silica were used as the stationary phase, and various chloroform–methanol mixtures were preferred as mobile phases [189–195], generally with acetic acid [189,192–195]. Addition of magnesium chloride to the mobile phase reduced polar interactions with the stationary phase and consequently the retention of doxorubicin and doxorubicinol [189,192,193], owing to complex formation between the Mg^{2+} ion and the anthracycline [195].

Gradient elution in normal-phase HPLC of anthracyclines was introduced in the 1970s. Thereafter is was readily established that mobile phases consisting of chloroform–methanol containing acetic acid [190,191] and acetic acid–water–triethylamine [198] produced acceptable peak shapes and sufficient selectivity in short isocratic runs Additionally, the mobile phases tried first [192,193,195,199] excluded gradient elution from methods amongst first choice.

Normal-phase HPLC is unable to separate aglycone fractions and appeared to be the main reason why 7-deoxyaglycone identification was lacking in pharmacokinetic data obtained with normal-phase HPLC [191,193].

SPI of anthraquinone glycosides from biological fluids proved to be an attractive alternative to LLE. Furthermore, reversed-phase HPLC proved to be successful in the identification of many aglycone metabolites [200–203].

On-column degradation has been discussed and a pH value of 4 was recommended [204], but degradation at pH 2 is thought to be less than 1% [171]. Methanol was used as modifier in most cases: with water [156,183,205], tetrabutylammonium phosphate or ammonium acetate or phosphate buffer [158,163,204], citrate buffer [206], acetonitrile–water [205], acetonitrile–water–phosphoric acid [207], formic acid or formate buffer [168,203] and with tetrahydrofuran–phosphate buffer, although acetone can also be used provided that UV detection can be omitted [181].

In reversed-phase HPLC several gradient elution systems have been described [160,164,172,201,202,208,209–212]. The acetonitrile–tetrahydrofuran–formate buffer system was considered to be most valuable [153,160,201,202,210]. Com-

pared with isocratic reversed-phase HPLC, is was concluded that gradient elution is not necessarily required for convenient HPLC analysis of anthracyclines.

In ion-pair chromatography of anthracyclines the ion-pairing agent (*e.g* alkyl sulphonate) forms a complex with the positivily charged anthracycline glycoside [168,174,176,186,204,213–218]. Ion-pair chromatography is not recommended for complex studies [219], and in general methods, ion-pair HPLC is not considered to be superior to other reversed-phase HPLC methods [153,204]. Sodium dodecylsulphate was found to be the optimal ion-pairing agent [215], but long retention times were encountered, *e.g.* 40 min for daunorubicin [174].

In contrast to the aglycones, retention of the anthracycline glycosides was found to be dependent on the pH and ionic strength of the mobile phase. Therefore, ion-exchange reversed-phase HPLC has been widely used in the analysis of anthracycline glycosides [169,215,220].

Direct injection into the chromatographic system of several biological fluids such as urine and bile has been described [164,167,172,208,221,238]. For urine, LLE and column clean-up procedures [158,165,172] have also been published. Several clean-up procedures have been reported for plasma: column clean-up [173,207,211,223–225,234], protein precipitation [168,216,217,219] with [159,161, 237] or without column clean-up and extraction [157,160,162,164–166,170,174, 190,191,198,201,202,208,213,227–231,253].

After the impressive results with the first anthracyclines, doxorubicin and daunorubicin, new anthracyclines with proven higher activity *in vitro* were introduced into the clinic, including 4′-epidoxorubicin, carminomycin, aclacinomycin A and idarubicin. In general, systems developed for the analysis of these agents were based on those developed for the first anthracyclines. Several reversed-phase HPLC systems were published for 4′-epidoxorubicin [172,208,221,228,231]. For idarubicin a separate summary of assays is described below.

In clinical drug monitoring with HPLC, detection has a key role and several techniques are available: visible light absorbance detection [161,174,181,183,198, 199,205], UV absorbance detection [163,174,176,186,204,206,218,220,232,233, 253], oxidative ED [173,203,225] and fluorescence detection [154–174,189–193, 195–198,200–202,207–211,213,216,217,219,223–225,228–231,234–237].

Visible light and UV absorbance detection is two to three times less sensitive than fluorescence detection. With fluorescence detection limits as low as 0.5 ng/ml can be achieved. The excitation wavelength is generally set at 480 nm and the fluorescence is recorded in the 500–600 nm range. An excitation wavelength of 250 nm has also been reported [156,193,207,229,234,253]; however, in drug monitoring sessions this cannot be recommended because of the interference from other compounds in the biological matrix.

Nowadays many systems are used in anthracycline HPLC. Clear-cut recommendations for mobile phases and columns cannot always be given, as the quality and the amount of the samples to be analysed will determine the system. When extensive identification of metabolites is required, isocratic reversed-phase HPLC

is amongst the methods preferred [203], but ion-pair chromatographic systems are effective for the fast and adequate separation of doxorubicin, daunorubicin and carminomycin [187].

Reversed-phase HPLC is the most important tool in the monitoring of anthracycline levels. It can be expected that this will continue in the next decade, although promising data from CE are already available. Laser-induced fluorescence detection can lower detection limits to 10 pg [238]. ED may be subject to interference owing to adsorption on and contamination of the glassy carbon electrode [173,203,225].

Gas chromatography

The use of GC in the analysis of anthracyclines is limited owing to their poor volatility. Derivatization of aglycones and the sugar moiety was required for GC [239]. In combination with EI-MS, GC allowed characterization of the aglycones whereas α and β anomeric forms of trimethylsilyl daunosamine could be separated by GC.

Thin-layer chromatography

Qualitative TLC was used for the analyses of anthracycline aglycones [169] and bis-anhydro aglycones [235]. Furthermore, semi-preparative TLC of 7-deoxyaglycones of doxorubicin and daunorubicin has been detailed [187].

TLC was also applied to (semi-)quantitative purposes: the systems described were generally not designed for true quantitative purposes owing to the risk of artefact formation during extraction. TLC proved to be useful in the separation and identification of unchanged drugs and their metabolites, as well as several anthracycline derivatives [172,206,240,241].

Nevertheless, quantitative TLC has been frequently reported. Quantification includes separation of parent compounds, metabolites and degradation products. Degradation of the anthracycline chromophore is the main reason for generation of false results [164]. Detection was performed by fluorometric scanning [242], and fluorometric measurement after the removal of zones of interest and subsequent extraction [201,202,243].

For pharmacokinetic studies with radiolabelled anthracyclines, scanning of radiating areas on the TLC plate was applied [244]. Dornberger *et al.* [245] used TLC for the analysis of daunorubicin and adriamycinone in fermentation broths. Comparison of results from TLC and HPLC showed some inconsistencies, presumably due to artefact formation during TLC development or, for some compounds, to a higher separation capacity of TLC. TLC proved to be able to indicate the presence of doxorubicinolone [201,202], whereas HPLC failed to elute doxorubicinone and 7-deoxydoxorubicinolone separately. Brenner *et al.* [202] found higher levels of aglycones in human plasma with TLC, which might be due to hydrolysis of the glycosidic bond in mobile phases with low pH [162,234].

4.2.2. Idarubicin

Idarubicin (Imi 30; 4-demethoxydaunorubicin) is a synthetic anthracycline that has activity against adult and paediatric acute leukaemia, malignant lymphoma and multiple myeloma (Fig. 10) [246–250] Imi 30 is characterized by its sufficient absorption from the gastrointestinal tract upon oral administration [251]. Its cardiotoxicity has been reported to be less than that of other anthracyclines [251]. Imi 30 is metabolized to its 13-hydroxy derivatives (Imi 30-OH; 4-demethoxydaunorubicinol); *in vitro* test systems revealed high cytostatic activity for this metabolite [252]. Several assays for Imi 30 based on reversed-phase HPLC in isocratic or gradient mode have been published [248, 253–255]. Eksborg and Nilsson [256] demonstrated the importance of the proper handling of plasma samples and optimization of the extraction procedures in a recent paper on idarubicin determination. The chemical stability of Imi 30 in aqueous buffers was found to be strongly dependent on pH; degradation of Imi 30 under alkaline conditions was somewhat faster than that of doxorubicin. Imi 30, like doxorubicin and daunorubicin, is enzymically reduced in whole blood samples, but this process can be inhibited by immediate cooling on an ice-bath prior to plasma separation. Separation was performed by reversed-phase HPLC. Photometric detection at 484 nm offered a detection limit of the same order of magnitude as those reported for fluorescence detection [248,253–256].

De Graaf *et al.* [257] developed an HPLC assay for the determination of Imi 30 and Imi 30-OH requiring only 100 μl of plasma per injection. Fluorescence detection (250/550 nm) enabled detection of concentrations down to 0 1 ng/ml, the lowest reported thus far. The need for only 400 μl of plasma allows paediatric pharmacokinetic studies, as well as drug monitoring in small laboratory animals.

4.3. Aminoanthraquinones

The amino(ethylamino)anthraquinones have been derived from studies in dye chemistry and are not anthracyclines *per se* (Fig. 10). The antitumour activity was discovered in the drug-screening programme of the NCI, which led to a wide-ranging synthetic effort. Aminoanthraquinones intercalate DNA owing to the quinoid chromophore. Ametrantone, bisantrene and mitoxantrone are amongst the aminoanthraquinones subjected to clinical evaluation. Thus far, mitoxan-

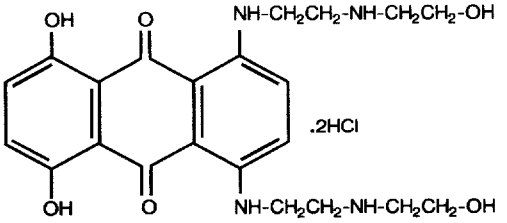


Fig 10 Structure of mitoxantrone

trone has proved to be the most promising one, which is reflected in the number of bioassays developed in the 1980s.

Mitoxantrone, 1,4-dihydroxy-5,8-bis((2((2-hydroxyethyl)amino)ethyl)amino)-9,10-anthracenedione dihydrochloride (CAS 70476-82-3; NSC 301739, Novantrone, Fig. 10), was recently (1987) approved by the FDA for treatment of leukaemia and is currently used against a wide variety of neoplastic diseases in humans.

Methods available for the determination of aminoanthraquinones are mainly based on HPLC, although radioimmunoassay (RIA) [258] and enzyme-linked immunosorbent assay (ELISA) [259] were reported too

The present review is focused on the analysis of mitoxantrone because of its increasing role in clinical oncology. Although many pharmacokinetic studies have been carried out, the reported estimated pharmacokinetic parameters have proved controversial.

High-performance liquid chromatography

Most of the data were obtained by HPLC analysis, with minor differences between chromatographic conditions [259–273]. Sample pretreatment procedures varied widely: LLE [273], SPI with a hydrophobic resin [265] or with reversed-phase silica gel [262] or by a column-switching technique [267,271] were included. Lin *et al.* [272] compared the various sample clean-up procedures and found that mitoxantrone bound strongly to silica gel and to laboratory glassware. Besides photometric detection [260,261,267,273], fluorometric [263] and electrochemical detection [264] can be used. The lowest sensitivity was at the 1 ng/ml level [262,272] with absorbance detection at 546 and 658 nm. Fluorometric [263] or electrochemical detection may offer better sensitivity [264].

Stability

Besides the problem of adsorption onto surfaces of glassware and injection systems, the instability of aminoanthraquinones in (biological) fluids also needs special attention. Oxidation of the phenylenediamine moiety to the corresponding quinoneimine is followed by hydrolysis to yield the quinone [261,262]. A decrease of the pH increases the stability, because these reactions are pH-dependent; acidification of the plasma samples concurrently with addition of ascorbate was sufficient to stabilize mitoxantrone.

Fluorescent room light proved to promote degradation of bisantrene and therefore samples of interest should be protected from exposure to this radiation [260].

4 4. Amsacrine

Amsacrine, 4'-(9-acridinylamino)methanesulfon-*m*-amiside (AMSA), is a recently developed cytostatic agent, which is active against acute leukaemia (Fig. 11). Different procedures have been described for the determination of AMSA in body fluids and cells [274–279].

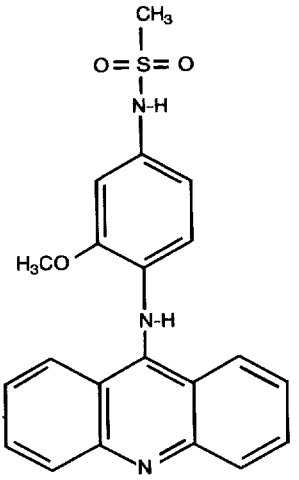


Fig. 11 Structure of amsacrine

Liquid column chromatography

Brons *et al.* [274] recently described the determination of AMSA in whole blood, plasma, white blood cells and (indirectly) in red blood cells. The method was a modification of the method of Jurlina *et al.* [275,276], who studied the plasma pharmacokinetics. An isocratic reversed-phase HPLC system was chosen, with detection at 265 nm, generating determination limits down to 6 ng/ml. Whole blood, plasma, white blood cell and red blood cell concentrations (0.005–0 06 ng per 10^6 cells) were reported [274]. The clean-up procedure was not essentially different from those reported earlier: lipid materials were removed by extraction with hexane of the acidified plasma The pH was adjusted to 9, and the AMSA was transferred to the organic phase.

Gas chromatography

AMSA (MW 394) is a relatively large and complex molecule for straightforward analysis by GC Nevertheless Emonds *et al.* [279] demonstrated that elution of unchanged AMSA on capillary support-coated open tubular (SCOT) columns was possible. The limit of determination was 50 ng/ml using NPD.

4.5. Bleomycin(s)

In the 1960s the isolation of a number of small glycopeptides from culture broths of the fungus *Streptomyces verticillis* was already reported. The highest antitumour activity in the broths was observed with a mixture of peptides now known as bleomycin Bleomycin has proved to have clinical activity in the treatment of squamous cell carcinoma, lymphoma and testicular carcinoma. Used in combination with vinblastine and CDDP, it generated a high rate of complete

remissions and cures in patients suffering from germinal neoplasms of the testis.

Bleomycin is a mixture of water-soluble glycopeptides, differing only in their terminal amines. At least 80% of the total weight of the commercially available preparations is represented by the A_2 and B_2 fractions (Fig. 12); the A_1, A_5, B_1, B_4, demethyl-A_2 fractions and bleomycin acid are minor components. Bleomycin is one of those anticancer agents known to be unstable in solution owing to conversion and/or degradation in the pharmaceutical phase. Its metabolism may also be extensive, but the mechanisms remain to be investigated; the 24-h recovery was found to be 45–70% [280,281]. Thakrar and Douglas [282] demonstrated the sensitivity of bleomycin to photolysis. The role of metabolites in the therapeutic efficacy of bleomycin has not yet been detailed, however, it cannot be excluded that the therapeutic index of bleomycin is determined by non-pharmacokinetic factors.

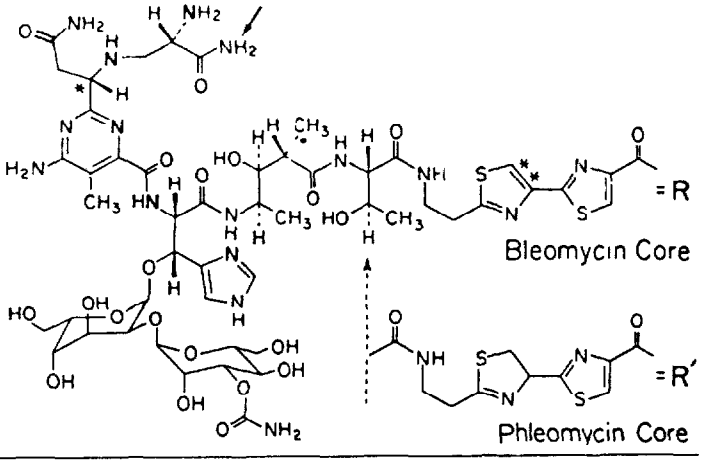
A variety of techniques has been developed for assaying bleomycin in body fluids, including microbiological, HPLC, biochemical, enzyme inhibition assay [283] and RIA [284].

High-performance liquid chromatography

Separation of bleomycin components was achieved by ion-pair chromatography using gradient elution [285–287]. Reversed-phase HPLC with UV detection at 254 nm permitted the determination of bleomycin A_2, in plasma and urine [288]. TCA was used for precipitation of plasma proteins; bleomycin was isolated from urine by SPI. Limits of determination were 500 ng/ml and 5 μg/ml for plasma and urine, respectively. Lazo and Humphreys [289] applied lyophilization of labelled samples after LLE. Unlabelled bleomycin A_2 standards and $CuSO_4$ was added to each sample. The bleomycin A_2 peak was located by monitoring the fluorescence of the column effluent (292/355 nm), whereas the radioactivity of bleomycin(s) of *in vivo* origin in the effluent was quantified by fraction collection and scintillation counting. An average recovery of 75% was reported for labelled bleomycin A_2 added after homogenization but prior to precipitation with TCA, lyophilization and HPLC [289,290].

A rapid, linear gradient chromatographic technique for separating and quantifying copper(II)-chelated bleomycin congeners was concluded to be suitable for studies of bleomycin tissue distribution, pharmacokinetics and metabolism [286,287]. Epi, iso, desamido and unmodified analogues could also be determined [287].

Bleomycin was among a series of antitumour compounds in fermentation broths that were separated and identified by an integrated biological–physicochemical system [291]. The system relies on preliminary fractionation of the broth by liquid–solid extraction and gradient HPLC with photodiode-array (PDA) detection of the compounds, and automated bioassay confirmation of compound identity was executed by thermospray LC–MS. It can be expected that parts of



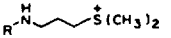
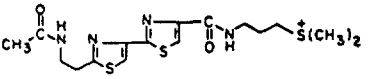
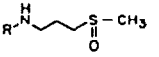
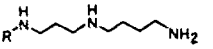
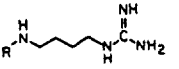
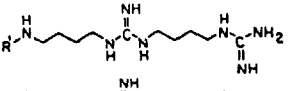
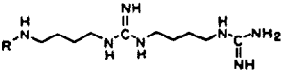
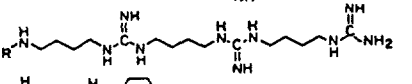
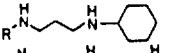
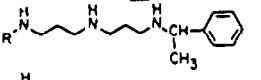
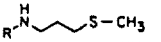
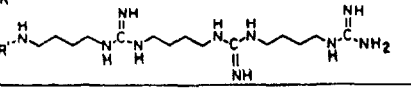
Bleomycin or phleomycin congener	Terminal structure
Bleomycin or phleomycinic acid	R–OH, R'–OH
BLM A_2	
BLM A_2-BT	
BLM A_1	
BLM A_3	
BLM B_2	
PHLM E	
BLM B_4	
BLM B_6	
BLM CHP	
BLM PEPP	
BLM DM-A_2	
PHLM G	

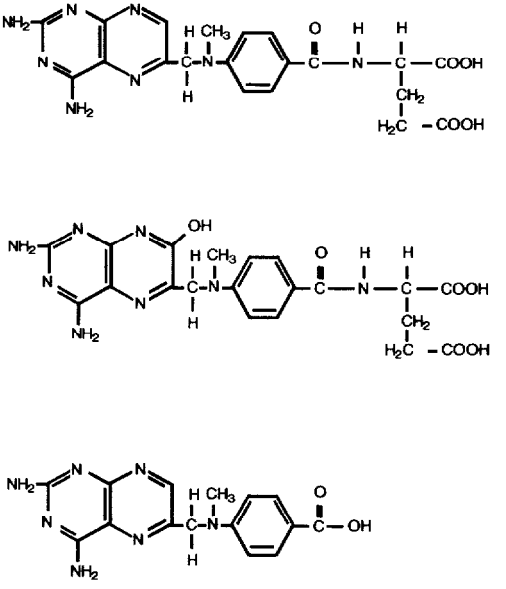
Fig 12 Structures of bleomycin and phleomycin (Reproduced with permission from ref 286)

the system, *e.g.* PDA and LC–MS, will be included in future assays capable of determining bleomycin and possible metabolites in biological samples.

5. ANTIMETABOLITES

5.1. Methotrexate

Methotrexate (MTX) is a potent anticancer agent of proven benefit in the treatment of a wide variety of neoplastic diseases (Fig. 13). Its effects are due primarily to inhibition of the enzyme dihydrofolate reductase (DHFR). As a consequence DNA and RNA synthesis is blocked, resulting in cell death. Severe toxicity has been associated with DHFR inhibition since normal cells are also affected, particularly following high doses. MTX is metabolized in a dose-dependent manner; in humans and primates, 7-hydroxymethotrexate (7-OHMTX) is excreted as the main urinary metabolite. This metabolite is less water-soluble than the parent compound and crystallization in the tubules is reported to be the main cause of renal damage. Patients at high risk of toxicity include patients receiving high-dose therapy and those with dysfunctions related to prolonged MTX residence times. Among these conditions are: renal dysfunction or prior use



A methotrexate (MTX)
B 7-hydroxy Methotrexate (7-OH MTX)
C 2,4-diamino-N-10-methylpteroic acid (DAMPA)

Fig. 13. Structures of methotrexate (MTX) and its main metabolites 7-hydroxymethotrexate (7-OH-MTX) and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA).

of nephrotoxic drugs· pleural effusion or ascites (accumulation!); gastrointestinal obstruction and poor hydration and acidic urine Leucovorin (folinic acid) is co-administered as a 'rescue agent' (antidote) in high-dose MTX treatments. In this way development of toxicity can be prevented and the determination of the leucovorin dose, resulting in enhanced therapeutic index of MTX is critical. Therefore, proper monitoring of MTX is of crucial importance in MTX therapy and has generated a great need for a selective and sensitive method for MTX analysis in biological samples [292,293].

Techniques for the measurement of MTX in biological samples have frequently been reviewed and compared [294,295] Procedures available for monitoring MTX and metabolites include: fluorometry, RIA, enzyme immunoassay, enzyme inhibition assay and competitive protein binding assay, HPLC, MS, isotachophoresis [296] and capillary electrophoresis (CE) [297]. Details on MTX stability, sample pretreatment, conditions for separation when applied, accuracy, selectivity and detection have been reviewed and compared [298]. According to the principles of method evaluation as detailed by Eksborg [299], large deviations are apparent between different methods.

During the past five years the number of MTX assays in which generally higher accuracy and sensitivity was claimed has increased [300–320].

High-performance liquid chromatography

Labat *et al.* [300] reported a comparative study of results obtained by two chromatographic methods: ion-exchange chromatography and reversed-phase HPLC with UV detection at 340 nm The comparative data demonstrated that both techniques permitted the identification and the dosage of MTX, its impurities and metabolites in biological samples. Salamoun and Frantisek [301] reported HPLC involving post-column cleavage and fluorometric detection for the determination of MTX and its metabolites in biological fluids. The detection was based on the post-column photooxidation of MTX and its metabolites to highly fluorescent products in a PTFE capillary irradiated by UV light. MTX and the metabolites 7-OH-MTX and 2,4-diamino-N-10-methylpteroic acid (DAMPA) were determined by this assay in plasma, urine and ascitic fluid samples at concentrations as low as $2 \cdot 10^{-8}$ *M*.

Slordal *et al.* [294] described a comparative study between EMIT, FPIA and HPLC. There was no indication for a clear prevalence of EMIT, fluorescence polarization immunoassay (FPIA) or HPLC for MTX quantification in the clinical laboratory. Furthermore, no evidence of decay of MTX concentrations in samples stored at −20°C for 1.5 years was found. Anzai *et al.* [302] coupled HPLC and RIA to separate and identify MTX and its polyglutamate forms in biological specimens. In order to separate and quantify MTX and its metabolites, clinical specimens were chromatographed over 60 min, and 1-ml fractions of HPLC eluates wete collected for RIA MTX ^{125}I RIA kits were used to quantify polyglutamates in HPLC eluates. With this technique the presence of MTX,

7-OH-MTX, and MTX polyglutamate forms G1–G4 in tumours and normal tissues of a patient who received a high-dose MTX infusion was demonstrated. The data acquired were in close agreement with those obtained by Samuels *et al.* [303], who used HPLC with direct UV detection at 313 nm and an enzyme titration assay.

Nuernberg *et al.* [304] developed a rapid isocratic HPLC method with detection at 303 nm for the quantification of MTX and its metabolites in human serum, urine and bile, using SPI. The method allowed rapid determination of MTX and its major metabolite 7-OH-MTX in patient serum, bile and urine at the low ng/ml level. DAMPA was not found to occur at levels above 5 ng/ml.

Palmisano *et al.* [305] determined MTX in untreated body fluids by micellar HPLC. The method was developed in order to possess the advantages of EMIT (one-step assay, *i.e.* no sample pretreatment) and HPLC (lack of interference, wide linear range and high sensitivity). Earlier work of Stratton *et al.* [306] proved that micellar HPLC offered interesting possibilities. A one-step HPLC method should involve direct injection of serum samples, which is feasible by using micellar mobile phases, because of their unusual protein-solubilizing power and ability to free the protein-bound drug to the stationary phase. Moreover, in micellar HPLC selectivity can be readily increased by proper selection of the surfactant type and its concentration, as well as by optimization of the pH of the mobile phase.

A comparison between data obtained with micellar HPLC and EMIT, including twenty serum samples from cancer patients, demonstrated sufficient correlation (C.C. 0.994).

5.2 Fluoropyrimidines

Fluorouracil (FUra) is still considered amongst the most active antineoplastic agents in the treatment of advanced colorectal cancer and malignancies of head and neck. After the development of FUra, several analogues have been produced based on data metabolic activation (Fig. 14). The latest fluoropyrimidine is 5′-deoxy-5-fluorouridine (dFUrd). Conversion to the nucleotide level is required in order to attain cytotoxic activity. The formation of 5-fluoro-2′-deoxy-5′-uridine monophosphate (FdUMP) is the main mechanism of FUra cytotoxic action, whereas FUra incorporation into RNA and, to a less extent, into DNA contributes to the efficacy of the antimetabolite. FUra has been known for several decades [1], and data on biochemical activation, including inhibition of thymidylate synthase by FdUMP and FUra incorporation into RNA and DNA, resistance to FUra, biochemical modulation, pharmacokinetics and bioanalysis, have been reported extensively [321–323]. Recently FUra was again amongst the topics in clinical oncology: an increased efficacy was demonstrated for the combination of FUra and levamisole *versus* FUra alone in the treatment of colorectal cancer [324].

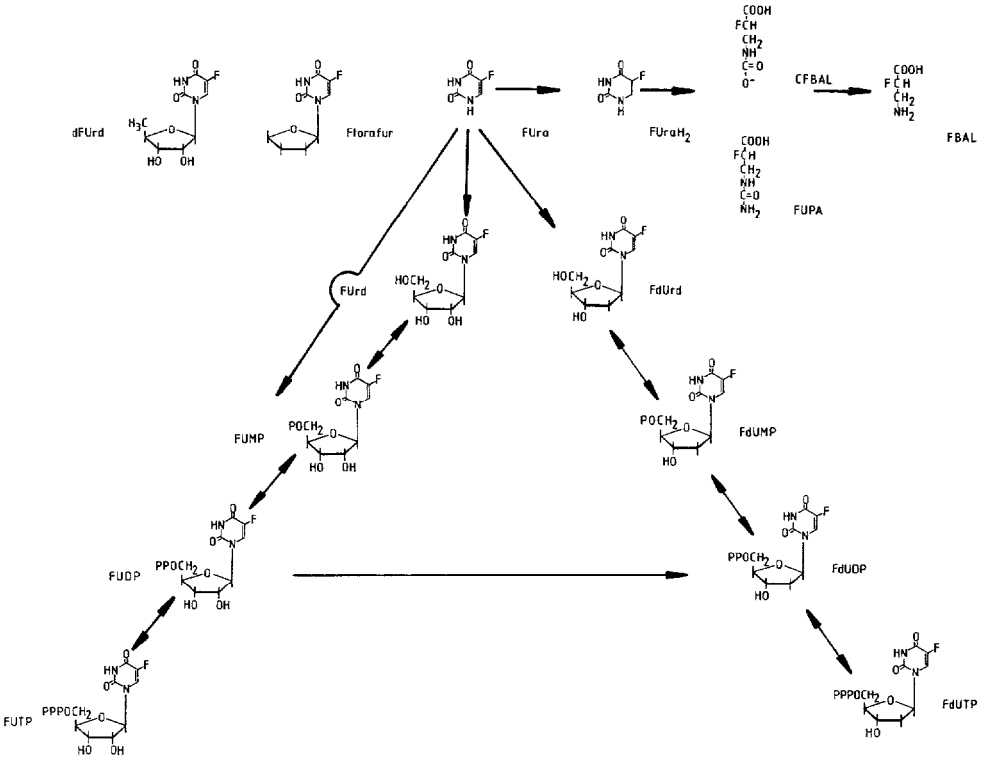


Fig 14 Structures of fluoropyrimidines and possible metabolites

A large number of papers on FUra analysis based on non-chromatographic and chromatographic procedures are available [325–345]. Interesting recent advances in the analysis of FUra and its congeners were the application of ^{19}F NMR [346–355] and SFC [356]

High-performance liquid chromatography

Reversed-phase HPLC is the most widely applied technique in the analysis of fluoropyrimidines. Peters *et al* [332] showed that use of PRP-1 (a co-polymer of styrene and divinylbenzene) as packing material at high pH led to sufficient separation of FUra and uracil, because it makes the most of the modest differences between ionization and ion-pairing. The ion-pairing agent used was the cetyltrimethylammonium ion. The technique seems advisable in clinical studies of the combination of FUra and uridine, since the latter is subject to catabolic conversion into uracil.

In most cases of HPLC, UV detection at 254–280 nm was associated with limits of determination in the range 10–100 ng/ml of plasma. Iwamoto *et al.* [335] applied fluorescence detection (346/395 nm) for FUra and 1-(tetrahydro-2-furanyl)-5-fluorouracil (FT, ftorafur) determination after derivatization with 4-bromomethyl-7-methoxycoumarin using 18-crown-6 as a catalyst. The detection limits were 100 and 384 fmol for FUra and FT, respectively, which could be lowered 100-fold by reducing the reaction volume to 10 μl. The sensitivity of this method puts it amongst the most sensitive assays based on HPLC and GC.

Schaaf *et al.* [336] used a relatively simple and sensitive HPLC method for the simultaneous determination of FUra and its prodrug dFUrd in biological fluids. Sample pretreatment consisted of the addition of 3% phosphoric acid and LLE with ethyl acetate–2-propanol. 5-Bromouracil was used as internal standard, and recoveries of 66 and 61% for FUra and dFUrd, respectively, were obtained. The method proved simpler than a method based on isotachophoresis [333] and included the possibility of simultaneous determination of dFUrd and FUra, in contrast to the method of Sommadossi and Cano [334]. Biondi and Nairn [338] developed an HPLC analysis for FUra, 5-fluorocytosine and their possible decomposition products. 5-Methylcytosine was used as internal standard. Applications of the assay in clinical settings with the fluoropyrimidines were not described in this paper. Stetson *et al.* [339] were able to monitor FUra concentrations in peritoneal fluids at concentrations of *ca.* 25 ng/ml. 5-Chlorouracil was used as internal standard Sample preparation included deproteination with ammonium sulphate and LLE with ethyl acetate. FUra concentrations were determined routinely in the concentration range 50–2000 ng/ml, in plasma and peritoneal fluids. Sequential cation and anion exchange as described by Williams *et al.* [337] was used to isolate the analytes. The limits of determination of the fluoropyrimidines tested were in the range 10–20 ng/ml using 5-chlorouracil as internal standard. Day and Sadée [341] reported on the determination of radiolabelled 5-fluorouridine diphosphate glucose, a FUra metabolite, in mouse T-lymphoma (S-49) cells using Aminex A-29 anion-exchange columns with gradient elution preparative C_{18} reversed-phase columns.

Briggle *et al.* [343] exploited HPLC for the determination of 5-fluoro-2′-deoxycytidine, 5-trifluoromethyl-2′-deoxycytidine and their related metabolites. The assay proved to be applicable in a complex and extremely important study of the activation mechanisms of 5-fluoro-2′-deoxycytidine [357] and comparative studies with FUra [358]. Tjaden *et al.* [344] applied HPLC with valve switching and PRP-1 columns as stationary phase in the simultaneous determination of dFUrd and FUra. Biological samples were pretreated with a simple LLE, and the sensitivity and selectivity of the procedure enabled determination of the analytes at the 5 ng/ml level.

Gas chromatography

GC of fluoropyrimidines has been performed on both packed and capillary

columns with and without derivatization of FUra Several derivatization reactions have been described, including methylation using flash alkylation with trimethylanilinium hydroxide [359], derivatization with diazomethane [325] and with methyl iodide, and derivatization by butyl, pentyl [327] and hexyl alkylation. Trimethylsilylation and the generation of a chloromethyldimethylsilyl derivative has also been reported. GC enabled determination of FUra below the ng/ml level, as described by Kok *et al.* [340]. GC–EI-MS was used with 5-chlorouracil as internal standard. Pentafluorobenzyl derivatives of 5-fluoro- and 5-chlorouracil were prepared by extractive alkylation with pentafluorobenzyl bromide with dichloromethane as solvent and the tetrabutylammonium ion as counter-ion. The limit of detection was 50 fg on-column.

Williams *et al.* [337] developed a GC analysis of fluoropyrimidine nucleosides and FUra in plasma and urine. On-column alkylation was employed for the derivatization of 5-fluoro-2′-deoxyuridine, 5-fluorouridine and FUra. 5-Chloro-2′-deoxyuridine and 6-methyluracil were used as internal standards for nucleoside and FUra analysis, respectively. Sample pretreatment included cation-exchange and anion-exchange chromatography; recoveries were 71–95% over the concentration range studied. After elution and drying, the residue was dissolved in *p*-toluyltrimethylammonium hydroxide in methanol before injection into the GC column. Correction (4–7%) of FUra concentrations was required when it was co-determined with 5-fluoro-2′-deoxyuridine, owing to minor on-column degradation of the nucleoside into FUra

Capillary GC is as yet the only technique for monitoring FUra catabolism at the ng/ml level without the use of radiolabelled FUra [326,360]. The impact of chemical and enzymic degradation of the first product of FUra catabolism, *i.e.* 5,6-dihydro-5-fluorouracil ($FUraH_2$) was discussed in refs. 361 and 362. In order to prevent $FUraH_2$ degradation, sample pretreatment should include adjustment to pH 4.0 and/or handling of samples at temperatures between 4 and 10°C. Degradation of $FUraH_2$ during sample pretreatment might explain the observation of the absence of $FUraH_2$ in patients monitored for the catabolite [361–363].

5.3. Cytosine arabinoside

Arabinose nucleosides are a unique class of compounds, which were discovered in isolates of the sponge *Cryptothethya crypta.* The presence of a β-OH group in the 2′ position of the sugar (Fig. 15) distinguishes them from physiological nucleosides identified in animal tissues. Antitumour and antiviral activity was demonstrated for several arabinose nucleosides; the chemically synthesized cytosine arabinose (Ara-C) appeared to be the most potent antileukaemic agent. The active intracellular form of Ara-C is the triphosphate, whereas Ara-C itself lacks activity Triphosphate Ara-C (Ara-CTP) exerts its effect by blocking DNA synthesis and (primarily) by incorporation of Ara-C into DNA. There is growing evidence that the accumulation of Ara-CTP in cells, a necessary preclude to DNA

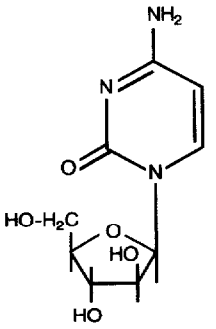


Fig. 15. Structure of cytosine arabinoside

incorporation, is determined by the capacity of active drug uptake by a membrane carrier [364–366]. Ara-C is metabolized by hepatic enzymes to uracil arabinoside, a compound without proven antitumour activity.

Non-chromatographic methods as well as chromatographic procedures are available for Ara-C monitoring. chromatography includes both HPLC and GC methods. The biological, enzymic and spectrophotometric methods are time-consuming and have a low selectivity. The sensitivity of the biological and enzymic methods is *ca.* 20 ng/ml; that of spectrophotometry is 2–5 ng/ml. The RIA methods are almost free from interferences, and the sensitivity for the determination of Ara-C is *ca* 20 ng/ml.

High-performance liquid chromatography

The most convenient method for the quantification of Ara-C in biological fluids is based on a reversed-phase HPLC [367] Sample pretreatment included addition of a deaminase inhibitor, tetrahydrouridine, prior to centrifugation. Direct injection of the plasma fraction into the liquid chromatograph resulted in sufficient selectivity and sensitivity, related to a limit of determination of 50 ng/ml. The limit of determination of Ara-C in ultrafiltered plasma was lower: 2–10 ng/ml. The assays developed were utilized, with or without modifications, in studies of both extra- and intracellular behaviour [364–366,368,369].

Gas chromatography

The introduction of GC in the analysis of Ara-C enabled selective and sensitive determination of the antimetabolite [370]. However, the methods required time-consuming multiple extraction and derivatization steps. The limits of detection subsequently obtained were 100 ng/ml, 40–70 ng/ml using NPD and 1 ng/ml with MS [370,371]. Sample pretreatment procedures included protein precipitation with ethanol or TCA and ultrafiltration, the latter being superior.

5.4. *Hydroxyurea (hydroxycarbamide)*

Hydroxyurea (HU) is a urea analogue (Fig. 16) that was first synthesized more than 100 years ago [372]. The drug entered clinical trials in the early 1960s following recognition of its antitumour activity. Clinical applications are limited, as are assay methods. The pharmacokinetic behaviour of HU was reported between 1963 and 1965, from colorimetric assays that measured the reaction product of HU with either picryl chloride or diacetylmonoxime and sodium *p*-diphenylaminesulphonate: a limit of determination of 5 μg/ml was obtained. Later on, Belt *et al.* [373] examined the pharmacokinetic and cytokinetic effects of HU. Data on the behaviour of HU in the pharmaceutical phase are limited; Pluscec and Yuan [374] described an LC assay for the determination of HU in pharmaceutical formulations.

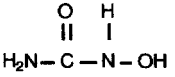


Fig. 16 Structure of hydroxyurea

5.5. *Purine antimetabolites*

The thiopurines, 6-thioguanine (6-TG), 6-mercaptopurine (6-MP) and azathiopurine (Fig. 17) are important agents in the treatment of leukaemia. Azathiopurine is also widely used as an immunosuppressive agent The cytotoxic effects of thiopurines are related to the depletion of purine nucleotides or incorporation into DNA as "false" nucleotides. DNA incorporation was found to be more

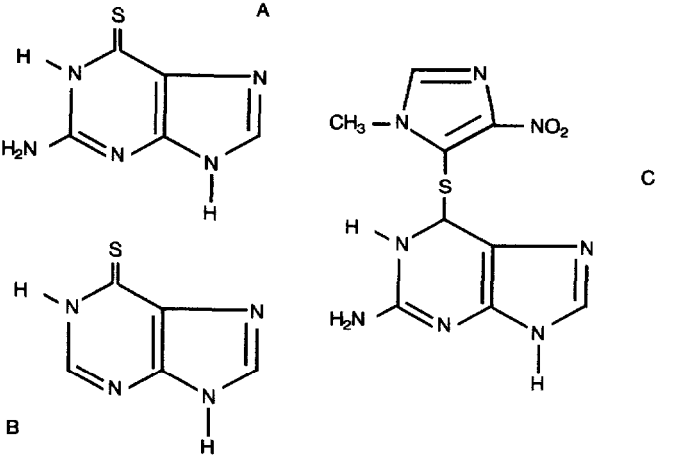


Fig. 17 Structures of the thiopurines 6-thioguanine (A), 6-mercaptopurine (B) and azathiopurine (C).

related to 6-TG activity, whereas 6-MP cytotoxicity is more probably due to purine starvation [375,376]. The agents are amongst the oldest antineoplastic agents, and by the mid-1980s several non-chromatographic and chromatographic procedures for their determination were described: spectrophotometry, fluorometry, HPLC [377–382] and GC. Clinical drug monitoring appeared to be important because 6-MP was found to be absorbed incompletely following oral administration, which might explain maintenance therapy failure. This demand, as well as the need for assays suitable for monitoring thiopurine metabolism and activation, resulted in more methods being developed, mostly based on HPLC [384–395].

During sample pretreatment, care should be taken to prevent metabolic and chemical conversion. Oxidation of the thiol group can be prevented by dithioerythritol; autoxidation of 6-MP can be limited by conversion of 6-MP into 6-MP-N-ethyl maleimide [377–379].

High-performance liquid chromatography

Isolation of thiopurines was performed by precipitation of plasma proteins with TCA, PCA or acetonitrile, but resulted in loss of the compounds of interest. LLE with ethyl acetate gave recoveries close to 100%. Direct injection of plasma onto a reversed-phase column proved to be possible for small amounts of plasma, ultrafiltration of plasma and direct injection enabled 6-TG monitoring in plasma. Azeemuddin *et al.* [395] used a rapid and simple SPI of 6-MP. They found that 6-MP solutions in water were stable for two months if protected from light and stored at or below 4°C.

Fluorescence detection following pre- or post-column oxidation, as well as UV detection, were applied after HPLC; for fluorescence detection determination limits reported were at the low ng/ml level whereas the levels for UV detection were in the range from 3 to 200 ng/ml. For surveillance of compliance in patients treated with 6-MP, the method of Azeemuddin *et al.* [395] seems most attractive because it does not involve tedious methodology and prescribes simple extraction and offers sufficiently low limits of determination in plasma (10 ng/ml) and urine (50 ng/ml).

Gas chromatography

After isolation, the separation technique of choice might be either GC or HPLC. Floberg *et al.* [383] introduced a sensitive GC assay in 1981, which can still be recommended for purine antagonist monitoring. Extraction and derivatization was directly performed from plasma samples by means of extractive alkylation with pentafluorobenzyl bromide and determination by MS with SIM. The detection limit of the method was 2 ng/ml, which places this GC analysis among the most sensitive. FID can be used also, but the limit of determination is higher.

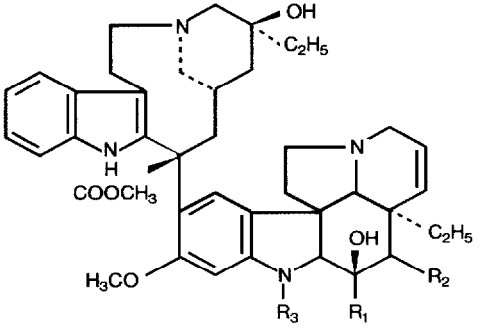
6 VINCA ALKALOIDS

The vinca alkaloids, vinblastine, vincristine (Oncovin) and vindesine, are among the most effective agents in the treatment of childhood and adult malignancies, including leukaemias and lymphomas (Fig. 18). The vinca alkaloids block the polymerization process and inhibit the progression of cells through mitosis

Until the mid-1980s, methods for vinca monitoring were limited and included RIA [396], flow cytometry and ELISA [397]. Desacetylvinblastine was already established as the main metabolite of vinblastine, and higher biological activity had been demonstrated in comparison with the parent drug [398]. More data on vinca alkaloid stability and drug monitoring became available in the second half of the 1980s [399–407]. In that period the most important contribution to vinca monitoring came from HPLC [399–405]

High-performance liquid chromatography

De Smet and co-workers [399,400] were able to determine vinblastine in MO4 mouse fibrosarcoma cells using HPLC. Later on Vendrig and co-workers [402,403,405] introduced assays based on HPLC, which enabled vinca alkaloid monitoring in plasma and urine of cancer patients. In clinical settings the normal dose of vincristine, vinblastine and vindesine of 1–6 mg/m^2 results in active plasma concentrations below 10 ng/ml several hours after administration. Sensitive detection is therefore needed, and it was demonstrated by Vendrig and co-workers that both electrochemical [403] and fluorescence detection [405] following HPLC were capable of vinca monitoring at the low ng/ml level. Sample clean-up



	R1	R2	R3
Vincristine	$COOCH_3$	$OCOCH_3$	CHO
Vinblastine	$COOCH_3$	$OCOCH_3$	CH_3
Vindesine	$CONH_2$	OH	CH_3
Desacetylvinblastine	$COOCH_3$	OH	CH_3

Fig. 18. Structures of the vinca alkaloids vincristine, vinblastine, vindesine and desacetylvinblastine

consisted of SPI on a cyanopropyl cartridge. The extraction recovery (>70%) was determined for several batches because different recoveries with the columns were observed [402]. Intra- and inter-assay variabilities of the extraction were determined, and all data indicated that it is possible to isolate the vinca alkaloids from biological matrices very selectively using CN-bonded phases for the extraction. Recycling of the extraction columns was possible, but care has to be taken when using the same column for (unknown) different concentrations [403]. The volume of methanol used for elution of vinblastine might affect the recovery for some batches [403], and therefore internal standardized and batchwise control can be recommended. The limit of sensitivity using ED was 100 pg on-column for all vinca compounds of interest; the limit of determination was 1 ng/ml. Desacetylvinblastine could also be determined by this assay [403]. Chromatography was also performed on silica in the ion-exchange mode with the tetramethylammonium ion as the competing ion [405]. In contrast to the reversed-phase system used with ED [403], the latest ion-exchange chromatographic system was suitable for fluorescence detection. The limits of determination were 0.5 and 2.5 ng/ml in plasma and urine for vinblastine and vindesine, respectively. This method could not be used to follow desacetylvinblastine clearance over prolonged periods owing to insufficient separation from compounds appearing upon isolation from biological fluids.

Vincristine could be used as internal standard in the assay of vinblastine, vindesine and desacetylvinblastine, whereas vinblastine fulfilled the role of internal standard in the vincristine analysis. Pharmacokinetic results showed that the sensitivity and selectivity are adequate for drug analysis. It is to be expected that the latest developments in HPLC determination of vinca alkaloids will increase the understanding of their *in vivo* behaviour.

7 PODOPHYLLOTOXIN DERIVATIVES

Etoposide (VP16) and teniposide (VM26) (Fig. 19), two semisynthetic epipodophyllotoxin derivatives, were developed in the 1970s and proved to have activity in the treatment of several malignancies, including larger-cell bronchial carcinoma, small-cell anaplastic bronchial carcinoma, acute and chronic leukaemias, lymphomas, testicular teratomas and central nervous system malignancies. The clinical results prompted to extensive studies of the pharmaceutical, pharmacokinetic and pharmacodynamic phases of the (semisynthetic) podophyllotoxin, subsequently raising an intrinsic demand for bioanalytical trials.

Till the mid-1980s, the methods available for the determination of non-radioactive etoposide and teniposide in biological fluids were based on reversed-phase (phenyl, C_8 or C_{18}) HPLC [408–412]. Absorbance [410,411], fluorometric [408,412] and electrochemical detection [409] were introduced and enabled pharmacokinetic analysis. Absorbance and fluorometric detection were less sensitive than ED (30 *versus* 1 ng/ml). Holthuis *et al.* [409,411] created assays to study

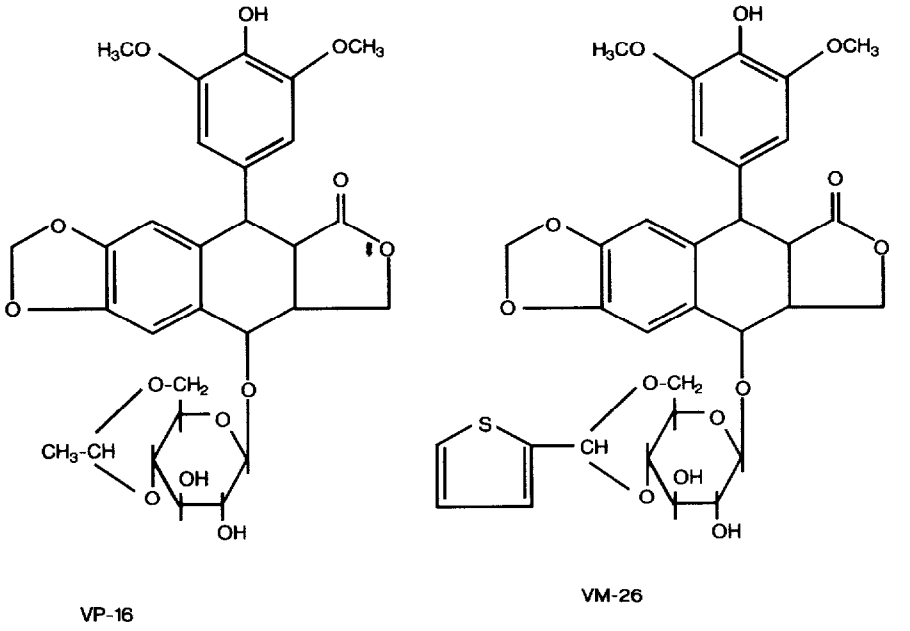


Fig 19 Structures of etoposide (VP-16) and teniposide (VM-26)

metabolism: etoposide, its *cis* isomer and the *cis* hydroxy acid of the aglycone were separated by elution on a phenyl reversed-phase column.

In the second half of the 1980s improvements were described at the level of sample pretreatment (automation) and detection in order to increase the selectivity and sample throughput An interesting although not chromatographic development was the introduction of assays based on flow injection analysis as reported by Van Opstal and co-workers [413–416]. On-line electrochemical derivatization combined with UV–visible diode-array detection in flow injection systems greatly enhanced selectivity. A detection limit of 6 ng for both VP16 and VM 26 was recorded. The surplus value was the increased injection value: 40 h^{-1}. In a comparative study, including HPLC analysis of VP16, it was concluded that flow injection analysis (FIA) of etoposide in plasma offers a good alternative to HPLC analysis, provided that the levels are at least 1.5 μg/ml. FIA, including on-line immunoaffinity sample clean-up in flow injection analysis with PDA, might provide a further increase of selectivity but needs additional research [416].

Fully automated analysis of podophyllotoxin derivatives has been described by Werkhoven-Goewie *et al.* [417]. Subtilisin A was used to hydrolyse blood samples, subsequently on-line preconcentration was performed and, after a post-column clean-up step on-line, fluorescence detection was used with a detection limit of 10 ng/ml. Automated reversed-phase chromatographic analysis involving

direct plasma injection by using on-line surfactant-mediated sample clean-up and column switching was described by Van Opstal *et al.* [415] The anionic surfactant sodium dodecyl sulphate was added to plasma at a final concentration of 38 m*M*. Plasma samples were loaded on to a clean-up column with an aqueous mobile phase with which the analyte was retained, whereas the solubilized plasma proteins were flushed to waste. Column-switching techniques enabled introduction onto the chromatographic system. The recovery increased considerably from 10–30 to 90–95% when the surfactant was added to the plasma prior to the analysis. The limit of determination with 100-μl injections was 0.10–0.15 μg/ml for UV detection and 0.01–0.02 μg/ml for ED In a comparative study with micellar HPLC and LLE, this surfactant-mediated sample clean-up procedure was considered to be a competitive technique for the conventional LLE approach. To date, micellar LC seems to lack the sensitivity of surfactant-mediated sample clean-up and LLE.

8 MISCELLANEOUS

8.1. Suramin

Suramin is a polysulphonated naphthylurea (Fig. 20) that has been used for Rhodesian and Gambian trypanosomiasis and Onchocerca volvules since the early 1920s [418]. Later suramin was discovered to exhibit anti-human immunodeficiency virus activity, both *in vitro* [419] and *in vivo* [420]. In one of the AIDS trials with suramin, a response in an HIV-associated Kaposi's sarcoma was noted whereas cancer cell growth was also demonstrated *in vitro* [421]. The cytotoxic effect of suramin might be related to its inhibition of growth factor activity, including that of platelet-derived growth factor, transforming growth factor β, and epidermal growth factor [422–424]. Furthermore, the influence of suramin on glycosaminoglycans might have an important effect on tumour cell differentiation and tumour angiogenesis [425,426]. Suramin has so far shown greatest efficacy in the treatment of advanced prostate cancer; in these trials the importance of clin-

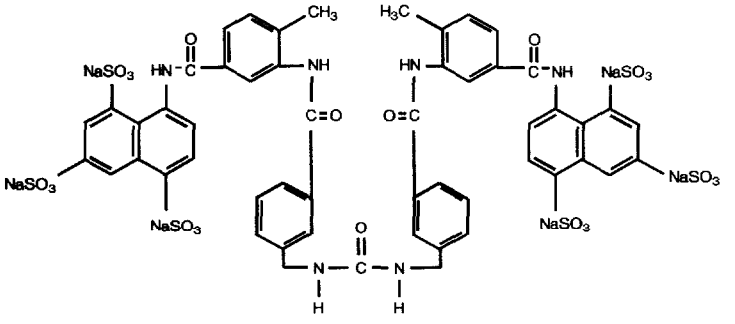


Fig. 20. Structure of suramin

ical drug monitoring became evident owing to the demonstration of a relationship between the development of toxicity at plasma levels above 300 μg/ml [427]. It can be expected that this agent will be subjected to further clinical studies and that analogues will be developed that exhibit more favourable behaviour in the pharmaceutical and pharmacokinetic phase. Therefore, we decided to devote some space to the HPLC methods described for suramin monitoring.

High-performance liquid chromatography

The assays reported are generally based on reversed-phase ion-pair HPLC [428–432]. Only one assay exploited gradient elution [428], the others applied isocratic chromatography. The assays reported by Ruprecht *et al.* [430] and Teirlynck *et al* [431] were convenient but linearity was limited to 200 μg/ml only. Tjaden *et al.* [432] achieved linearity in the range 5–500 μg/ml; this range is assumed to be relevant in clinical oncology. A one-step sample pre-treatment method was used. defrosted plasma samples were mixed with 1 *M* tetrabutylammonium bromide. After addition of methanol, the sample needed stabilization for 30 min at 4°C prior to centrifugation. It should be noted that incubation of plasma with suramin for 8 h or more is needed in order to ensure complete protein binding when constructing calibration curves. The column effluent can be monitored at different wavelengths: suramin has two pronounced absorption maxima, at 237 and 313 nm, with molar absorptivities of 126 000 and 28 000, respectively. In spite of the lower detectability, 313 nm has to be preferred owing to a lower risk of interferences. At this wavelength the minimum detectable concentration in plasma was 0.1 μg/ml [432].

8.2 1-Hydroxy-3-aminopropylidene-1,1-bisphosphonate

Analysis of polyphosphates and compounds with P–O–P and P–C–P bonds has become of interest during the past decade owing to the use of these drugs for Paget's disease and malignant hypercalcaemia [433]. One of these compounds, 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate (APD), is amongst standard chemotherapeutic regimens in the treatment of breast cancer metastatic to the bone (Fig. 21). APD shows a high affinity for the crystal surfaces in mineralized bone matrix. When present on this surface it inhibits its resorption by osteoclasis.

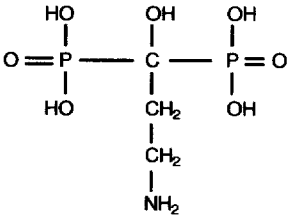


Fig 21 Structure of 3-amino-1-hydroxypropylidene-diphosphonate

Subsequently, it operates in two ways: (1) suppression of excessive bone resorption; (2) prevention of secondary bone disease through alteration of bone remodelling characteristics. The first activity inhibits outgrowth of already existing bone metastasis whereas the second might decrease the bone anchorage capacity of cancer cells.

Several methods developed for the analysis of polyphosphonates and compounds with P–O–P and P–C–P bonds have been published [434,435]. These methods, however, were not designed for the measurements of low concentrations in plasma and urine. Wingen and Schmäl [436] used radiolabelled APD and autoradiography in their study of APD pharmacokinetics in mammals. Flesch and Hauffe [437] reported on the determination of APD in urine only, the method was based on precolumn derivatization with fluorescamine prior to HPLC. Daley-Yates *et al.* [438] recently described a method that enables APD to be determined in both urine and plasma. High-performance, metal-free, anion chromatography was used to separate the sample components Samples were spiked with 1-hydroxy-5-aminopentylidene-1,1-bisphosphonate as an internal standard, and calcium chloride was added to precipitate the bisphosphonates. After centrifugation the precipitate was redissolved in acetic acid, and the bisphosphonates were separated on a Dionex AS 7 column coupled to an AG 7 guard column. A Dionex MFC-1 column was used to remove trace metal ions from the eluent prior to its introduction into the guard and analytical columns. The bisphosphonates were oxidized to orthophosphate using post-column addition of ammonium persulphate, which was followed by a post-column reaction with molybdenum ascorbate to generate a phosphomolybdate chromophore. Detection was executed at 820 nm; the limit of determination (10 ng/ml) proved to be sufficient for routine drug monitoring in patients receiving APD infusions, although the method is laborious.

8.3. Tamoxifen

Tamoxifen is an antiestrogenic compound commonly used now in the treatment of advanced breast cancer (Fig. 22). Its role in adjuvant therapy of breast cancer has been highlighted; it was concluded that a clear reduction in mortality could be achieved with tamoxifen, especially in women aged 50 or older [439,440]. Tamoxifen is thought to competitively block estrogen receptors, resulting in the inhibition of key role metabolic pathways. In addition to its antiestrogenic effects, tamoxifen enhances natural killer function and the capacity of peripheral blood cells to lyse autologous tumour cells. The pharmacokinetics of tamoxifen have not been extensively investigated; however, methods for its determination are now available.

Sane *et al.* [441] developed a GC method for the determination of tamoxifen in pharmaceuticals. Lien *et al.* [442] identified 4-hydroxy-N-desmethyltamoxifen as a metabolite of tamoxifen in human bile, thus raising demands for selectivity of

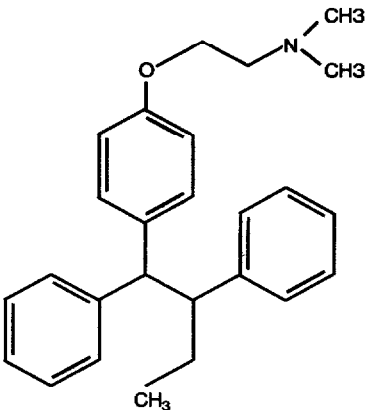


Fig 22. Structure of tamoxifen

the assays to be used in tamoxifen monitoring. Jalonen [443] used HPLC for the simultaneous determination of tamoxifen and its *E* isomer impurity in the bulk drug and tablets. Murphy *et al.* [444] applied GC–MS in the SIM mode for the analysis of tamoxifen and 4-hydroxytamoxifen levels in immature rat uterine cytoplasm, as well as for the analysis of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen levels in cytosol and nuclear extracts of breast tumours from tamoxifen-treated patients [445].

A more detailed description of the methods available for tamoxifen monitoring, together with similar data for other hormonal agents, will be presented elsewhere [446].

9 CONCLUSIONS

During the past decade several new treatment modalities were introduced and experimented in clinical oncology. These included new drugs in an old jacket (*i.e.* analogues of established drugs with an expected higher therapeutic efficacy), regional treatment modalities with old and new drugs, as well as chemotherapeutic regimens exploiting the dose intensity hypothesis. Most promising probably was the introduction of lymphokines and cytokines, such as interferons, tumour necrosis factor and interleukins, and of biological response modifiers and colony-stimulating factors. Furthermore, monoclonal antiobodies and immunoconjugates were added to the armoury of the clinical oncologist. It remains to be seen whether the agents used in these new treatments become as established as the drugs included in the present review. Methods available for routine monitoring of agents such as α-, β- and γ-interferons and interleukin-2 are detailed elsewhere, as they were beyond the scope of this article [446].

What can be expected for anticancer drug monitoring in the future? In order to increase the insight in anticancer drug action, drugs and/or active metabolites

need to be determined both at the site of action (generally intracellular) and systematically. Techniques based on MS [*e.g.* FAB-MS–(MS)] and HPLC already proved to be successful in the determination of the drug of interest at the site of action, as has been described for MMC. The study of Tomasz and coworkers [108,109] demonstrated the need for combining several analytical techniques. Systemic and locoregional anticancer drug monitoring will hold its place in the proper determination of the benefit of locoregional administration. HPLC, generally with UV detection, seems the most suitable technique for the latter purpose. Nevertheless, the interesting progress of CE might result in a clear competition between HPLC and CE at this front. Furthermore, there was a noticeable trend during the 1980s towards the use of larger thermolabile compounds, so it cannot be expected that the role of GC will increase, on the other hand, CE seems an interesting technique for these compounds.

10 SUMMARY

The present review on the methods for the analysis of anticancer drugs should be seen as an addition to the excellent work of Eksborg and Ehrsson published half a decade ago in this journal (Vol. 340, p. 31). The style and format have been followed closely, with the focus again on chromatographic techniques. We felt it important to add a list of compound (group) structures as a service to the reader.

Methods have been reviewed for alkylating agents, platinum compounds, antitumour antibiotics, antimetabolites, alkaloids, suramin, 1-hydroxy-3-aminopropylidene-1,1-bisphosphonate and tamoxifen.

11 ACKNOWLEDGEMENTS

Financial support for the production of the review by the 'Saal Van Zwanenberg Stichting' and 'Arti-Science Milano–New York–Paris' was greatly appreciated. The authors thank Greet Pattyn, Eric Gheuens and Patty Hendrix for their editorial assistance.

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