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Review

Separation methods for alkylating antineoplastic compounds

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Abstract

The separating method for alkylating neoplastic compounds were reviewed based on the classification of the Merck Index (12th Edition). Each section, whenever available or relevant, was subdivided according to the following approach: stability studies, extraction methods, gas chromatography, high-performance liquid chromatography and capillary electrophoresis. At the end of each chapter a separate table summarizing the main characteristics of the separating method were established. In particular LODs and/or LOQs were expressed as quantity to facilitate comparison between methods. This review highlights the problems to measure trace levels of these compounds into biological fluids with respect to their instability, adsorption to glass and plastic or derivatization requirements. Over the last decades, HPLC seems to be more popular than GC for separating the alkylating agents. The development of narrow- or microbore LC coupled to MS is certainly the way to further improve both separation and sensitivity obtained in the different papers surveyed for this review.

Keywords: Reviews; Alkylating antineoplastic compounds

Contents

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1. Introduction

Since nitrogen mustard was first used in the treatment of a patient with lymphosarcoma in 1942, many alkylating agents have been synthesized and tested for their antitumor activity. All these compounds are capable of reacting in such a manner that an alkyl group or a substituted alkyl group becomes covalently linked to the cellular constituents. The reaction involved is alkylation giving the name for this group of drugs. The nitrogen mustard is one of the five major classes of anticancer drugs which act as DNA cross-linkers. These drugs include the nitrogen mustard, the aziridines, the alkane sulfon-

ates and the nitrosoureas. The mechanism of action of nitrogen sulfur mustards were studied before the other alkylating agents, and these compounds are used to describe the alkylation reaction and the biological consequences of alkylation. The other alkylating agents react with DNA in a similar way with similar consequences. These agents are mutagenic and carcinogenic as well as they are cytotoxic. 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. As a matter of fact, the authors have paid attention to the stability of the drug in plasma and in vitro solutions.

The present review concentrates on the chromatographic determination of alkylating agents and some of their metabolites in various biological fluids mainly plasma and urine samples. Many of these drugs are prescribed at such dosages that the resulting plasma levels are extremely low. In the last decade, improvement in extraction from biological fluids and in detection modes such as mass spectrometry and tandem mass spectrometry, allows the quantitation of very small amounts of active compounds for pharmacokinetics studies.

We chose to separate the extraction from the biological fluids and the different chromatographic techniques (i.e., liquid chromatography (LC), gas chromatography (GC) and thin-layer chromatography (TLC)). For most of alkylating agents, the various methods reported in the literature are summarized in tables containing, the derivatization reagent (when used), limit of detection (LOD) and/or limit of quantitation (LOQ) and the retention time of the considered compound. The LODs and LOQs were expressed as quantity of drug injected on column whenever possible to allow easier comparison for the reader.

2. Alkyl sulfonate

2.1. Busulfan

Busulfan (1,4-butanediol dimethanesulfonate) (Fig. 1) is a bifunctional agent that has been used in clinical routine since 1959 either at high doses for preparative regimens for bone marrow transplantation (16 mg/kg) or at low doses (60 μg/kg) for a mainstay of treatment for chronic myelogenous leukemia.

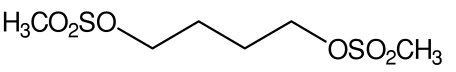


Fig. 1. Structure of busulfan (BU).

Clinically, oral busulfan (BU) causes hematological toxicity, hyperuricemia, bronchopulmonary dysplasia, hepatic dysfunction, neurotoxicity and gastrointestinal irritation (nausea, vomiting) leading to erratic bioavailability. Furthermore, when administered at high doses with cyclophosphamide, the major dose-limiting toxicity is hepatic veno-occlusive disease. The risk of developing the latter could be dose-related toxicity [1]. In this context, analytical techniques are required to enable the monitoring of the plasma level of busulfan after the first administration as soon as possible. Unchanged busulfan has been measurable in human plasma only since 1983 and the description of the first GC methods [2,3]. The HPLC was reported in 1987 [4] and has been extensively studied the last 5 years [5–11].

BU is converted into three metabolites, i.e., sulfolane, 3-hydroxysulfolane and tetrahydrothiophene-1-oxide, which are eliminated via the kidneys. No attention has been paid to the determination of these metabolites as it was demonstrated that they show no cytotoxicity to V79 cells [3]. However, some authors checked that these metabolites do not interfere with the BU assay developed [12]

2.1.1. Drug stability

Different studies have been carried out to determine adequate short-term storage conditions [1,4,11]. BU undergoes degradation at 37°C with half-time of approximatively 14 h and is stable at 22°C for 22 h [4]. Thus, plasma samples must be frozen as soon as possible. There are two controversial reports for the time that samples could be kept frozen at −20°C. Rifai et al. [11] recommends to freshly prepare new controls every 3 weeks and stored frozen sample for also up to 3 weeks as they observed a 12–15% degradation after 10 weeks of storage. By contrast, Grochow et al. [1] observed a

short-term stability of 6 months which allows more flexibility for the clinical laboratory.

2.1.2. Extraction from biological fluids

For both GC and HPLC methods LLE extraction has been extensively used. Ethyl acetate [1,4,6,7,13–18] was mainly employed with methylene chloride [2,14] and a mixture of diethylether:methylene chloride [19]. Recovery are quite satisfactory going from 80% (ethyl acetate) to approximatively 100% for methylene chloride and the diethylether:methylene chloride mixture.

2.1.3. Derivatization

Unless using an MS detector, BU requires derivatization prior to chromatography either to make it measurable with ECD, UV or fluorescent detectors depending on the technique used.

Every GC method reported has used the derivatization processes first described by Ehrsson et al. [2] with NaI, or by Chen et al. [13] using 2,3,5,6-tetrafluorothiophenol (TFTP) as derivatizing agent.

In 1983, Ehrsson and Hassan added to BU NaI at alkaline pH to form 1,4-diiodobutane. This method was successfully applied by other authors [16,17,20]. Derivatization yield of 91% was reported by Hassan and Ehrsson [3] after optimizing conditions. The addition of high iodine concentration (4 *M*) in a two-phase system, where the ratio $V_{org}/V_{aq}=0.2$ favors BU conversion into 1,4-diiodobutane makes the further concentration step unnecessary. However, this method raised some problems as the reaction rate was found to be different between 1,5-pentanediol dimethanesulphonate, the internal standard and BU [3]. The two compounds are not extracted and converted into diiodoalkanes to the same extent. To circumvent this, other authors have used deuterated BU as internal standard [16,20] as it undergoes the same process as the parent drug BU. Although it was reported by some authors [16] that iodine derivatives were unstable, Lai et al. [14] demonstrated that they were actually stable for 5 days.

In the second method described by Chen, TFTP was used to derivatize BU. Derivatization yield is about 90% according to Burns et al. [12]. This method allowed handling time reduction compared to iodine avoiding the shaking and washing steps [16].

Both derivatization processes seem to be suitable for the detection of BU [2,3,12–14,16,17,20,21]. A good correlation was found between the two techniques [16]. Extraction from plasma were done using ethyl acetate with an extraction efficiency of about 80% [13,16,21].

Diethyldithiocarbamate (DDTC) has been widely used for derivatization of anticancer alkylating agents [4–7,11,15,19,22]. It was first applied for the determination of BU by Henner [4]. DDTC is usually added to plasma to form in alkaline medium (NH_3 or NaOH) with BU the 1,4-bis(diethyldithiocarbamoyl)butane (DDCB). The latter was shown to be stable when dried and 6 h in solution [4]. Derivatization yield was reported to be about 100% [4,5]. Further steps before injection onto the chromatograph include a protein precipitation [4,8], or LLE [7] or SPE [15]. However, it was demonstrated that this derivatization was not reproducible and determination in serum was described as clearly impossible owing to the interferences mostly derived from DDTC [19,22]. To overcome these problems observed with DDTC several solutions have been proposed. MacKichan et al. [15] added a SPE extraction step to further purify the reagent and avoid solvent front but no recovery were reported for BU. Similarly, Chow et al. [6] got rid of DDTC interferences by combining plasma deproteinization with ACN, LLE with ethylacetate and SPE which yielded to a better chromatogram and a better solvent front as well. Funakoshi et al. [19] have proposed on line derivatization associated successively to a clean-up column for DDTC and column switching to increase reproducibility of this process and get a chromatogram free of interferences. The use of Novapak® stationary phase allowed elimination of co-eluting peaks related to DDTC avoiding the solid-phase extraction sample and clean-up described [6,15]. Lastly, Rifai et al. [11] only purchased a purer DDTC from a different chemical supplier to obtain better chromatograms.

Two other derivatization processes were reported [1,9] as the methylsulfonate moieties of BU can undergo nucleophilic substitution by electrophilic molecules such as *p*-thiocresol or 8-mercaptoquinoline. *p*-Thiocresol derivatization was conducted at 70°C in NaOH for 2 h. Derivatization yields were

about 60% [1] and the BU derivative exhibits absorbance at 254 nm. More recently, 8-mercaptoquinoline was added to BU at alkaline pH in presence of tri-*n*-butyl phosphine to avoid the oxidation of the reagent [9]. Hence, the BU derivative exhibits attractive fluorescence allowing improvement of sensitivity.

In conclusion, five different derivatization modes have been described using TFTP, NaI, DDTC, *p*-thiocresol or 8-mercaptoquinoline. TFTP, NaI methods only could be applied to GC while all were coupled to HPLC separation. Regarding GC methods, both NaI and TFTP give convenient results for both ECD and MS detectors [2,3,12–14,16, 17,20,21]. DDTC has been the most extensively used derivatization method before HPLC separation [4,5,7,8,11,15,19,22]. It seems that the DDTC related interferences can be simply circumvented by using purer reagent [8] or certain stationary phases [7]. *p*-Thiocresol gave poor derivatization yield compared to DDTC [1]. NaI was also used as a pre-column derivatization agent leading to 1,4-diiodobutane, which was chromatographed and detected after on-line post-column photolysis.

2.1.4. Gas chromatography (GC)

It seems that using NaI gave interference peaks with ECD detection suggesting that MS should be preferred for pharmacokinetic studies in human biological samples. By contrast, MS is more expensive than ECD, but it should be emphasized that BU monitoring only represents 1% of the total cost of bone marrow transplantation [17]. Best LOD and LOQ were 40 and 2 pg, respectively, according to Lai et al. [14].

GC–MS could be regarded as the definitive methodology for BU analysis in the clinical laboratory considering its specificity. The applications of GC in pharmacokinetic studies have been long established for their sensitivity. However, for the last 6 years, HPLC has been the most used method. Details of the different techniques are summarized in Table 1.

2.1.5. High-performance liquid chromatography (HPLC)

HPLC separating BU as DDTC derivative was successively improved to yield to quite a simple and sensitive assay without any interference related peak [8]. The method described by Funakoshi et al. who used on line DDTC derivatization is pretty smart in terms of chromatographic settings [19]. However the latter do not seem to be necessary when chromatographing on certain stationary phases [6,7] or adding purer DDTC reagent to BU [8].

The only HPLC-FLUO method described seems to be very attractive considering its sensitivity performance [9]. The method using post-column photolysis with NaI seems to be quite unspecific (large peak width of about 8 min, detection set at 226 nm) and requires a reactor which is technically critical to set up in any laboratory for routine analysis [23]. The only assay using TFTP requires a prohibitive time analysis [10].

The LC–MS should be considered as it requires no time-consuming derivatization steps and exhibits sensitivity comparable to other reported assays [24].

It could be very useful when synthesizing it to choose an internal standard (I.S.) with a shorter alkyl chain to make elution earlier [11]. Pap et al. [8] have simply used BAD (1-bromo-1-deoxy-3,6-anhydrogalactilol) as I.S. which is commercially available.

All HPLC assays fulfill requirements for pharmacokinetic measurements in terms of sensitivity and sample handling time except the initial work reported by Henner [4]. However, time analysis, sample handling procedure, sensitivity make the methods developed by Pichini et al., Pap et al. and Peris et al. [8,9,24] the most attractive. Details of the different techniques are summarized in Table 1.

2.2. Treosulfan [25]

Treosulfan (Fig. 2) was synthesized in 1961 and represents a prodrug of an alkylating agent with activity in ovarian carcinoma and other solid tumors; its alkylating potency is therefore related to the active mono- and diepoxybutane. Hilger et al. [25] reported an RP-HPLC assay with refractometric detection to measure treosulfan.

Separation was achieved on Nucleosil C_{18} column using mobile phase composed of phosphate buffer (pH 5.0):EDTA. LOQ was 10 ng in plasma and 200

Table 1
Summary of the different methods to assay busulfan in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–MS	Plasma	LLE	NaI	Q: 20 pg	4	[2]
GC–ECD	Plasma	LLE	NaI	Q: 10 pg D: 1 pg	5	[3]
GC–MS	B fluids	LLE	NaI	D: 10 pg	3.5	[20]
GC–ECD	Plasma	LLE	TFTP	D: 20 pg	3	[13]
GC–ECD	Plasma	LLE	TFTP	D: 60 pg	3	[21]
GC–ECD	Plasma	LLE	TFTP	D: 20 pg	3	[12]
GC–MS	Plasma	LLE	TFTP	Q: 20 pg D: 10 pg	12	[16]
GC–MS	Cells	LLE	NaI	Q: 625 pg D: 25 pg	3.9	[17]
GC–MS	Plasma	LLE	NaI	Q: 40 pg D: 2 pg	4.5	[14]
LC–UV	Plasma	LLE	DDTC	D: 30 μg	12	[4]
LC–UV	Plasma	LLE	p-thiocresol	D: 5 ng	11	[1]
LC–UV	Plasma	LLE/SPE	DDTC	Q: 1 ng	4.3	[15]
LC–UV	Plasma	SPE	NaI	D: 1 ng	20	[23]
LC–MS	Serum/CSF	LLE	–	Q: 2 ng D: 1 ng	2	[24]
LC–UV	Serum	LLE	DDTC	Q: 1 ng	16	[19]
LC–UV	Pharm Prep Plasma	LLE/SPE	DDTC	D: 3 ng	7.5	[6]
LC–UV	Plasma	LLE	DDTC	D: 0.6 ng	8.3	[7]
LC–UV	Plasma	LLE	DDTC	D: 5 ng	2.8	[11]
LC-FLUO	Plasma	LLE	8-MCQ	Q: 0.2 ng D: 0.4 ng	12	[9]
LC–UV	Plasma	LLE	TFTP	Q: 2.5 ng D: 1.25 ng	27	[10]
LC–UV	Plasma	LLE	DDTC	Q: 2.5 ng D: 1.25 ng	8.4	[8]

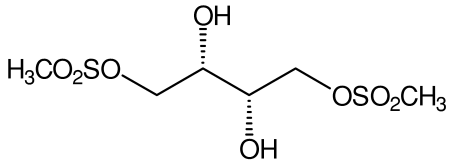


Fig. 2. Structure of treosulfan.

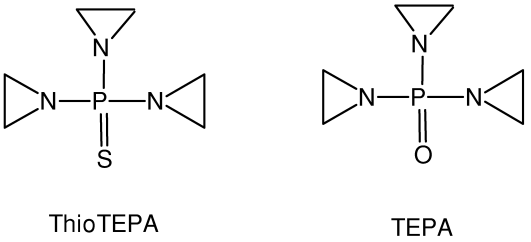


Fig. 3. Structures of ethylenimines (ThioTEPA and TEPA).

ng in urine. Plasma was separated by centrifugation and then microfiltration.

3. Ethylenimines and methylmelamines

3.1. ThioTEPA and TEPA

The alkylating agent thioTEPA (Fig. 3) has been applied in cancer therapy for more than 40 years. ThioTEPA possesses a broad spectrum of antitumor activity and is employed in high dose with combination regimen for breast cancer, ovarian cancer and other solid tumors [26]. ThioTEPA is metabolized to TEPA (Fig. 3) which also acts as an alkylating agent and other metabolites which have been recently identified [27]. The toxicity at standard doses appears to be confined to the bone marrow [28].

Pharmacokinetic studies are required to establish optimal administration regimen and several analytical methods have been developed since 1985 [27,29–34]. On the other hand, thioTEPA is very unstable and much attention has been paid to compatibility and stability studies in solution [35,36].

3.1.1. Drug stability [27,30,35–38]

Biological samples can be kept frozen for 2–3 months to guaranty stability of thiotepa, TEPA and thiotepa mercapturate [27,30,37]. Two freeze–thaw cycle on biological samples did not show significant differences on drug and related product concentrations. Thiotepa contained extract can be kept for 24 h while TEPA should be assayed in less than 14 h when extracted from plasma.

A recent paper from Van Maanen describes the degradation process of thiotepa in solution leading to the formation of mono and dichloro adducts as well as the 1,2,3-trichloroethythiophosphoramide [38].

3.1.2. Extraction from biological fluids

Extraction has been shown to be one of the key step of thiotepa determination in biological fluids. Both LLE [28,29,31,32] and SPE [31,33,34,39] were reported with contradictory results.

The extraction procedure involving ethyl acetate has proved to be satisfactory in pharmacokinetic studies although the recovery (when reported) was low [29,32,40,41]. When chloroform was used [29,40], recoveries were slightly improved (getting about 60%). However, it should be considered that lower recoveries were obtained when organic layer was evaporated under nitrogen to complete or near dryness [33]. More recently, Van Maanen investigated different ways of increasing extraction recoveries for LLE [30,37]. To gain a good sensitivity, they tried protonating thiotepa or using other solvent and/or mixtures of solvent. Best results were obtained with propan-1-ol–chloroform (25:75, v/v) including evaporation to a final volume of approximatively 20 μl at room temperature under nitrogen. Extraction recoveries were of 70–80% for thiotepa and TEPA in plasma or urine [30].

SPE was carried out using different column (C_{18} or ion-exchange) [31,33,34,37,39] but required approximatively 1 ml of sample. Overall recoveries were about 40% [33,42].

3.1.3. Derivatization

There is only one report by Sano et al. [33] which developed a fluorogenic reaction of thiotepa based on condensation with *o*-phthaldehyde. Both thiotepa and TEPA yield to fluorescent derivatives although TEPA derivative was two times less fluorescent than the thiotepa one. The major drawback of this reaction was the poor stability of the product formed (less than 20 min) which could explain why this method was only described once.

3.1.4. Gas chromatography (GC)

GC–NPD has been the most extensively described method the [28–31,37,39–41], whereas GC–MS was only reported by Van Maanen [30] for determination of thiotepa and TEPA in biological fluids. Internal standards commonly used are diphenylamine [40,41] or hexaethylphosphoramide [31]. Relative sensitivities are pretty uneasy to compare between the different methods due to erratic extraction recoveries reported. Best LOD were obtained with NPD detectors. LOD as low as 1.5 ng/ml for TEPA and 2.5 ng/ml for thiotepa were obtained in plasma [30]. Chromatographic details are summarized in Table 2.

3.1.5. High-performance liquid chromatography (HPLC)

In all papers [33–36], thiotepa and TEPA were separated on a C_{18} column with simple mobile phase composed of water and methanol or acetonitrile. Best chromatographic profile was reported by Sano et al. corresponding to the OPA derivatives of thiotepa and TEPA [33]. The stability indicating method reported by Murray et al. [35] allowed to chromatograph thiotepa and the chloroadducts which are the result of a chemical reaction between thiotepa and sodium chloride. Considering the few HPLC methods published and the related LOD obtained, it seems that HPLC is more suitable to carry out stabilities studies. However, the work developed by Sano et al. [33] exhibited very good sensitivity but was limited by the sample processing step. Chromatographic details are summarized in Table 2.

3.2. Altretamine [43]

A isocratic HPLC method has been developed and validated for the determination of altretamine (Fig.

Table 2
Summary of the different methods to assay thiotepa in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–NPD	Plasma/CSF	LLE		D: 1 pg	?	[40]
GC–NPD	Plasma	LLE		D: 10–100 pg	4.9 Tt 5.4 T	[29]
GC–NPD	Plasma	SPE		D: 1–5 pg	3.5 Tt 3.1 T	[31]
GC–NPD	Plasma	LLE		D: 10–100 pg	4.9 Tt 5.4 T	[28,41]
GC–NPD	Plasma Urine	SPE		D: 1–5 pg	?	[39]
LC–FLUO	Plasma	SPE	*o*-PA	D: 0.2 ng Tt D: 0.4 ng T	17 Tt 4 T	[33]
LC–UV	Plasma	SPE	–	D: 2.5 ng Tt	11 Tt	[34]
LC–UV	Pharm prep	–	–	–	6.5 Tt	[36]
LC–UV	Pharm prep	–	–	–	7.5 Tt	[35]

Tt, thiotepa; T, TEPA.

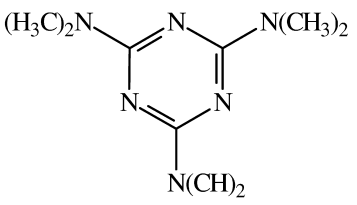


Fig. 4. Structure of altretamine.

4) in human plasma using a conventional C_{18} column. Extraction was carried out with acetonitrile at 4°C and the precipitate removed by filtration. Clean-up procedure is rapid but not satisfactory considering the impurities related to plasma. Although the altretamine peak is well separated, the determination of the metabolites (i.e., pentamethylmelamine and 2,2,4,6-tetramethylamine) seemed to be very critical. LOQ was 3 ng which allowed measurement of altretamine up to 360 min after ingestion. This method demonstrated that altretamine peak concentration was lowered by food intake.

4. *N*-Mustards

4.1. Mechlorethamine (aliphatic mustard)

Mechlorethamine or nitrogen mustard (HN_2, Fig. 5) is an antineoplastic agent used in the treatment of a wide variety of malignant tumours such as Hodgkin and non-Hodgkin lymphomas. It has been largely replaced in combination chemotherapy regimens by more stable bi-functional nitrogen mustard such as melphalan and cyclophosphamide, which have a better therapeutic index. However, another role has emerged for this drug in the treatment of psoriasis, as the reason is found in literature is a long-term stability study in ointment. Kirk et al. [44] described in 1986 a LC technique with electrochemical detection for the study of long-term stability of reconstituted mechlorethamine in various solvents (0.9% NaCl, water) and stored in syringes or PVC infusion bags. No extraction was required for this purpose.

As Cummings et al. [45] have described, mechlorethamine can be determined by HPLC with ultraviolet detection using a derivatization procedure with diethyldithiocarbamic acid (DDTC). The nitrogen mustard reacts with two equivalents of DDTC leading to disubstituted DDTC adducts of NH_2, which are then extracted from aqueous solutions or plasma samples using a solid-phase extraction on Bond-Elut minicolumns containing phenyl-bonded silica gel.

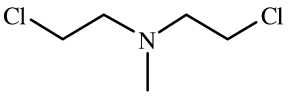


Fig. 5. Structure of mechlorethamine.

After activation with 2 ml of methanol, followed by 2 ml of water (wash), the samples are poured onto the columns. The derivatives were eluted with 2 ml of acetonitrile which were evaporated to dryness. A solid-phase extraction from plasma for HN_2–$DDTC_2$ was set with more than 90% efficiency but when the drug is derivatized in plasma, the recovery dropped to 50% for HN_2. The retention time of HN_2–$DDTC_2$ was 13 min with a limit of detection on column found at 0.5 ng for HN_2. Due to the high instability of HN_2 in plasma, it is essential to trap the reactive mustard in whole blood immediately after the samples collection. The identification of HN_2–DDTC adducts was done using FAB-MS. This derivatization procedure leads to the production of disubstituted adducts via monosubstituted adducts.

Due to the lack of chromophore, the analytical determination is not frequently described in the literature. To our knowledge, these two methods seem to be the only ones used for HN_2. For an ultraviolet detection, derivatization is obviously required, but the method is complicated and not suitable for a routine analysis and shows a lack of sensitivity. The electrochemical detection used by Kirk et al. [44] avoided derivatization of HN_2 but no details of the method validation was communicated by the authors.

4.2. Chlorambucil (aromatic mustard)

Chlorambucil (CLB) is one of the nitrogen mustard derivatives series. The chemical combination of the reactive bis-(2-chloroethyl)amino group with an aromatic carboxylic acid system was built to facilitate the passage of the potent alkylating function through membranes and hence between tissues. The drug has become a valuable antineoplastic agent, widely used in the treatment of lymphatic disorders as well as ovarian cancer. CLB is rapidly and completely absorbed from the gastrointestinal tract. A significant portion of the drug is oxidized at the butyric acid side chain to yield phenylacetic acid mustard (PAAM). The latter, is also an active DNA cross-linking agent and contributes significantly to the activity of the drug. CLB and its major metabolite are shown on the Fig. 6.

4.2.1. Extraction from biological fluids

4.2.1.1. Protein precipitation (PP) and liquid–liquid extraction (LLE). Most reported methods in the literature describe a liquid–liquid extraction for chlorambucil and its metabolites. A method developed by Leff et al. [46] involved LLE using chloroform and derivatization by esterification with various alcohols. Jakhammer et al. [47] reported a LLE procedure using chloroform–hexane (3:7) and a back-extraction using a pH 9.0 buffer solution to allow the separation of two alkylating agents; prednimustine (PDM) and chlorambucil. The first one was collected in the organic layer and CLB in the aqueous phase. In 1980, Ehrsson et al. [48] reported a LLE of chlorambucil from plasma with methylene chloride at pH 3 for 30 min. The authors have shown that the pH of the mixture has its importance with 60% yield at pH 1, 56% at pH 7 and total recovery at pH 3.2. Ahmed et al. [49] used acetonitrile for the extraction of chlorambucil while Chandler et al. [50] reported a solvent extraction of chlorambucil from rat liver microsomes using methylethylketone (MEK) with an extraction recovery of 99.1%. Deproteinization was obtained after thorough mixing and centrifugation, leading to a supernatant removed and dried under vacuum with 91% recovery. Greig et al. [51] described a LLE of plasma samples for the determination of chlorambucil and its main metabolite using a mixture of organic solvents (3 ml acetonitrile, 1 ml methylene chloride and 2 ml of

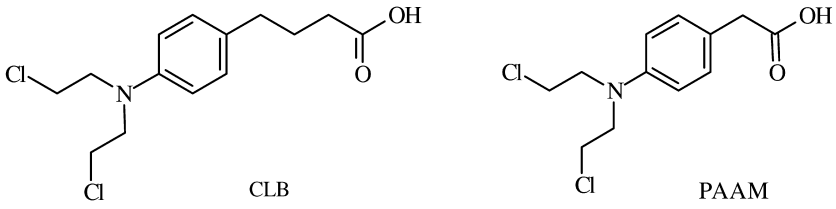


Fig. 6. Structures of chlorambucil (CLB) and phenylacetic acid mustard (PAAM).

hexane). After shaking and centrifugation, the top two phases were removed and evaporate to dryness. Most of these liquid–liquid extractions offer good recoveries with low investment even though they are time-consuming procedures. Workman et al. [52] extract chlorambucil from plasma (100 μl) by the addition of 1 ml methanol.

4.2.1.2. Solid-phase extraction (SPE). Few solid-phase extraction procedures were reported in the literature. Adair et al. [53] described a combination of protein precipitation using concentrated perchloric acid and a SPE procedure using C_{18} Sep-pak columns. Oppitz et al. [54] used column Extrelut 3 for the extraction procedure of chlorambucil from plasma. After 15 min, the analyte was eluted using 10 ml ethyl acetate followed by 10 ml 85% *n*-hexane–15% isoamyl alcohol with a mean recovery rate ($n=10$) of 95%.

Recently, Davies et al. [55] reported a 96-well SPE. Samples were extracted using 96-well Microlute solid-phase extraction blocks packed with 15 mg of C_{18} sorbent per well. The wells were activated with 150-μl aliquots of acetonitrile followed by 0.1% (v/v) formic acid for conditioning; 200 μl of sample were then loaded. The wells were washed with further 150-μl aliquots of 0.1% (v/v) formic acid and the compounds were then eluted from the sorbent with 100 μl of mobile phase containing 0.1% (v/v) formic acid in acetonitrile mixed in the ratio 30:70 (v/v). The mean recovery rate ($n=8$) for CLB was determined to be at 88.9% for chlorambucil and 91.5% for PAAM at concentrations of 50 ng/ml.

In this method, the 96-well SPE reduced significantly the sample preparation time but this expensive equipment is not available in each routine laboratory especially in hospital environments.

Various analytical procedures are reported for the quantification assay of chlorambucil including the colorimetric determination of 4-(*p*-nitrobenzyl)-pyridine derivatives using TLC, LC with either ultraviolet or electrochemical detection and GC with mass spectrometry associated or not to a derivatization procedure.

4.2.2. High-performance liquid chromatography (HPLC)

In the earlier study, the sensitivity of the developed method was no less than 100 ng/ml (1 ng on column) due to the extraction recoveries and the sensitivity of the detection. At the end of the 1980s, improvement in extraction procedures from biological fluids led to lower limits of detection: as low as 25 ng/ml (0.25 ng on column). But the most important improvement in analytical method was the possibility to use LC methods coupled to tandem mass spectrometry which lowered the limit of detection at ng/ml level of plasma (10 pg on column). The automatization of the extraction procedures using SPE reduces considerably the time consumption of these determination methods but the analysis material cost for has considerably increased. An alternative to LC–MS methods is the use of GC–MS but the derivatization procedure is complicated and also time consuming. For these nitrogen mustards carried by aromatic compounds, no derivatization procedures were developed except to increase the volatility of the compounds when a GC method is used.

Leff et al. [46] reported a simple HPLC method with ultraviolet detection operating at 254 nm for the determination of the drug levels in plasma and the pharmacokinetics of CLB in ovarian carcinoma. The authors reported a LOQ of 50 ng/ml (0.5 ng on column). Newell et al. [56] reported a gradient HPLC method for CLB and its major metabolite, phenyl acetic mustard, using a RP C_{18} column. The retention time was 8 min and the LOQ 0.05 μ*M* (0.15 ng) with an extraction efficiency of 77.5%.

Chatterji et al. [57] described a stability specific high-performance liquid chromatography method for the kinetic study of CLB hydrolysis. The system described is a reversed-phase column with methanol–acetonitrile–0.01 *M* acetate buffer, pH 4.5, using an ultraviolet detection set at 245 nm. The retention time for CLB was found at 10.6 min and the LOQ was found at 10 μg/ml (100 ng on column). The same year, Ehrsson et al. [58] reported a similar high-performance liquid chromatography method using UV detection at 257 nm and methanol–water containing 0.01 *M* phosphoric acid as mobile phase for the same purpose, to follow the degradation kinetic of CLB in aqueous solutions. Zakaria et al. [59] have developed a method for the determination of CLB and its major metabolite in plasma with a retention time at 15 min and the LOD was found at 3.6 ng. These methods with ultraviolet

detection do not provide the required sensitivity to study the drug pharmacokinetics or its metabolism in humans.

Workman et al. [52] reported an isocratic, ion-pair RP high-performance liquid chromatography method suitable for the assay of CLB and various analogues in biological fluids with a twin-channel UV detection set to record at 254 and 280 nm. The mobile phase was methanol–water–tetrabutylammonium hydroxide adjusted to pH 7.4 with orthophosphoric acid. The retention time for CLB was 4 min and the LOD was found at 60 ng/ml of plasma (0.55 ng on column).

Oppitz et al. [54] described a LC–UV method to study simultaneously the pharmacokinetic of CLB, its metabolite (PAAM) and prednimustine (PDM) in patients. The time-analysis increased to 12 min due to the number of analytes but the LOD was 30 ng/ml (1.2 ng on column with 40 μl injected).

A gradient high-performance liquid chromatography method with UV detection set at 254 nm was reported by Greig et al. [51] to allow the determination of CLB and CLB *tert.*-butyl ester as internal standard simultaneously to PAAM and phenyl propionic mustard as internal standard in plasma and tissue. The retention time was 14 min and the LOD 25 ng/ml (5 ng on column) for CLB. In 1994, Chandler et al. [50] reported a RP-HPLC method using UV detection to distinguish CLB *N*-oxide, a prodrug of CLB, from CLB and quantitate both after separation from biological samples like rat liver microsomes. The wavelength of 249 nm was chosen for the analysis of both compounds. After LLE, CLB was analysed in 30 min to allow the separation from *N*-oxide derivative with a LOQ found at 40 ng.

More recently, Davies et al. [55] reported a high-performance liquid chromatography tandem mass spectrometry (LC–MS–MS) method using a 96-well SPE to allow the rapid determination of CLB and its major metabolite in human serum and plasma. The time analysis did not exceed 3 min with a LOQ found at 4 ng/ml or 8 pg on column (20 μl injected, split 1:9). This high specificity is due to the nature of the detector used in selected reaction monitoring (SRM) mode. Obviously, this recent technique is the most sensitive and rapid method for the determination of this alkylating agent but this material is still very expensive even for the monitoring of antineoplastic treatment.

4.2.3. Gas chromatography (GC)

Gas chromatography–mass spectrometry satisfies analytical criteria, sensitivity and accuracy in the study of the pharmacokinetics of CLB and its metabolite. This method was used by Chang et al. [60] after LLE and derivatization with bis-(trimethylsilyl)trifluoroacetamide leading to trimethylsilyl derivatives and a LOD calculated at 0.5 ng. In 1977, Jakhammer et al. [47] described a GC–MS method for the simultaneous determination of prednimustine (PDM) and CLB using mass fragmentometry of CLB methyl ester obtained by transesterification of PDM or by esterification of CLB after previous separation. Transesterification of PDM and esterification of CLB were conducted using methanol and boron trifluoride diethyl etherate. Deuterated internal standard substances were used for the quantification analyses and the detection limit was found to be 8 ng/ml with 5 μl injected for both compounds (40 pg on column). Obviously, this method is not easy to employ for a routine use. Ehrsson et al. [48] described a GC–MS method with SIM mode offering a selective and sensitive method with a LOD of 1 ng/ml ca. 1–2 pg on column. CLB was converted to a thiazane derivative by reaction with sodium sulphide and the carboxylic group of CLB derivative is derivatized by alkylation with allyl bromide. As usual, GC is the most sensitive but also the most time-consuming method because of the need of derivatization before analysis.

4.2.4. Thin-layer chromatography (TLC)

In 1973, Norpoth et al. [61] described a TLC procedure for various alkylating agents. The method used 4-pyridinecarboxaldehyde-2-benzothiazolylhydrazone (4-PBH) as reagent and plates heating over an acetophenone bath for 20 min at 200°C. The LOD of this technique is 0.66 nmol for CLB corresponding to 200 ng injected.

Except TLC, which allows the simultaneous determination of numerous substances for a semi-quantitative purpose, chromatographic determination of CLB using GC or LC are numerous in literature. The use of mass spectrometry as detection mode leads to very low LOD/LOQ in contrast to UV detection. GC combined to MS allows picogram levels of LOD but the development of LC–MS coupling technique reaches similar sensitivity avoiding time-consuming and complicated derivatization procedures as for GC

Table 3
Summary of the different methods to assay chlorambucil in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)-	Ref.
GC–MS	Plasma Urine	LLE	TMS	D: 0.10 ng Q: 0.50 ng		[60]
GC–MS	Plasma	LLE	Transesterification/esterification	D: 40 pg	3.5	[47]
GLC–MS	Plasma	LLE	Thiazane alkylation	D: 1–2 pg	8	[48]
LC–MS–MS	Serum/plasma	96-well SPE		Q: 8 pg	2	[55]
LC–UV	Plasma	LLE	Alcohols	Q: 0.50 ng	3–6	[46]
LC–UV	Plasma	LLE		Q: 0.15 ng	8	[56]
LC–UV	Plasma	LLE		D: 3.60 ng	15	[59]
LC–UV	Aqueous sol.			Q: 100 ng	10	[57]
LC–UV	Plasma	LLE		D: 7.5 ng	8	[49]
LC–UV	Plasma	LLE/SPE		D: 2 ng	4	[67]
LC–UV	Plasma	LLE		D: 0.55 ng	4	[52]
LC–UV	Plasma	SPE		D: 1.20 ng Q: 4 ng	12	[54]
LC–UV	Plasma/tissue	LLE		D: 5 ng	14	[51]
LC–UV	Plasma/tissue	LLE		Q: 40 ng	30	[50]
TLC			4-PBH	Q: 200 ng		[61]

method. Some characteristics of the different methods are summarized in Table 3. LLE procedures are numerous for CLB and provides good extraction recoveries while SPE procedures are less often reported in literature. Nevertheless, the reported recoveries are superior by 88% for the Oppitz et al. [54] and Davies et al. [55] procedures.

4.3. Melphalan (aromatic mustard)

In contrast to CLB, the bioavailability of melphalan (L-PAM, Fig. 7) after oral administration is extremely variable and the drug undergoes rapid chemical degradation with little active metabolism. Melphalan shows limited in vitro stability, which is affected by the pH and chloride ion concentration. Methods applied to L-PAM analysis include LC, GC with various detection mode (UV, fluorescence, EC, MS). A large number of analytical methods for the determination of melphalan levels have been reported in the literature.

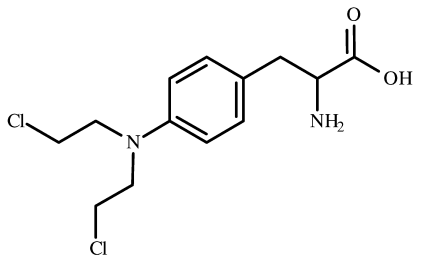


Fig. 7. Structure of melphalan (L-PAM).

4.3.1. Extraction from biological fluids

4.3.1.1. Protein precipitation (PP) and liquid–liquid extraction (LLE). Davis et al. [62] described a method with protein precipitation of 1 ml of plasma with concentrated perchloric acid, followed by direct injection while Sweeney et al. [63] reported a methanol protein precipitation and a derivatization procedure using *o*-phthalaldehyde for a fluorescent detection. Silvestro et al. [64] also reported a protein precipitation by 0.1 ml of perchloric acid (60%) followed by chloroform washing of the supernatant which is then directly injected onto the LC system. The method reported by Pinguet et al [65] involved a simple treatment of the samples with cold methanol and protein denaturation with full speed vortex mixing and centrifugation for 10 min at 4°C, as described by Wu et al. [66].

4.3.1.2. Solid-phase extraction (SPE). Adair et al. [67] reported a similar acidic protein precipitation clean-up procedure followed by solid-phase extraction of the supernatant using a C_{18} reversed-phase Sep-pak. The melphalan was eluted from the cartridge with 2 ml of cold methanol and stored at −20°C prior to injection onto the column. This extraction procedure was described previously by Egan et al. [68]. In the same way, Bosanquet et al. [69] used SPE with automation procedure using for the extraction step, XAD-2 resin columns eluted with methanol. A recent work reported by Davies et al. [70] describes the use of rapid automated 96-well solid-phase extraction using samples and solvent volumes of less than 200 μl. Muckenschnabel et al. [71] described a solid-phase extraction procedure for the determination of melphalan in various biological fluids such as serum, plasma, limb perfusate samples or foetal calf serum. To 400 μl of those samples, 300 μl of ice cold mobile phase buffer were added, and passed through an activated 200-mg LiChrolut reversed-phase RP-18 cartridge. After washing with 2 ml of mobile phase buffer, the cartridges were left at room temperature for 10 min. The analytes were then eluted with 1.5 ml methanol. The authors tried, at first, to adapt the extraction procedure previously developed for plasma using Sep-pak C_{18} cartridge with no satisfactory results. They compared the recoveries of melphalan from mobile phase buffer on four different cartridges; Adsorbex 100 mg, LiChrolut packed with 100 and 200 mg of reversed-phase RP-18 material and LiChrolut 200 mg. The reported recoveries were, 91.5±3.3, 36.9±2.2, 58.4±2.5 and 46.8±3.0%, respectively. De Boeck et al. [72] described the extraction of L-PAM from plasma performed by solid-phase extraction over trifunctional C_{18} and cyanopropyl silica columns. The authors have compared the extraction efficiency of L-PAM from samples buffered at pH 7 and reported higher extraction recovery with C_{18} than with cyanopropyl columns with more than 80% for both.

The separation of melphalan is hampered by its instability and by the very high solubility of the compound in water. In addition, the amphoteric nature of the molecule makes solvent extraction unsatisfactory. Consequently, the described chromatographic methods have depended on protein precipitation followed by direct measurement in the supernatant which inevitably involves a dilution of the original biological sample. Kato et al. [73] reported an automated column-switching HPLC method whereby 100 μl of plasma were injected into the HPLC system and the endogenous material separated on a pre-treatment column. Plasma samples are loaded onto this precolumn with an aqueous mobile phase, retaining the sample to be analyzed and flushing the solubilized plasma proteins to be discarded. The retained compound is eluted onto the analytical column using the chromatographic mobile phase.

4.3.2. High-performance liquid chromatography (HPLC)

HPLC with UV detection mode is the most widely used as analytical tool for L-PAM detection or quantitation but these described methods lack in sensitivity [63,69,74–78]. The method reported by Cummings et al. [74] for the determination of L-PAM, presents a derivatization procedure using diethyldithiocarbamate (DDTC) to allow an ultraviolet detection at 276 nm with a LOD at 1 ng for L-PAM. This derivatization procedure which presents a 93% yield reaction lead to the formation of two products; the disubstituted adduct and an intermediate form which appears in first and persists out to 4 h. The authors have modified the conditions to improve the formation of the disubstituted adduct, with a reaction time set at 90 min and the temperature set at 50°C instead of 37°C for the other drugs reported to be derivatized with DDTC. Using this derivatization, it might be important to assess the derivatization reproducibility and to control the formation rate of each compound. Furthermore, this method was reported for the determination of L-PAM and HN_2 but unlike the latter, no extraction data were reported for L-PAM. Recently, Brightman et al. [79] described a gradient HPLC method for the determination of melphalan and related impurity content in a pharmaceutical form with a ultraviolet detection set at 260 nm.

Fluorescence detection with HPLC enables monitoring of L-PAM levels at the low ng/ml [66,68,71]. Egan et al. [68] used, for the quantitation of melphalan and its hydrolysis products, a phenyl column and fluorescence detection with a detector set at 265 nm for the excitation wavelength and 350 nm for the

emission one. The LOQ was found at 0.5 ng on column with a injected volume of 20 μl. Wu et al. [66] described a similar procedure for the quantitation of L-PAM and its hydrolysis products in various biological samples. The LOQ were similar to those found by Egan et al. [68]. Ehrsson et al. [80] described a HPLC procedure using fluorimetric detection for the quantitative determination of melphalan after derivatization with *N*-acetylcysteine which allows the determination of L-PAM concentrations exceeding 5 ng/ml of plasma (0.5 ng on column). In 1997, Muckenschnabel et al. [71] described a RP 18 ion-pair HPLC method with fluorimetric detection for the determination of melphalan from aqueous solutions and from biological materials involved in hyperthermic isolated limb perfusion. The LOQ was found at 2.6 ng with a injected volume of 30 μl.

Silvestro et al. [64] reported a reversed-phase HPLC method with electrochemical detection. The advantage of this detection mode is the high sensitivity of the electrode redox reaction involving the amino groups present in melphalan molecule, which allowed to omit the derivatization and allowed the evaluation of blood levels in patient receiving conventional oral doses of the drug. The detection limit was ca. 100 pg injected, corresponding to a plasma sample concentration of 2 ng/ml. This very sensitive detection system can be used for routine analyses avoiding sample pre-treatment procedure except protein precipitation.

In the last decade, liquid chromatography with mass spectrometry detection has been described. Bean et al. [81] reported on solvent–sample interactions in thermospray MS with a low limit of detection for L-PAM at 150 ng on column. Recently, similarly to CLB, melphalan was chromatographed by Davies et al. [70] in less than 2 min since the mass selective nature of the detection instrument allowed the resolution of the isotopically labelled internal standard. The use of MS–MS provides greater specificity than UV detection. The mean recovery was determined to be 74% with this 96-well SPE procedure and the LOD of the technique was found at 2 ng/ml (4 pg on column).

4.3.3. Gas chromatography (GC)

L-PAM analysis using GC with MS (SIM) method has already been developed at the end of the 1970s by Pallante et al., as Tjaden and de Bruijn reported in their review [82]. The method described a complicated procedure with derivatization of L-PAM with trifluoroacetic acid (TFA), followed by esterification with diazomethane. Before, Goras et al. [83] described a GLC–FID procedure with bis-(trimethylsilyl) acetamide (BSTFA) to afford trimethylsilyl derivatives (L-PAM-TMS). More than one derivative can be found with this derivatization procedure. In fact, a mixture of hydrophilic decomposition products was prepared, silylated and chromatographed. Three compounds have been identified. This means that the method determines the amount of those three derivatives but no evidence of control of the relative proportion of those decomposition products was furnished. More recently, de Boeck et al. [72] described a GC–MS procedure for the determination of melphalan and its hydrolysis products in body fluids. L-PAM levels were determined after trifluoroacetylation with TFA of the amino formation while the carboxylic function is methylated with diazomethane. The retention time of 11 min was found and the LOQ was 40 pg on column.

GC is not the most frequently used chromatographic method for the analysis of this alkylating agent according to the literature since 1970.

4.3.4. Thin-layer chromatography (TLC)

For the quantitation of CLB, Norpoth et al. [61] have used a TLC procedure for the quantitation of L-PAM. The derivatization reagent was the same (4-pyridinecarboxaldehyde-2-benzothiazolylhydrazone (4-PBH)) and the LOD of this technique was 0.66 nmol for L-PAM corresponding to 200 ng sprayed.

In contrast to CLB, SPE procedures are more elaborate and better described than PP/LLE for L-PAM. Although, SPE cartridge are expensive, SPE should be used because of their repeatability and their recoveries. As for CLB, LC methods using fluorescence or mass spectrometry detection avoid derivatization procedures as in GC methods. The use of LC–EC methods allows picogram levels of LOD. Moreover, MS and gas chromatography–chemical ionization-mass spectrometry provide good results, but they involve the use of expensive equipment and

Table 4
Summary of the different methods to assay L-PAM (melphalan) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)-	Ref.
GC–MS	Plasma	SPE	TFA	Q: 40 pg	11	[72]
GLC–FID	Pharm. prep.		TMS		13	[83]
LC–EC	Plasma	PP/LLE		D: 100 pg	15	[64]
LC–FLUO	Plasma/tissue	PP/LLE	*o*-Phthalaldehyde	D: 10 ng	4.5	[63]
LC–FLUO	Plasma	PP		Q: 1.4 ng	9	[66]
	Tissue			Q: 7.2 ng		
LC–FLUO	Plasma/tissue	PP/SPE		D: 0.5 ng	15	[68]
LC–FLUO	Blood	SPE		D: 100 pg	10	[162]
	Plasma			Q: 600 pg		
LC–FLUO	Plasma	PP	*N*-Acetylcyst.	Q: 0.5 ng		[80]
LC–FLUO	Biol. material	SPE		Q: 2.6 ng	8	[71]
LC–MS						[81]
LC–MS–MS	Serum/plasma	96-well SPE		Q: 4 pg	2	[70]
LC–UV	Plasma	PP		D: 1 ng	18	[62]
LC–UV	Plasma	PP	DDTC	D: 1 ng	15	[45]
LC–UV	Plasma	PP		D: 0.4 ng	4	[65]
				Q: 1 ng		
LC–UV	Plasma	PP/SPE		D: 1 ng	9.5	[67]
LC–UV	Plasma	SPE		Q: 1 ng		[69]
LC–UV	Perfusate	PP		Q: 160 ng	6	[78]
LC–UV	Blood	PP		Q: 1 ng	12	[76]
LC–UV	Plasma	PP/LLE		D: 0.5 ng	4	[77]
LC–UV	Drug subst.				10	[79]
LC–UV	Plasma	Pre-column		D: 1 ng	15	[73]
TLC			4-PBH	Q: 200 ng		[61]

time-consuming sample pre-treatment and are not easily available for routine drug monitoring. Details of the different methods are summarized in Table 4.

4.4. Prednimustine (carbamoyl mustard)

Prednimustine (PDM, Fig. 8) can be considered as a prodrug of chlorambucil as it is the prednisolone ester of this alkylating agent used for the treatment of various malignancies including chronic lymphocytic leukemia (CLL). Chlorambucil has been widely used in the therapy of CLL and malignant lymphomas and in order to improve its clinical antitumor efficacy, CLB was often combined to prednisolone. Subsequently, the prenisolone ester of CLB, prednimustine was introduced into therapy

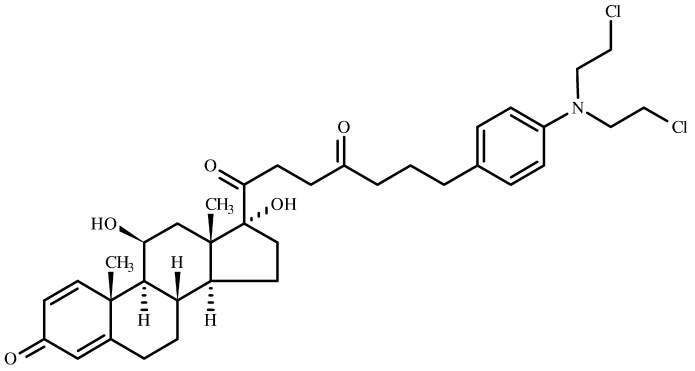


Fig. 8. Structure of prednimustine (PDM).

Table 5
Summary of the different methods to assay prednimustine (PDM) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–MS	Plasma	LLE	Transesterification/ esterification	D: 40 pg	3.5	[47]
LC–UV	Plasma	LLE		Q: 22 ng/ml	11	[56]
LC–UV	Plasma	SPE		D: 30 ng/ml Q: 100 ng/ml	18	[54]

with the expectation that the steroid group would facilitate the uptake of the alkylating compound into tumour cells via cellular glucocorticoid receptors. After administration, no intact prednimustine was found in plasma whereas its constituents CLB and prednisolone could be detected. In addition, phenyl acetic mustard, the β-oxidation product of chlorambucil, was found after the oral administration of prednimustine. Many analytical methods have been described for the determination of CLB in biological fluids but very few methods have been reported for the analysis of prednimustine.

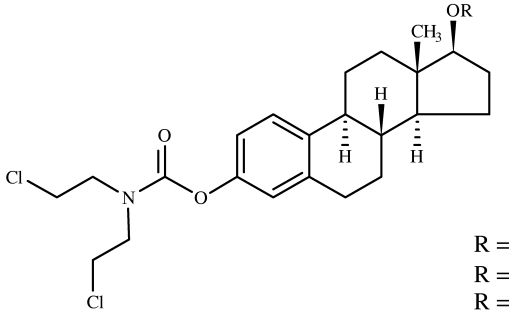
We have seen previously that Jakhammer et al. [47] reported a GC–MS method for the determination of CLB and PDM with a LOD also found to be 8 ng/ml (40 pg on column) for PDM.

In 1979, Newell et al. [56] reported an HPLC method for the estimation of prednimustine, CLB and phenylacetic acid mustard in human plasma. The method involved an ultraviolet detection set at 254 nm and the compound separation was accomplished on an RP C_{18} column. Elution occurred by running a linear gradient from methanol–0.175 *M* acetic acid (60:40, v/v) to 100% methanol at 2 ml/min. The retention time of prednimustine was 11 min and the extraction recovery was found at 95.4% after LLE from 1 ml of plasma with 2 ml ethyl acetate twice. The LOQ was found at 0.05 μ*M* corresponding to 0.3 ng on column. In 1989, Oppitz et al. [54] reported an HPLC assay to study the pharmacokinetics of prednimustine, CLB and PAAM. As previously described, the authors developed an LC–UV method which allows the simultaneous determination of these three compounds in less than 20 min with a LOD of 30 ng/ml (1.2 ng on column) after solid-phase extraction.

Both LLE and SPE are described for PDM also references are few.As previous remarks, UV detection provides nanogram levels LOD while GC–MS method reaches a 40-pg LOD. Details of these methods are summarized in Table 5.

4.5. Estramustine (carbamoyl mustard)

Estramustine phosphate (EMP, Fig. 9), a nor-nitrogen mustard derivative of oestradiol, has been used for several years in the treatment of prostatic carcinoma. After oral administration, EMP is rapidly dephosphorylated in the gastrointestinal tract to yield estramustine (ESM). ESM and its 17-keto analogue, estromustine, are the major metabolites of EMP found in plasma (Fig. 9).



R = PO_2H, Estramustine phosphate
R = H, Estramustine
R = [o], Estromustine

Fig. 9. Structures of estramustine (ESM) and its metabolites.

In 1980, Brooks et al. [84] reported the determination of estramustine and its 17-keto metabolite in plasma by HPLC using fluorescence detection with excitation and emission wavelength set at 195 and 250 nm, respectively. The mobile phase was a hexane–ethanol (92.5:7.5, v/v) mixture. The extraction from a buffered plasma sample at pH 9 was done with hexane with an overall recovery for estramustine at 75% and the minimum detectable amount for both ESM and its 17-keto metabolite was found to be 10 ng injected on column.

In 1982, Anderson et al. [85] developed a GC method for the same purpose; the determination of estramustine and four of its metabolites levels in plasma. A LLE with diethyl ether and a purification by partition between hexane and acetonitrile. This LLE is time-consuming and the recovery was determined to about 75% for estramustine. Using 2 ml of plasma, the limit of detection was 20 ng/ml for estramustine (80 pg on column). This GC method used an alumina column and estramustine was determined by flame-ionization detection after silylation in 9 min.

More recently, a LC method with fluorescence detection combined with a GC method with nitrogen-phosphorus and mass spectrometric detection was reported by Edman et al. [86] for the determination of estramustine phosphate and its metabolites (estromustine, estramustine, estrone and estradiol) in human plasma. The samples were purified by a C_{18} SPE and LLE procedure followed by derivatization by silylation with bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with almost 70% of recovery and a LOQ at 13 ng/ml (13 pg on column) and LOD at 3 ng/ml (3 pg on column) for estramustine. The authors have studied the possibility of using LC–MS–MS technique for the determination of these compounds and found that it has been not possible to achieve as low LOD (10 ng/ml with LC–MS–MS) for estramustine as with the described GC–NPD technique.

LLE and SPE seem to provide similar recoveries (about 75%) while LC-Fluo allows rapid determination of nanogram levels of ESM. GC with various detection mode leads to the quantitation of picogram levels of derivatized ESM. Details of the methods are summarized in Table 6.

4.6. Oxazaphosphorine drugs and their metabolites (cyclic phosphoramide N-mustard)

Oxazaphosphorine drugs are alkylating antineoplastic substances used in various cancer chemotherapy regimens. Cyclophosphamide (CPM) and ifosfamide (IFM) are members of this family and are widely used for the treatment of sarcoma [87]. More particularly, high doses of IFM (up to 9 g/m^2) are sometimes administered to children suffering from osteosarcoma and soft tissue sarcoma [88,89]. Actually, IFM and CPM are non-cytotoxic prodrugs which require a bioactivation step that occurs in the liver via a cytochrome P450-mediated ring oxidation. Indeed, after oxidation at position 4, true alkylating moieties (phosphoramide (PAM) or isophosphoramide mustards (IPAM)) are spontaneously and concomitantly formed with the release of acrolein (Fig. 10). This latter is responsible for the urotoxic side effects of the therapy [90,91] and its involvement in CPM-induced cellular toxicity has also recently been proposed by Friedman et al. [92]. Despite the use of large quantities of mesna (sodium mercaptoethanesulfonate) to prevent acrolein toxicity, haemorrhagic cystitis [87–89] frequently occurs with alarming severity. Concomitantly, a competitive deactivation pathway occurs by oxidation of the chloroethyl groups and leads to the liberation of chloroacetaldehyde (CAA) which is suspected of

Table 6
Summary of the different methods to assay estramustine (ESM) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–FID	Plasma	LLE	BSTFA	D: 80 pg	9	[85]
GC–NPD	Plasma	SPE/LLE	BSTFA	D: 3 pg Q: 13 pg	10	[86]
LC–UV	Plasma	LLE		D: 10 ng	7	[84]

Table 7
Summary of the different methods to assay cyclophosphamide (CPM) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–MS	Urine	LLE	TFAA	D: 12.5 pg	10	[107]
GC–MS	Plasma	LLE/SPE		D: 3 pg		[112]
GC–MS	Plasma	Two-step SPE	TFAA	D: 6 fg	24	[117]
GC–NPD	Plasma	LLE	TFAA	D: 10 pg	4	[163]
GC–NPD	Plasma	LLE	TFAA	Q: 20 pg	10	[110]
LC	Plasma	Two-step precolumn	Diastereo. pair	Q: 10 ng	20	[126]
LC–MS	Plasma	SPE		Q: 300 pg	13	[118]
LC–MS–MS	Urine	LLE		D: 0.5 pg Q: 2 pg		[125]
LC–UV	Plasma	LLE		D: 300 pg	20	[124]
LC–UV	Plasma	LLE		Q: 1.4 μg	30	[96]
LC–UV	Plasma	SPE		Q: 20 ng	12	[115]
LC–UV achiral–chiral	Serum	SPE		D: 0.6 μg/ml Q: 1.25 μg/ml	8	[115]
TLC–PD	Urine	SPE	4-NBP	D: 30 ng		[120]

Table 8
Summary of the different methods to assay 4-OH-CPM in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
GC–MS	Blood	LLE	4-NBP	D: 25 ng		[127]
LC–UV	Plasma	LLE	2,4-DNPH	D: 0.9 ng	10	[128]
LC–UV	Serum	LLE	SCZ	D: 2.5 ng	10	[104]
LC–UV	Plasma Erythrocytes	LLE	SCZ	D: 2.5 ng	30	[101,103]

Table 9
Summary of the different methods to assay ifosfamide (IFM) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
LC–UV	Plasma	SPE		D: 20 ng	10	[116]
LC–UV	Urine	LLE		D: 25 ng	11	[97]
TLC–PD	Urine/ Cerebrospinal fluid/plasma	SPE	4-NBP	D: 30 ng		[105]
GC–NPD	Urine/plasma	LLE		D: 1 pg	4	[99]
Chiral GC–MS	Urine/plasma	LLE		D: 250 pg	20	[108]
LC–UV achiral–chiral	Serum	SPE		D: 50 ng	12	[114]
GC–MS	Plasma	LLE		Q: 50 pg	9	[109]
GC–MS	Plasma	Two-step SPE	TFAA	Q: 7 fg	21	[117]
Chiral GC–NPD	Urine Plasma	LLE		D: 200 pg	24	[98]
LC–MS	Serum	LLE		D: 30 pg	7	[106]
LC–UV	Plasma Erythrocytes	LLE	DDTC	Q: 5 ng		[102]

Table 10
Summary of the different methods to assay 4-OH-IFM in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
LC–FLUO	Plasma	LLE	3-Aminophenol	D: 100 pg Q: 150 pg	8	[164]
LC–UV	Plasma Erythrocytes	LLE	SCZ	D: 10 ng	12	[100]

Table 11
Summary of the different methods to assay acrolein in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
LC–FLUO	Liver microsomes	LLE	3-Aminophenol	D: 250 pg Q: 500 pg	8	[129]
GC–MS	Urine	Headspace	4-NBP	D: 2.8 pg	3	[165]
LC–FLUO	Plasma	LLE	Luminarine 3	D: 120 pg	7	[134]

Table 12
Summary of the different methods to assay chloroacetaldehyde (CAA) in various biological matrices

Method	Matrix	Extraction	Derivatization	LOD/LOQ	RT (min)	Ref.
LC–UV	Plasma	SPE	Thiourea	D: 1.6 ng	6	[166]
LC–FLUO	Plasma/ liver microsomes	LLE	$1N^6$- Ethenoadenosine	D: 200 pg	6	[135]
LC–FLUO	Plasma	LLE	Luminarine 3		11	[134]

being responsible of the observed neurotoxicity, urotoxicity and cardiotoxicity of the oxazaphosphorines. Since oxazaphosphorine metabolism is sensitive to auto-induction [93], as well as to inter- and intra-individual variations [94], an accurate measurement of the metabolites levels (active and inactive) in plasma should ease individual pharmacokinetic and metabolism studies of these drugs

Table 13
Summary of the different methods to assay Dacarbazine in various biological matrices

Method	Matrix	Extraction	Compound	LOD/LOQ	RT (min)	Ref.
LC-UV	Plasma	Ultrafiltration	DTIC	D: 250 ng	14.5	[145]
	Urine		AZA/AIC	D: 25 ng	3/5	
LC–UV	Plasma		DTIC	D: 1 ng	?	[143]
LC–UV	Plasma	PP	DTIC	D: 50 ng/ml	4	[144,151]
			AIC	D:125 ng/ml	6	
LC–UV	Plasma	PP	DTIC	D: 23 ng/ml	14	[142]
	Urine		AIC	D: 345 ng/ml	6	
LC–UV	Plasma	SPE	DTIC	D: 0.5 ng	15	[146]
LC–UV	Pharm. prep.	–	DTIC	D: 0.5 ng	5.7	[149]
LC–UV	Pharm. prep.	–	AIC		4.5	[148]
			AZA		10.8	
			DTIC		25	
LC–UV	Plasma.	LLE	AIC	D: 1 ng	2.6	[167]
			AZA		3.1	
			DTIC		6.8	
LC–UV	Pharm. prep.	LLE	TMZ	D: 1 ng	2	[147]

Table 14
Summary of the different methods to assay temozolomide (TMZ) in various biological matrices

Method	Matrix	Extraction	Compound	LOD/LOQ	RT (min)	Ref.
LC–UV	Urine	LLE	TMZ/DTIC		5	[153]
LC–UV	Plasma	PP	MTIC	D: 4–6 ng	?	[154]
LC–UV	Plasma	LLE	TMZ	?	2	[155]
LC–UV	Plasma	SPE	TMZ	Q: 6 ng(P)	3.2	[152]
	Urine			Q: 60 ng(U)		
LC–UV	Plasma	PP	MTIC	D: 2.5 ng	4.9	[157]
LC–UV	Plasma	PP	MTIC	Q: 0.2 ng	4.5	[158]
LC–UV	Plasma	PP	MTIC		4.5	[156]
LC–UV	Plasma	LLE	TMZ	D: 1 ng	2.7	[159]

and thus lead to a better understanding of the variation in the metabolism.

The bio-analysis of ifosfamide and cyclophosphamide is hampered by their high reactivity, polarity and lack of a UV chromophore. Their high solubility makes it difficult to purify the analyte effectively from biological fluids. Earlier studies reported laborious LLE and SPE or a combination of LLE and SPE as sample pre-treatment of IFM or CPM assay. Extraction of IFM and CPM and their biological metabolites from biological fluids have been described in several methods. *N*-Deschloroethylated metabolites and parent drugs are frequently extracted from biological fluids and analysed with the same procedures. The simultaneously determination of these compounds is frequently found out. In contrast, ended metabolites from the activation way of cytochrome P450 oxidation of oxazaphosphorine drugs are small and polar molecules with different physicochemical characteristics. They need to be derivatized before extraction. The derivatization also allows the determination of those compounds without chromophore or specific moieties.

In 1990, Tjaden and De Bruijn [82], reviewed the chromatographic methods for these alkylating *N*-mustards. Liquid chromatography with UV detection and gas chromatography with azote-phosphorus detection were found to be the principal methods. In the last decade, mass spectrometry took an important place in chromatographic analysis, especially for the molecules with a lack of chromophore as oxazaphosphorine drugs. The separation methods did not change a lot; only solid-phase extraction showed improvement in the purification of samples and the availability of mass spectrometry.

A review of the analysis of CPM and its metabolites have been published recently by Malet-Martino et al. [95]. This works is very helpful for the determination of the metabolites of CPM involving various separation methods with various detection modes and includes the ^{31}P NMR for the determination of phosphorus compounds from biological fluids. This analytical tool allows the direct study of any intact sample without prior extraction or separation of compounds avoiding the problems encountered in extraction recovery and chemical derivatization. Furthermore, each phosphorus-containing metabolite can be detected simultaneously and quantified in a single run. However, the lack of this method is its low sensitivity and the fact that NMR spectrometers are rarely available in clinical units due to their cost.

4.6.1. Extraction from biological fluids

4.6.1.1. Liquid–liquid extraction (LLE). In 1989, Masurel et al. [96], used the chloroform as an organic phase for the liquid–liquid extraction for the determination of IFM, CPM and trofosfamide. The LLE method of Goren [97], also using chloroform, is a rather elaborated method including various filtration steps, but was validated only for 3-ml urine samples. Kaijser et al. [98] described a LLE of N^2- and N^3-DEC-IFM (Fig. 10) from alkalinized urine and plasma samples with ethyl acetate, already validated for the extraction of IFM [99]. Kerbusch et al. [99,100] in 1998 used the same organic solvent for the extraction of 4-OH-IFM (Fig. 10) from human plasma and erythrocytes, but they used acetonitrile for the extraction of the derivatized

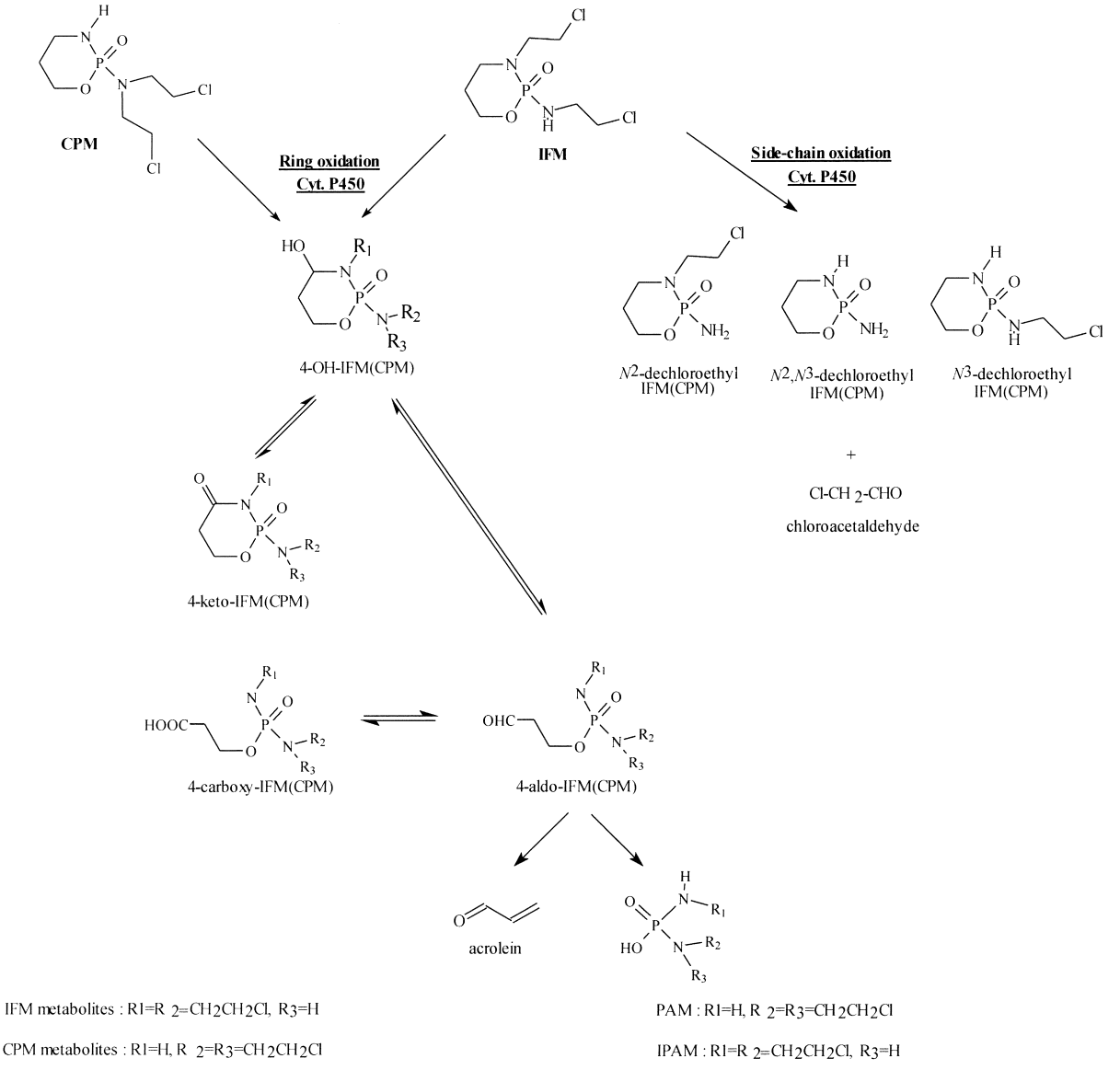


Fig. 10. Structures of oxazaphosphorine drugs: cyclophosphamide (CPM) and ifosfamide (IFM) and its metabolites.

product of IPM (Fig. 10) with diethyldithiocarbamate [101,102]. Ethyl acetate was also used by Huitema et al. [101,103] for the extraction of the semicarbazide derivatized product of 4-OH-IFM from plasma and red blood cells while Belfayol et al. [104] mixed ethyl acetate and chloroform (75:25, v/v).

Boddy et al. [105] added cold acetonitrile to an equal volume of plasma for a LLE and protein precipitation procedure as Siethof et al. [106]. Sessink et al. [107], have used for the determination of CPM in urine by GC–MS, after derivatization with trifluoroacetic anhydride, a liquid–liquid extraction using diethyl ether.

For the LLE, some authors described a simple procedure using chloroform after alkalinization of

samples (urine or plasma samples). In fact, Goren et al. [97] have shown that urine acidification reduced the recovery of the metabolites to less than 1%. At neutral pH, they found that the recovery of N^2- and N^3-DEC-IFM was diminished by about half. They reported the most efficient procedure using chloroform and addition of 0.2 ml of 1 *M* sodium hydroxide followed by addition of an excess of crystalline sodium chloride. This procedure has been used by Granville et al. [108] for the preparation of both plasma and urine samples before the enantioselective determination of ifosfamide and its N^2- and N^3-dechloroethylated metabolites. The recoveries found by these latter were increased to 80–90% for the N^2- and N^3-DEC-IFM.

Methylene chloride can also be used for LLE of oxazaphosphorine drugs as reported by Wang et al. [109] and Kalhorn et al. [110] who described a most elaborate procedure using methylene chloride as a first step leading to the extraction of non-polar compounds as CPM and *N*-deschloroethylated metabolites and the remaining aqueous fraction was deproteinated with acetonitrile–methanol prior to analysis. Recently, a LLE method for plasma samples using chloroform after alkalinization with sodium hydrogen carbonate has been validated for IFM, CPM, N^2- and N^3-DEC-IFM in our laboratory with good recoveries, respectively, 97, 95, 72 and 68% (A. Paci, unpublished data, 1999).

Non-polar metabolites are generally more stable and easily extractable from biological fluids with parent drugs with most of the common organic solvents. In contrast, more polar compounds (acrolein, CAA and IPAM) (Fig. 10) contained in the extracted aqueous phase are analysed by LC procedures with UV, fluorescence or mass-spectrometry detection.

4.6.1.2. Solid-phase extraction (SPE). The liquid–liquid extraction (LLE) first described by Margison et al. [111] modified by Goren, and the solid-phase extraction (SPE) method of Momerency et al. [112] appeared to have the highest extraction recovery for those *N*-deschloroethylated metabolites (75 and 80%, respectively), whereas all LLE and SPE methods have comparable extraction recoveries for IFM and CPM (>95%). The SPE method developed by Hardy et al. [113] showed a recovery of 91% for IFM, but only 40% recovery for both N^2- and N^3-DEC-IFM. A procedure described by Boddy et al. [105] used a XAD-2 Spe-Ed SPE cartridge for urine samples. After the cartridge was washed with water and dried for 3 h with flushing air, IFM and their metabolites were eluted with three 1-ml volumes of methanol. No recovery data was reported with this procedure. In contrast, Corlett et al. [114,115] have described an SPE method using cyclohexyl (CH) cartridges previously reported by Burton et al. [116]. with an extraction recovery between 85.3 and 90.2% for racemic IFM and between 90.6 and 101.7% for CPM.

Bohnenstengel et al. [18] have used C_8 cartridge conditioned with 2 ml of methanol and 2 ml of water, for the extraction of N^2- and N^3-DEC-IFM while Steger-Hartmann et al. [117] have used a similar SPE with samples applied to a C_{18} SPE cartridge for IFM, CPM and TFM and Bauman et al. [118] for CPM. Kaijser et al. [119] used the same coated SPE cartridge for the extraction of IPAM after derivatization.

4.6.2. Thin-layer chromatography (TLC)

Boddy et al. [105] have used this chromatographic technique for the quantification of IFM and its metabolites in urine, cerebrospinal fluid and plasma. They used a combined thin-layer chromatography–photo-densitometry with spot visualization using 4-(4-nitrobenzyl)pyridine (NBP) described previously for the quantitation of CPM in urine by Hadidi and Idle [120]. Struck et al. [121] have used the same derivatization procedure for the 4-OH-IFM quantification in plasma.

The sample preparation for TLC was described previously with a XAD-2 solid-phase extraction and the use of precoated high-performance thin-layer chromatography glass-backed silica gel 60 for a saturated elution. After chromatography, the plates were sprayed with 5% NBP acetone solution and then heated at 150°C for 10 min. The plates were dipped in 3% methanolic potassium hydroxide to reveal the blue spots formed from alkylated NBP, and then photographed because of the unstable nature of the chromophore. The photographs of the plates were scanned on a densitometer in the reflection/absorption mode at 500 nm to allow the quantification after calibration curves plotting.

TLC is a relatively simple and non-expensive assay method which does not require the use of radiolabelled drug, complex derivatization or expensive gas or high-performance liquid chromatographic equipment. But the accuracy and repeatability of this analytical method is not as good as the two most common chromatographic methods. Nevertheless, Hadidi and Idle [120] described the determination of CPM and several metabolites (PM, 4-keto-IFM,4-carboxy-IFM) using this TLC method with an intra-assay variation coefficient for each compound of less than 6% and a limit of detection as low as μg/ml level.

4.6.3. Gas chromatography (GC)

Most of the gas chromatography procedures use a nitrogen-phosphorus detector because of the presence of both azote and phosphorus atoms in the oxazaphosphorine structure drugs. This detector is highly specific of these atoms and therefore is responsible of the high sensitivity of these methods. Nevertheless, for quantitation purposes it can be found in literature, several other detection techniques such as flame-ionization detection (FID), electron-capture detection (ECD) or mass spectrometry with selected ion-monitoring mode (SIM-MS).

Most reports deal with prior derivatization of the oxazaphosphorine, especially with trifluoroacetic anhydride (TFA) or heptafluorobutyric acid (HFBA). For de Bruijn et al. [122] special attention has to be paid to the derivatization. Although a proper choice of temperature programming enables the elution of some metabolites without derivatization, trifluoroacetylation is recommended. However, Kaijser et al. [98] described, in 1992, a GC–NPD procedure completed by a GC–MS method to determine the plasma and urine levels of N^2- and N^3-DEC-IFM. The LOD when using a split ratio of 1:10 and an injection volume of 1 μl, appears to be 10 ng/ml (1 pg on column) for N^3-DEC-IFM and 30 ng/ml (3 pg on column) for N^2-DEC-IFM. This technique allows the direct determination of IFM and metabolites and their quantification in biological fluids without derivatization. This technique was completed by the same authors in 1997 for the enantioselective determination of IFM and some of its metabolites in plasma and urine using a Chirasil–L-val gas chromatographic column [99]. The LOD for both IFM enantiomers in plasma were 20 ng/ml with a split ratio 1:10 and 50 ng/ml with a split ratio 1:25 in urine. In our laboratory we found that for the analysis of oxazaphosphorine drugs and their related metabolites a derivatization procedure was not mandatory (A. Paci, unpublished data, 1999). Kalhorn et al. [110] also described a GC–NPD method for the determination of CPM and *N*-deschloroethylated metabolites.

A GC–MS procedure have been employed by Granville et al. [108] for the enantioselective separation of IFM enantiomers and its dechloroethylated metabolites using a chiral stationary phase based upon heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin. The detection used was electron-impact and selected-ion monitoring mode. This analytical method was applied to the simultaneous determination of the enantiomers of IFM and its *N*-dechloroethylated metabolites in plasma and urine samples. A similar analytical method was employed by Wang et al. [109] to determine IFM, 4-OH-IFM, and its N^2- and N^3-dechloroethylated metabolites as well as isophosphoramide mustard which is the active metabolite of this oxazaphosphorine drugs. The technique described a GC–MS with an ion-trap mass spectrometer after silylation of each compound before GC–MS analysis with a LOQ found at 50 pg injected.

Steger-Hartmann et al. [117] also used an electron-impact SIM mode GC–MS method after a derivatization procedure of IFM, CPM and TFM using trifluoroacetic anhydride (TFA) to quantitate those drugs in sewage water samples. This technique allowed to quantitate less than 10 ng/l of each compound (6 fg on column for CPM and 7 fg for IFM). This GC–MS method with previous derivatization with TFA has already been developed by Sessink et al. [107] for the determination of CPM in urine samples.

A GC method was also used with an electron-capture detector (ECD) for the biological fluids determination of chloroacetaldehyde, one of the potential neurotoxic metabolites of IFM and CPM [123].

4.6.4. High-performance liquid chromatography (HPLC)

LC is not the most suitable analytical method for the analysis of oxazaphosphorine drugs. Goren et al.

[97] described in 1991 a HPLC procedure with an UV detection at 190 nm. Several works deal with a LC-UV method for the biological fluid determination of CPM and its principal metabolites [96,101, 104,115,124] This technique was judged to lack the required sensitivity for drug monitoring.

In 1994, Corlett et al. [114] described a reversed-phase achiral–chiral coupled high-performance liquid chromatographic method to determine the concentration of *R*- and *S*-IFM. Racemic ifosfamide was separated from its metabolites and interfering serum components on an achiral C_1 column and the eluent was transferred onto an α_1-glycoprotein column for the enantiomers quantification. The detection used an UV detector set at 195 nm with a LOD found at 2.5 μg/ml (50 ng) which was not satisfactory for bioanalytical and pharmacokinetic research. A similar LC-UV technique was set by Kaijser et al. [119] for the analysis of isophosphoramide mustard, the active metabolite of IFM after direct derivatization with diethyldithiocarbamate. The reaction product was less polar than IPAM which increased the extractability of the analyte by a C_{18} SPE. The LC procedure used ion-pair chromatography with cyclohexylamine and a detection at 280 nm.

LC–MS techniques were used more recently, as Kalhorn et al. [110] have described in 1999 for the determination of several polar metabolites of CPM. Bauman et al. [118] have described a similar procedure using LC–MS which allowed the simultaneous determination of CPM with a LOD at 15 ng/ml (300 pg on column) and most of its metabolites (i.e., 4-OH-CPM, aldo-CPM, 4-keto-CPM, carboxy-CPM). Sottani et al. [125] described a HPLC/ion spray-tandem mass spectrometry procedure to quantify CPM in human urine from hospital personnel involved in drug preparation and administration of antineoplastic alkylating agents. This method used LLE with ethylacetate and led to 0.5 pg LOD and 2 pg LOQ.

A two-step pre-column chiral derivatization has been described by Reid et al. [126] using a reversed-phase LC method resulting in the separation of (−)-CPM and (+)-CPM. A chiral variant LC procedure was developed by Kaijser et al. [98] in 1997 to determine the enantiomer of IFM. The LC system consisted in a Chiralcel OD column which allows the resolution of the racemic compounds for identification purposes and not for quantitation. The same authors have also used a LC method for the determination of 4-OH-IFM in biological matrices, the primary metabolite of these oxazaphosphorine drugs, after derivatization with 7-hydroxyquinoline which can be detected fluorometrically with a LOD found at 100 pg on column. First steps oxazaphosphorine drugs metabolism lead to 4-hydroxylated metabolites which are very unstable. Therefore derivatives are often formed to allow their quantitation. Many substances for the derivatization are used, such as *p*-nitrophenylhydrazine [127,128], semicarbazide [104], potassium cyanate [110] and *o*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine [123].

In 1997, Bohnenstengel et al. [129] described an indirect fluorimetric method measuring acrolein, a decomposition product of 4-hydroxy-CPM–aldophosphamide for the quantification of 4-hydroxy-CPM. This method, based on the formation of the fluorescent derivative 7-hydroxyquinoline by condensation of acrolein with 3-aminophenol, showed high sensitivity with a LOD of 250 pg (5 ng/ml). Different methods have been described to achieve the quantitative determination of acrolein in ambient air [130,131] in biological samples or urine [129,132,133]. Among these methods, the formation of an hydroxyquinoline by condensation of acrolein with 3-aminophenol allowing fluorimetric detection after HPLC has been reported [85,86]. However, this method only described for urine or liver microsome extracts analysis, requires drastic conditions (samples heating at 100°C) that might modify the real kinetic of the aldehyde formation, by a thermodynamically induced mechanism, in the plasma of patients receiving multitherapy. Another HPLC method including the derivatization of acrolein with 2,4-dinitrophenylhydrazine leading to UV detection has also been proposed, but the necessary 254 nm detection dramatically limits its sensitivity and specificity in the case of plasma samples. Facing the crucial need of possessing an accurate method of the quantification of acrolein in plasma, we were eager to develop an high-performance liquid chromatographic technique with fluorescent detection using only mild conditions and hence suitable for the quantitative determination of acrolein in human plasma [134].

Condensation of acrolein with Luminarin® 3 afforded a fluorescent derivative that could be specifically detected and quantified. Chromatographic conditions involved a C_{18} RP column Uptisphere and a gradient elution system to optimize resolution and time analysis. The method showed high sensitivity with a LOD of 100 pmol/ml (120 pg on column) and a LOQ of 300 pmol/ml (360 pg on column). This technique is particularly suitable for pharmacokinetic studies on plasma of patients receiving oxazaphosphorines.

An LC-FLUO method was set in 1999 by Huang et al. [135] to determine chloroacetaldehyde, a neurotoxic metabolite based on the formation of the highly fluorescent adduct 1-N^6-ethenoadenosine from the reaction of CAA with adenosine. The LC method with a fluorimetric detection developed in our laboratory for acrolein can be used for the determination of CAA after derivatization with Luminarin® 3.

Various organic solvent have been used for LLE of oxazaphosphorine drugs and their metabolites with good recoveries (>80%). LLE and SPE methods have comparable extraction recoveries for parent drugs but SPE do not have the efficiency of LLE for more polar compounds as most of their metabolites. Oxazaphosphorine drugs are determine by TLC after derivatization with NBP. This technique is non-expensive and allows the simultaneous determination of several samples. Nevertheless, TLC is still a qualitative analytical tool with a lack of sensitivity for quantitative purpose. The technique of choice of oxazaphosphorine drugs and their non-polar *N*-deschloroethylated metabolite, is GC–NPD method which allows very low LOD/LOQ due to the high specificity of this detector. This analytical method is successfully completed by GC–MS for the quantitation of more polar compounds. Powerful analytical methods have been described also they need time-consuming derivatization procedures. Furthermore, because of the lack of chromophore, few LC methods have been described for the oxazaphosphorine drugs determination. Nevertheless, the availability of LC–MS coupling allows the determination of these compounds and their metabolites leading to low LOD/LOQ and without the need of derivatization. LC methods using fluorimetric detection are often used for the determination of the toxic aldehydic metabolites of oxazaphosphorine drugs with high sensitivity. Details of these methods are summarized in Tables 7–12.

5. Nitrosoureas

5.1. Tallimustine [136]

Tallimustine (Fig. 11) was separated by HPLC with ODS column. Extraction yield obtained using SPE was about 70%. No LLE could be employed because alkaline pH was mandatory for this purpose causing rapid degradation of tallimustine. Tallimustine solution must be kept acidic to avoid strong adhesion to the glassware and to plastic container. The tallimustine quantitation was made possible with UV detection set at 314 nm. This method was successfully applied for the determination of tallimustine in plasma. LOQ was of 0.5 ng/ml plasma.

5.2. Tauromustine [137,138]

Polacek et al. have developed two HPLC–UV methods to quantify tauromustine (TM, Fig. 12) and/or metabolites with two different strategies; the most recent one allowed determination of demethylated metabolites (DDMTM and DMTM) as well.

LLE was used to extract TM with an extraction recovery of 95% [137]. SPE was proved to be effective for TM and metabolites [138]. Separation of TM was achieved on ODS column whereas cyano column was employed to discriminate both metabolites from interferences. The pH of mobile phase was

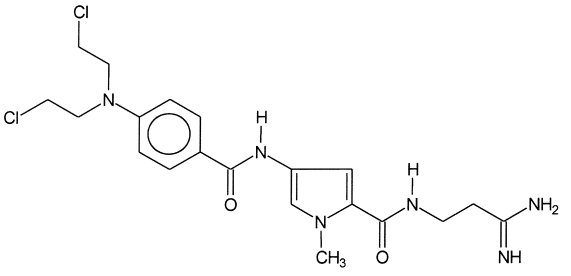


Fig. 11. Structure of tallimustine.

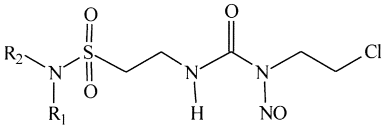


Fig. 12. Structures of tauromustine (TM) and its metabolites.

optimized to get rid of disturbance on separation. This modification had no effect on k'. The LOD was about 30 pg for tauromustine and 50 pg for both DMTM and DDMTM at 230 nm.

TM and metabolites in plasma are stable for at least 6 months when kept at a temperature below −65°C.

5.3. Lomustine/carmustine

Several papers have been published on monitoring carmustine (Fig. 13) or lomustine (Fig. 14). Most of them were reported and summarized in the excellent paper of Tjaden and De Bruijn [82]. An isocratic HPLC assay was described for the measurement of *trans*- and *cis*-4′-hydroxyCCNu plasma concentrations [139]. The latter are the alkylating moieties deriving from lomustine after biotransformation. An ultrasphere ODS column was employed to separate those two by-products with a MP consisting of methanol–phosphate buffer, pH 7.4 (50:50). RT were 7.7 and 8.7 min, respectively. In this conditions, carmustine and lomustine could be eluted as well after 9.5 and 48 min.

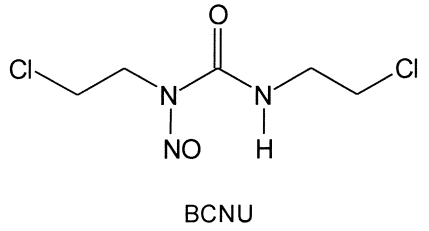


Fig. 13. Structure of carmustine.

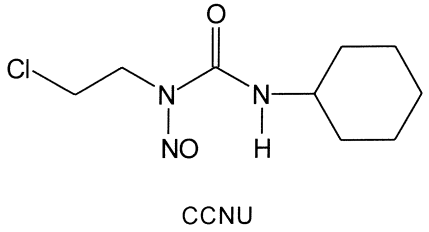


Fig. 14. Structure of lomustine.

Effluent was monitored at 254 nm with an UV detector allowing a LOD of 0.01 mg/l.

LLE extraction was carried out using dichloromethane with a recovery from plasma of about 90 to 95% for the two metabolites.

Stability studies showed that plasma samples or methanolic solution of lomustine were stable at least for 7 days when stored at −80°C.

Beitz et al. reported a short HPLC method for carmustine likely suitable only for pharmaceutical assay. Very recently Favier et al. [140] described a reversed-phase HPLC to determine both carmustine and its degradation product the 1,3-bis(2-chloroethyl)urea in pharmaceutical preparation. The mobile phase consisted of 30% acetonitrile in water. The retention times were 3.8 and 7.0 for BCNU and carmustine, respectively. The LOQ obtained were only suitable for carrying out stability studies.

5.4. SarCNU [141]

A HPLC–UV (240 nm) was described for the separation of SarCNU (Fig. 15). Chromatography was performed using mobile phase methanol–ammonium buffer, pH 3.7 (25:75, v/v) and a C_{18} column. The drug eluted at 5.8 min and the I.S. ProCNU at 9.1 min. LLE was carried out with *t*-butylmethylether allowing a 96% recovery.

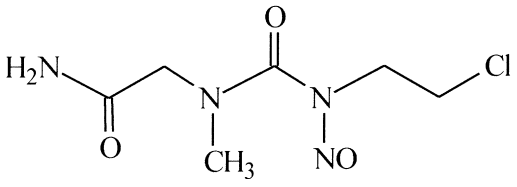


Fig. 15. Structure of SarCNU.

The switching valve set to prevent the build-up of extractable strongly retained UV absorbance was unnecessary for human plasma. Nevertheless, this method allowed drug measurement only up to 70 min after injection. Stability was also investigated. Optimal pH was 5.2. At this pH, SarCNU stability was 2.7 and 200 h, and 3 days, at room temperature, and 5 and −70°C, respectively.

6. Others

6.1. Dacarbazine

The anticancer drug dacarbazine (DTIC, Fig. 16) has been administered to patient singly or together with other drugs to treat malignant melanoma, soft tissues sarcomas and Hodgkin's lymphoma [142]. To decrease the duration of side effects produced during each cycle intermittent doses were developed and thus pharmacokinetics studies were mandatory. These investigations require analytical methods able to measure DTIC and its metabolite, the 5-amino-imidazole-4-carboxamide (AIC), in plasma and urine. The ones reported in the literature have used HPLC to separate the parent drug and AIC [143–146].

The photodecomposition of DTIC has been described leading to the formation of 2-AZA. To guaranty the quality of DTIC preparation several stability indicating methods were developed in the last 8 years [147–149].

6.1.1. Drug stability

When DTIC is exposed to light, the triazene bond dissociates yielding to dimethylamine and diazoimidazole-carboxamide which cycles to 2-AZA.

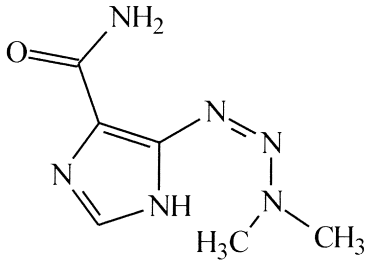


Fig. 16. Structure of dacarbazine (DTIC).

Therefore, the drug must be protected from light exposure when storing samples or pharmaceutical preparation.

6.1.2. Extraction from biological fluids

Different methods were reported using ultrafiltration [145], LLE [144] or SPE [146]. Little is described about recovery efficiencies. Only Miranda-Ordieres et al. [146] relates an extraction recovery of 47% with C_{18} cartridges.

6.1.3. Derivatization

Although this method has been coupled to any chromatographic assay, Loo and Stasswender [150] described a colorimetric method to quantify DTIC. It involves decomposition of DTIC by UV radiation to produce 5-diazo-carboxamide which was derivatized with the Bratton-Marshall reagent to produce an azo-dye measured at 540 nm. This method was extremely unspecific suffering from interferences and was also time consuming.

6.1.4. High-performance liquid chromatography (HPLC)

HPLC has been applied in the RP mode with C_{18} column coupled with UV detection to measure DTIC or AIC or both.

First, Benvenuto et al. [143] used methanol–acetate buffer as mobile phase but could only separate DTIC. Later on, Breithaupt et al. set up a paired ion HPLC on C_{18} column with a HPS contained mobile phase for AIC determination and with a tBA containing mobile phase at pH 7 for DTIC determination [144]. In 1985, Fiore at al [145] proposed an assay able to determine DTIC, AIC and 2-AZA simultaneously including an Internal standard 3-methylxanthine after setting up a pH and solvent gradient elution owing to polarity differences between DTIC and AIC. At pH 7, ionization of AIC was suppressed avoiding its elution in the solvent front. Then, pH was decreased to achieve the analysis of the three drugs and the internal standard in about 15 min. The most recent works conducted to elucidate pharmacokinetics of DTIC used successfully the methods reported by Breithaupt et al. [142,151]. It seems that it offered the best compromise between separation and sensibility. For all these methods authors have employed UV detection.

By contrast, Miranda-Ordieres designed an HPLC assay where DTIC was determined using anodic amperometric detection [146]. However, AIC was not detected and chromatograms obtained were quite messy.

The recent papers published [147–149] described pharmaceutical oriented methods. First, King and Stewart [149] chromatographed simultaneously DTIC, doxorubicin and ondansetron using a underivatized silica stationary phase. It appeared that with a mobile phase set at pH 3 the mechanism of retention of this basic analyte (i.e., DTIC) was cation exchange. Finally, Haque et al. [148] after failing to separate in a appropriate time analysis DTIC and its its by-product with an ODS column used an avidin protein column coupled with UV detection. This method can be used either to study the degradation of dacarbazine or determine impurities at the 1% (w/w) level. With this column, retention of AIC depends on ionic or other non-specific interactions, whereas DTIC and 2-AZA chromatography relates to hydrophobic interactions.

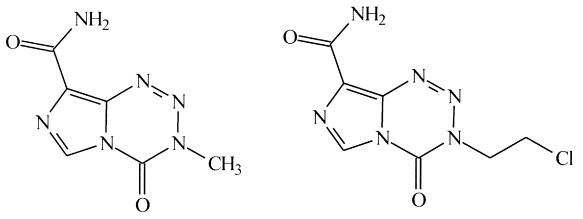
6.1.5. Capillary electrophoresis (CE)

Haque and Stewart [148] achieved separation of 2-AZA, DTIC and AIC by CE on a fused-silica capillary with an electrolyte buffer made of methanol–phosphate buffer, 0.025 *M*, pH 4 (2:98, v/v). However, the CE method could not be used for the quantitation of impurities because at high concentration, the DTIC peak overlapped the nearest impurity peak. Details of these methods are summarized in Table 13.

6.2. Temozolomide

Temozolomide (Fig. 17) is a prodrug and has little if any pharmacological activity until hydrolyzed in vivo to 5-(3-methyl triazen-1-yl)imidazole-4-carboxamide (MTIC), known as well as the active metabolite of DTIC. However, while DTIC generates MTIC via hepatic metabolic oxidation, temozolomide undergoes chemical degradation to MTIC at physiological pH [152]. A few analytical methods have been developed to either determine pharmacokinetic parameters of the drug [152–157] or to assess the drug stability [158,159].

Temozolomide is used in the treatment of refractory anaplastic astrocytoma in adults whose disease has relapsed after initial therapy with nitrosourea and procarbazine.



Temozolomide Mitozolomide

Fig. 17. Structures of temozolomide (TMZ) and mitozolomide (MTZ).

6.2.1. Drug stability

Due to the differences in the stability profiles of TMZ and DTIC, two separate blood samples must be collected at each time. Consequently, both samples must be treated differently [159]:

- for TMZ, ice immediately after collection, adjust to pH<4 and freeze at −20°C [152,159], then samples can be stored for 3 months in the dark;
- for MTIC, collect samples in prechilled tubes, centrifuge immediately, freeze and samples can be stored at −80°C for at least 70 days [157,159].

Moreover, as samples for MTIC analysis may not be stable, injections should be made manually one at a time [157].

6.2.2. Extraction from biological fluids

The prodrug TMZ has been extracted mainly with LLE method using ethylacetate [153–155,159]. Extraction described for TMZ was favoured when sample was acidified [153]. However, LLE led to low extraction recovery of about 50–58% [153,159]. By contrast, Shen et al. [152] obtained an overall recovery of about 95% when quantifying TMZ in biological matrix using SPE as a clean-up procedure. SPE not only prevents the drawbacks of repetitive solvent extraction but also offers a high recovery rate and the advantages of enabling simultaneous processing of a large number of samples.

For MTIC quantitation, efficicent deproteinization step was reported after methanol addition only [157], or combined with HCl [158] or acetonitrile [154].

6.2.3. High-performance liquid chromatography (HPLC)

All authors used HPLC as the separative tool for TMZ and/or MTIC [152–159]. Nevertheless, different attitudes have been described depending on whether TMZ or MTIC or both were separated. For TMZ, C_{18} columns were used by several authors [152–155,159]. For MTIC, C_{18}, cyano and deactivated support chain column (SCD 100) were assessed for their selectivity. Separation on the cyano column allowed simultaneous separation of TMZ, DTIC and MTIC [158]. Eventually, SCD 100 was evaluated leading to improvement of peak shape [157]. Consequently, LOD with this method was lower than the one reported by Reid et al. [158].

Different internal standards were described including metrodinazole [154], DTIC [158], ethozolastone [159] for TMZ quantitation or hydrochlorothiazide for MTIC determination [157].

TMZ is a strong chromophore as mitozolomide which facilitates interference-free traces to be obtained from control plasma. Details of these methods are summarized in Table 14.

6.3. Mitozolomide (Fig. 17)

Mitozolomide is an antitumor agent which presents an original chemical structure placing it in a drug-alkylating family. It has proved to be effective on a number of experimental murine tumors and human xenograft [160]. Stock and Godard [161] quantified mitozolomide in plasma by HPLC following ethyl acetate extraction. It was separated on a C_{18} column with eluant composed of methanol–5% acetic acid in water (3:7). Stability studies of the drug in solution showed that acidifying greatly enhanced stability of the drug. Therefore extraction was carried out right after acidification with 1 *M* hydrochloric acid.

More recently Gachon et al. [160] reported a study where both mitozolomide and metabolites were determined. By contrast, in this paper, the separation and quantitation was achieved on a Spherisorb normal-phase column at ambient temperature using a 15 min linear elution gradient from dichloromethane–ethanol (98:2, v/v) to (80:20, v/v). Effluent was monitored at 325 nm enabling pharmacokinetic analyses.

For the metabolites, different collection of the eluates were made using similar HPLC conditions. Nevertheless, the metabolites were quantified using ^{14}C radioactivity measurements. In this paper as well, Gachon et al. [160] related an exhaustive extraction method for mitozolomide and metabolites in plasma, urine and tissues combining LLE and SPE.

7. Conclusion and perspectives

Efforts at identifying new alkylating agents with adequate activity have not had much success. Therefore, optimizing the dose intensity and schedule of the existing agents have resulted in superior activity. These improvements have become possible owing to the development of new analytical techniques to monitor the different drug and/or metabolites.

Powerful analytical tools are now available for separating complex mixtures and quantifying components. High-performance liquid chromatography is the most extensively used technique, and it has largely replaced gas chromatography (GC). In fact, many molecules of pharmaceutical interest are polar compounds, not thermostable enough to be analyzed with GC so that time-consuming and tedious processes of derivatization or chemical conversion before analysis are required. Nevertheless, many alkylating agents that are small molecules without chromophore need to be derivatized to give low limits of detection for pharmacokinetics studies. Some of them are analyzed with GC method after derivatization with very low LOD and LOQ but time-consuming procedures. GC methods using mass spectrometry detection allows the quantitation of very small amounts of compounds like alkylating agents and their metabolites with a very high specificity due to the nature of this detection mode. The development of HPLC-mass spectrometry coupling has been a major advance, offering greater sensitivity than ultraviolet or fluorimetric detection and greater selectivity through selected ion monitoring (SIM) or by using tandem mass spectrometry (MS–MS) and

selected reaction monitoring (SRM) mode. In cancer chemotherapy, these techniques are efficient for metabolic studies. Eventually, the development of narrow and microbore LC coupled to MS is likely to further improves LODs of these techniques.

8. Nomenclature

CI	chemical ionization
ECD	electron capture detection
EC	electrochemical detection
EI	electron impact
FAB	fast atom bombardment
FID	flame-ionization detection
GC	gas chromatography
HPLC or LC	high-performance liquid chromatography
I.S.	internal standard
LLE	liquid–liquid extraction
MS	mass spectrometry
NMR	nuclear magnetic resonance
NPD	nitrogen phosphorus detection
PP	protein precipitation
SIM	selected ion monitoring
SPE	solid-phase extraction
SRM	selected reaction monitoring
TLC	thin-layer chromatography
UV	ultraviolet

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