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Journal of Chromatography A, 976 (2002) 239–247

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Hyphenated techniques in anticancer drug monitoring II. Liquid chromatography–mass spectrometry and capillary electrophoresis–mass spectrometry

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Abstract

High-performance liquid chromatography has become the separation technique of choice for the monitoring of generally thermolabile anticancer agents. With the introduction of electrospray mass spectrometry, the coupling of liquid chromatography and mass spectrometry has opened the way to widely and routinely applied anticancer drug monitoring. Real-time metabolism versus degradation can now be distinguished, since derivatization is no longer obligatory. This is important for the monitoring of the anabolic and catabolic pathways of the same agent, such as 5-fluorouracil. Detection limits almost equal to those obtained with capillary gas chromatography–mass spectrometry are realistic with the latest generation of mass spectrometers, enabling quantitative analysis of various anticancer agents and their metabolites down to the low ng/ml level. Furthermore, sample clean-up and chromatography can be downscaled markedly using the latest column technologies, such as the generally applied 10 cm×2.8 mm I.D. RP 18 columns. The coupling of capillary electrophoresis to mass spectrometry is today far from a routine application in anticancer drug monitoring. Nevertheless, interesting applications have been reported and are selected for the present review.

Keywords: Reviews; Drugs

Contents

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01227-X

1. Introduction

Anticancer drug monitoring is essential for the successful development of anticancer drugs and their optimal use in cancer treatment. During drug development, stability and degradation data, information on in vitro metabolism using artificial systems such as liver microsomes, and the determination of in vivo metabolic pathways in laboratory animals can all be obtained. In therapeutic drug monitoring in the clinical setting, several factors point to the need for sophisticated analytical techniques: a heterogeneous population of patients; the assessment of bio-availability; the low therapeutic index of most anticancer agents; the potential for drug interactions; the identification of therapeutic failure; and the often limited function of organs involved in drug clearance, such as the liver and kidneys, in cancer patients. Furthermore, when an anticancer agent is initially administered to humans, in phase I studies, it is a routine requirement to determine the levels of the parent compound and its metabolites. Most anticancer agents are highly reactive and rather unstable. Therefore, the parent drug, metabolites and degradation products need to be separated, identified and quantified, preferably using one analytical system; it is here that hyphenated techniques have an important role.

Anticancer agents are usually thermolabile and need derivatization prior to GC analysis. Only a small number of agents, with unique properties at higher temperatures, can be introduced into a GC system without derivatization. Liquid chromatography (LC) was recognized two to three decades ago as the separation technique of choice for anticancer agents, but its application was limited by the inadequate sensitivity and selectivity of the available detection systems. As shall be discussed, the combination of liquid chromatography (LC) with mass spectrometry (MS) is now becoming increasingly important in anticancer drug monitoring, especially after the revolutionary introduction of liquid-phase atmospheric pressure ionization (API) techniques, such as electrospray (ESI) and ionspray. Thermospray (TSP), which governed LC–MS coupling in the 1980s, has clearly become diminished in importance relative to the more modern API techniques, and LC–API-MS is now replacing capillary GC–MS in many cases of anticancer drug monitoring. LC–ESI-MS has greatly facilitated the qualitative and quantitative analysis of the highly polar molecules frequently used in anticancer pharmacotherapy. This has been possible without the need for intensive sample pretreatment, including derivatization.

Experience with LC–MS, both with microcolumn and capillary (c) LC–MS techniques and interfacing technologies, propelled the development of another separation technique with MS, CE–MS. For example, capillary zone electrophoresis (CZE) coupled to mass spectrometry can separate and identify DNA platinum adducts, generated by exposure to *cis*-diaminedichloroplatinum(II) (cisplatin) [1]. Cisplatin is one of the most widely used anticancer agents, and the search for DNA adducts has been, and remains, a research area of its own. Thus far, rather complicated techniques with various limitations have been required to study cisplatin–DNA adducts. CE–MS seems to be an interesting, less expensive, and less complicated alternative.

Hyphenated techniques such as LC–MS(–MS) continue to play an important role in pharmacological studies of cytotoxics. The analysis of antitumor antibiotics (e.g. doxorubicin, mitomycins), antimetabolites, and plant alkaloids (the new agents taxol, taxotere and campothecin) has been described [23]. This article focuses on recent developments in the analyses of tamoxifen, oxazaphosphorines and other alkylating agents, capecitabine, and platinum compounds.

2. Tamoxifen

Tamoxifen has demonstrated its potency in the hormonal treatment of breast cancer for more than three decades, and remains a first line agent in the

adjuvant treatment of breast cancer in postmenopausal women. However, it is known to induce hepatomas in small laboratory animals, and it is associated with an increased risk of endometrial cancer. Hyphenated techniques have been important in the elucidation of the mode of action, metabolism and toxicity of tamoxifen [2–10], and have aided the development of analogues with increased therapeutic efficacy [11]. New metabolites have been identified, such as tamoxifen acid, in which the side-chain of tamoxifen is changed to an oxyacetic acid moiety, and 4-hydroxytamoxifen acid. Using LC–MS–MS, phase I and II metabolites have been defined directly for the first time in human urine and plasma samples. In plasma extracts, in addition to the parent drug and *N*-desmethyltamoxifen, the *N*-oxide of tamoxifen has been identified, whilst the glucuronides of 4-hydroxytamoxifen, 4-hydroxy-*N*-desmethyltamoxifen, dihydroxytamoxifen, and a monohydroxy-*N*-desmethyltamoxifen have been isolated in 24 h post-treatment urine samples [3]. Tamoxifen metabolism was further unravelled by on-line LC–ESI-MS in a mouse liver microsomal model [4–6]. Mouse liver microsomes were chosen to demonstrate the applicability and superiority of the method, since mice metabolize tamoxifen faster, and produce more metabolites than rats or humans. The separation was performed on a Res Elute BD column (5 μm particle size, 250×4.6 mm I.D.) with 70% (v/v) methanol in 0.5 *M* ammonium acetate as the mobile phase. Eleven metabolites were detected, some of which were previously unidentified [5,6]. The hydroxylated derivatives, including 4-hydroxytamoxifen and α-hydroxytamoxifen, were thought to be detoxication metabolites. Conversely, oxides, or their free radical precursors or metabolic intermediates, were most likely to be involved in DNA–adduct formation.

Subsequently, LC–MS was used in the quantification of α-hydroxytamoxifen following 30 min incubations of tamoxifen with liver microsomal preparations from women, female CD1 mice, or female Sprague–Dawley rats [7]. It was shown that α-hydroxytamoxifen was glucuronated in rat liver. Glucuronidation was assumed to represent a means of detoxifying α-hydroxytamoxifen, and sulphonation a generator of electrophilic genotoxic intermediates [7]. Tamoxifen metabolite profiling in rat liver microsomes by LC–ESI-MS identified a dimeric metabolite (*m*/*z* 773) derived from free radical intermediates. The mass of this metabolite is consistent with a dimer of hydroxylated tamoxifen (*m*/*z* 388) [9].

Combinatorial chemistry techniques have been exploited extensively to produce many tamoxifen derivatives with potentially improved therapeutic efficacy. Time-saving cassette dosing experiments are used during pre-clinical drug development. Therefore, a rapid sample analysis turnaround time is required. Selected reaction monitoring (SRM) LC–MS has been applied elegantly to search for more selective estrogen receptor modulators [11]. Idoxifene, an halogenated tamoxifen derivative, has a greater affinity for the estrogen receptor than tamoxifen and is less uterotrophic (Fig. 1).

Idoxifene has also been shown to reduce bone loss and lower cholesterol levels, suggesting that it may be effective in the treatment of osteoporosis and other conditions associated with the menopause. A high-throughput robotic sequential sample injection system linked to a SRM-MS–MS analytical system has been used to determine idoxifene and its pyrrolidinone metabolite in human plasma samples. Small-bore columns (C_{18}, 3 μm particle size, 30 mm×1 mm), high flow-rates (0.7 ml/min), and an elevated LC column temperature (70 °C) were used to perform LC separations of idoxifene and its metabolite at 10 s/sample. The mobile phase consisted of acetonitrile–water–formic acid (85:14:1). LC–MS-SRM was executed on a PE sciex API 3000 triple quadrupole mass spectrometer. The Turbulon Spray source operated in the positive ion mode, and the gas was heated to 425 ° C at a flow of 7.5 L/min. The optimized spray voltage was 2000 V, and, at a collision energy of 42 V, the following transitions

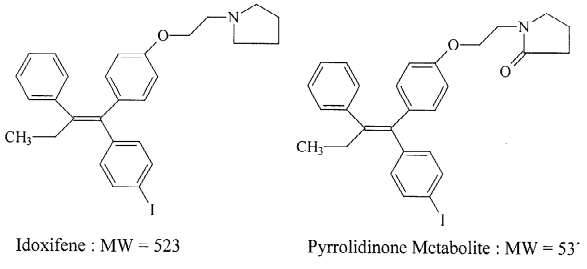

Fig. 1. Structures of idoxifene and pyrrolidinone metabolite. MW = molecular weight.

were monitored in the SRM mode: idoxifene, m/z 524→98; [$^2H_5$]idoxifene, m/z 529→98; and the pyrrolidinone metabolite, m/z 538→112. Coupling of the LC–MS-SRM system to a high-throughput robotic, sequential sample injection system allowed validated analysis of 613 plasma samples with an average run time of 23 s/sample. This validated high-throughput bioanalytical method was capable of analysing approximately 3900 samples in 24 h [11].

Information on the use of CE–MS in the analysis of tamoxifen is limited, and thus far only nonaqueous CE (NACE) has been reported [12,13]. A suitable and widely applicable NACE–MS analysis has been identified, which includes the use of an electrolyte buffer containing ammonium acetate (5–50 m*M*) and/or acetic acid (up to 100 m*M*), with a varying composition of organic solvents.

An alternative separation and/or isolation technique coupled to ESI-MS has been described by Pretty et al. [14], and used to analyse tamoxifen. In this system, retention and elution of tamoxifen and 4-hydroxytamoxifen are controlled by switching the working electrode potential, rather than changing the mobile-phase composition, as performed in more traditional hyphenated techniques incorporating ESI-MS (Fig. 2). One can argue whether this technique is hyphenated; strictly speaking, an electrochemically modulated preconcentration and sample matrix elimination device can be viewed as a separation technique combined with an absolute detection system.

Using a 5.0 min preconcentration period with SRM for tamoxifen (m/z 372→72), detection limits (DLs) were 0.010 n*M*<DL<0.025 m*M*.

3. Oxazaphosphorines

Cyclophosphamide (CP, 2-[bis(2-chloroethyl)-amino] - tetrahydro - 2*H* - 1,3,2 - oxazaphosphorine - 2-oxide, Endoxan) and ifosfamide (IF, *N*,3-bis(2-chloroethyl)tetrahydro - 2*H* - 1,3,2 - oxazaphosphorine - 2-amine 2-oxide (Holoxan) play a significant role in the treatment of various solid malignancies. Trofosfamide, mafosfamide, and sufosfamide are other oxazaphosphorines, while glufosfamide is a new compound, which can be given orally and is currently in clinical trials.

Mustard gas, or sulphur mustard, was first used as

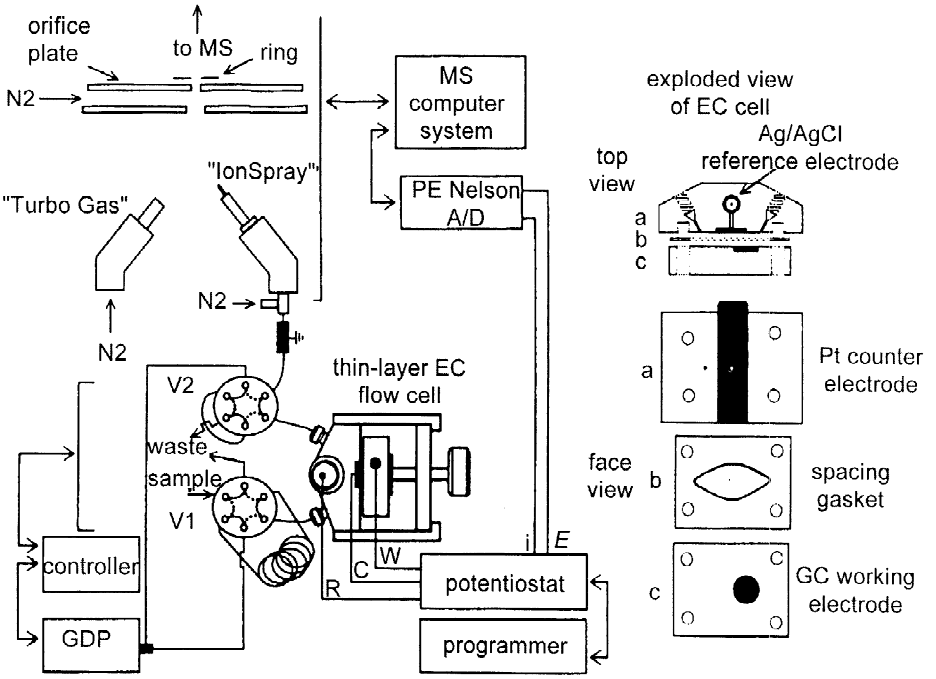

Fig. 2. Schematic of the EMPM/ESI-MS system.

a chemical warfare agent on the Belgian front at Ieper in 1917, following which it became known as ''Ieperiet''. Contact with the gas caused bone marrow suppression, a phenomenon which was again documented following the Bari incident in 1943, when sailors were exposed to the compound. These effects stimulated research into the potential anticancer effects of sulphur mustard, and nitrogen mustard was developed as a more readily formulated alternative. Nitrogen mustard was carefully investigated using transplanted lymphosarcoma in murine models, and, shortly thereafter, the first clinical trials were performed. The oxazaphosphorines and melphalan were developed as derivatives of nitrogen mustard in the 1950s.

Malet-Martino et al. [15] recently reviewed the analytical tools for CP and its metabolites. CP analytical methods can usually readily be adapted for IF, as these oxazaphosphorines are structural isomers. The use of LC–MS(–MS) and CE–MS(–MS) has been limited, but impressive results have already been obtained [16,17]. LC–MS(–MS) assays of CP, IF, and their sometimes labile metabolites have the advantage of sample introduction without derivatization, in contrast to GC–MS. LC–MS(–MS) has been exploited to monitor CP plasma [17,18] and urine [16] concentrations. The analytes can be extracted from plasma by liquid–liquid or solid-phase extraction. Fox et al. [18] eluted CP with methanol–0.1 *M* ammonium acetate buffer (pH 4.9) (60:40) at 1 ml/min. Detection and quantitation were performed by MS (Finnigan MAT TSQ 700, atmospheric pressure chemical ionization interfacing). The parent ions at *m/z* 261 and 265 (CP and d4 internal standard, respectively) were selected by quadrupole 1, collisionally dissociated in the octapole to daughter ions at *m/z* 120 and 124, then monitored via quadrupole 3. The utility of the validated assay was demonstrated in a clinical pharmacokinetic study of CP given as a 1000 mg/m^2 intravenous infusion over 1 h [18]. For LC–MS–MS analysis of CP at low concentrations in urine, a liquid–liquid ethyl acetate extraction was preferred [16], enabling the detection of 0.05 ng/ml in the urine of hospital personnel involved in the preparation and administration of antineoplastic agents [16].

Kalhorn et al. [17] quantitated three cytotoxic metabolites of CP in the plasma of cancer patients by LC–APIc-MS, phosphoramide mustard (PM), carboxyethyl phosphoramide (CEPM), and 3-hydroxypropylphosphoramide mustard (HPPM) (Fig. 3).

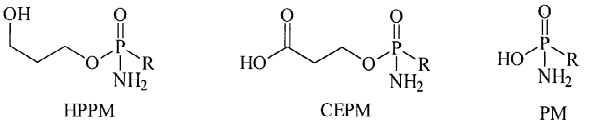

Fig. 3. Chemical structures of HPPM, CEPM, and PM.

A Shimadzu LD-10AD solvent delivery system and an Alcott 738 R autoinjector, equipped with a cooled (4 °C) sample tray, were used. Five percent (50 μl/min) of the column effluent was introduced into a Micromass II tandem quadrupole MS system using ESI. The ESI probe was maintained at 3.8 kV, the cone voltage at 30 eV, and the source temperature at 80 °C. For quantitation, SIR mode monitoring at *m/z* 221, 223 and 227 was used for PM and [$^2H_4$]PM, *m/z* 279 and 281 for MPPM, and *m/z* 293, 295 and 299 for CEPM and [$^2H_4$]CEPM. The quantitation limits of these crucial metabolites were at the lower μ*M* level, which enables the routine profiling of metabolite formation and clearance in cancer patients treated with CP [17].

4. Other alkylating agents

Melphalan (*p*-[bis(chloro-2-ethyl)amino]-L-phenylalanine, L-phenylalanine mustard, L-PAM) does not require metabolic activation by hepatic enzymes, unlike CP and IF. Over the last 40 years, L-PAM has become established as an agent with a wide spectrum of antitumor activity, in both leukaemias and solid tumors. It exerts a cytotoxic effect through the formation of inter-strand or intra-strand DNA cross-links, or DNA–protein cross-links, via the two chloroethyl groups of the molecule. Mono-adducts of mustards most frequently occur at the N^7 position of guanine, but such adducts of melphalan have also been demonstrated at the N^3 of adenine. The use of hyphenated analytical methodology in anticancer drug monitoring is not restricted to blood plasma. The group of Esmans et al. used capillary (cap) and nano-liquid chromatography (nLC)–ESI-MS–MS for the analysis of minor DNA–melphalan adducts [19]. The detection limit of a dAMP–melphalan adduct

was 395 fg using nLC–ESI-MS–MS under single-ion monitoring conditions at $S/N = 14$. Minor adducts, e.g. cross-linked adducts, could only be detected with nLC–ESI-MS–MS in an in vitro solution of 2′-deoxynucleotides (dNMP) exposed to melphalan. DNA from Jurkat cells exposed to melphalan was isolated and enzymatically hydrolyzed. Various modified dinucleotides were identified, and the most abundant adducts were pdG$^{Mel(Cl)}$ and pdC (m/z 453), and pdG$^{Mel(OH)}$ and pdC ring opened (m/z 453), at retention times (t_R) of 17.0 and 39.45 min, respectively (Fig. 4).

This study illustrates the capacity of a sophisticated hyphenated technique to detect the interaction of an anticancer agent with its site of action, tumor DNA.

Busulfan (1,4-butanediol dimethanesulfonate) is a bifunctional alkylating agent used in the treatment of chronic myelogenous leukemia at dosages of 0.065–0.1 mg/kg daily. It is also used at high doses (4 mg/kg daily) concomitantly with CY as a preparative regimen for bone marrow transplantation procedures, when profiling of blood plasma concentration–time curves is important to prevent serious side-effects such as veno-occlusive disease of the liver. LC–MS has been used for monitoring busulfan concentrations in the cerebrospinal fluid and serum of children undergoing bone marrow autotransplantation [20]. Two liquid–liquid extraction steps with dichloromethane were required for isolation of the analyte from biological fluids. Separation of busulfan was carried out by isocratic reversed-phase LC, and the MS system was operated in the ESI mode. Principal ions were observed for busulfan at m/z 175, 111 and 79, and m/z 175 was chosen for quantitation of the analyte. Busulfan was eluted within 2.5 min. A limit of detection of 100 ng/ml permitted busulfan concentration–time profiling of cerebrospinal fluid and serum during the 4 days of high-dose (1 mg/kg) treatment prior to autotransplantation.

Chlorambucil [4-(bis-2-chloroethylaminophenyl)-butyric acid] is an antineoplastic agent used in the treatment of chronic lymphocytic leukemia, lymphomas, and ovarian and breast carcinomas. An *N*-oxide derivative has been developed, which is reduced to chlorambucil in the hypoxic milieu of solid tumors. A hyphenated bioanalytical method [LC–MS(–MS)], including an automated solid-phase extraction of chloroambucil and its phenyl acetic acid mustard (PAAM) metabolite (Fig. 5) from human serum or plasma, has recently become available [21].

Automated solid-phase extraction of the analytes is performed with a C_{18} sorbent packed in a 96-well format microtitre plate, using a robotic sample processor. Isocratic RPLC with pneumatically and thermally assisted electrospray ionization (TurboIonspray) is employed. Using a sample volume of 200 μl, accurate and precise monitoring can be performed within the range of 4–800 ng/ml of biological fluid. The analytes are detected by SRM of the transitions m/z 304–192 for chlorambucil, m/z 312–172 for [$^{13}C^{2}{}_{4}H_{4}$]chlorambucil, m/z 276–

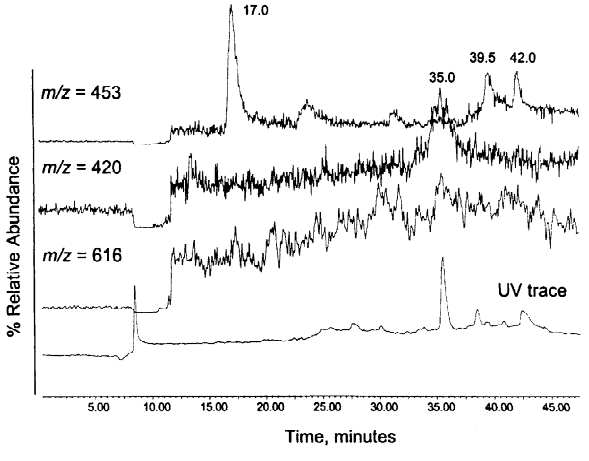

Fig. 4. Analysis of 10 μl of a DNA hydrolysate of melphalan-treated Jurkat cells by nanoLC–ESI-MS using column switching. Reconstructed ion chromatograms for the $[MH]^+$ ions corresponding to gua$^{Mel(Cl)}$ (m/z 420), dGMP$^{Mel(Cl)}$ (m/z 616), pdG$^{Mel(Cl)}$ pdC ($[M+H]^+ = 453$, $t_R = 17.0$ min) and pdG$^{Mel(OH)}$ pdC ring opened (m/z 453, $t_R = 39.0$ min).

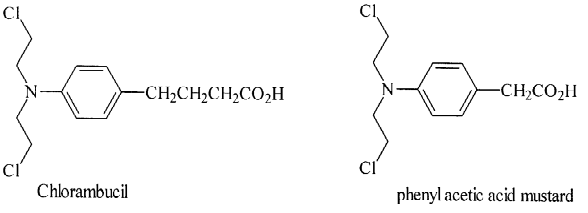

Fig. 5. Structures of chlorambucil and phenyl acetic acid mustard (PAAM).

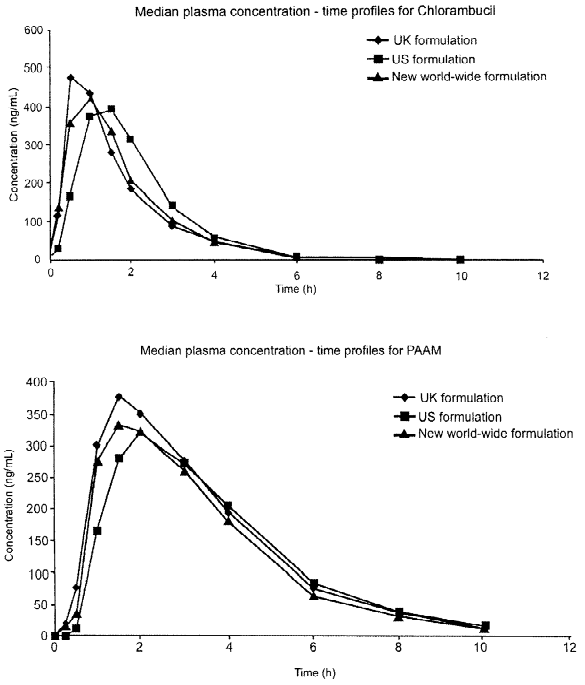

Fig. 6. Median plasma concentration–time profiles for chlorambucil and PAAM following a single oral dose of three chlorambucil formulations.

164 for PAAM, and *m*/*z* 286–167 for [$^{13}C^{2}{}_{4}H_{6}$]PAAM. Simulation of the blood plasma concentration–time curves of chlorambucil and PAAM following a single oral dose is given in Fig. 6, based on data acquired using this LC–MS(–MS) system [21].

The alkylating agent *N*,*N*′,*N*″-triethylenethiophosphoramide (thioTEPA) has been used for more than four decades (Fig. 7). It has a broad spectrum of antitumor activity, and is currently employed in high-dose combination regimens for breast cancer, ovarian cancer, and other solid tumors. The structural identification of thioTEPA-mercapturate, a novel metabolite of thioTEPA, has recently been performed by LC–MS [22]. The identification and monitoring of such metabolites is important for understanding pharmacokinetic and pharmacodynamic processes. ESI-MS was used following elution of the analytes over RP18. The *m*/*z* ranges 350–360 and 255–265 were scanned for thioTEPA mercapturate and the internal standard sulphadiazine, respectively.

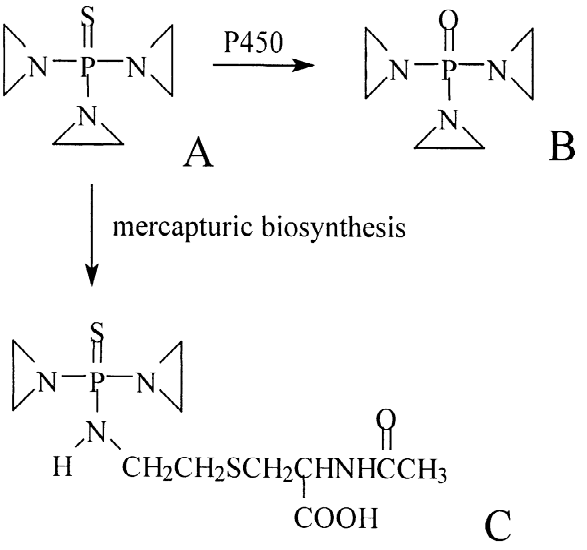

Fig. 7. Biotransformation of thioTEPA (A) to TEPA (B) and thioTEPA-mercapturate (C).

5. Capecitabine

Fluoropyrimidines, such as fluorouracil (Fura), have been an integral part of many cytotoxic regimens for more than 40 years, but their use has been limited by schedule-dependent effects and poor oral absorption. Absorption has been improved with the introduction of Fura and doxifluridine (5′-deoxy-5-fluorouridine), and, most recently, capecitabine {*N*-[1-(5-deoxy-β-D-ribofuranosyl)-5-fluoro-1,2-dihydro-2-oxo-4-pyrimidinyl]-*n*-pentyl carbamate} (Fig. 8).

Capecitabine is converted enzymatically in vivo to doxifluorouridine, which is metabolized more readily to Fura within tumor cells possessing an increased activity of PyNPase compared with normal cells. Fura is then metabolized into anabolites, some of which are cytotoxic, and catabolites such as FuraH_2 (Fig. 8) [24].

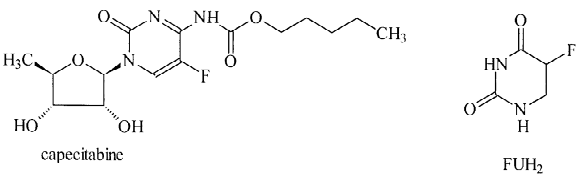

Fig. 8. Structures of capecitabine and FUH_2.

Various analytical tools have been developed to analyze anabolic and catabolic nucleosides and nucleotides concomitantly: GC, HPLC, supercritical fluid chromatography, and CE, with different modes of detection [25]. In a study of oral capecitabine, LC–MS could determine 5′DFCR (5′-deoxy-5-fluorocytidine) and 5′DFUR (5′-deoxy-5-fluorouridine) simultaneously, and Fura with 5-FUH_2. Compounds were eluted on C_{18} columns, using methanol–ammonium formate mixtures at flow-rates between 0.8 and 1.0 ml/min. LC–MS–MS can also be used to further explore Fura catabolism. Using these assays, the effects of food, hepatic dysfunction, age, gender, body surface area, and creatinine clearance on capecitabine pharmacokinetics have been investigated.

6. Platinum compounds

Platinum adducts are the critical cytotoxic lesions in DNA after platinum-containing anticancer therapy. Different adducts are formed by the interaction of platinum complexes with nucleotides, but the contribution of individual adducts to the antitumor activity and toxicity of platinum complexes has yet to be fully defined. The use of hyphenated techniques in this field has been limited, but the separation and identification of DNA nucleotide platinum adducts by capillary zone electrophoresis, and capillary zone electrophoresis coupled to MS, has been reported recently [1]. The method was used to analyse the formation of adducts between *cis*-diaminedichloroplatinum (cisplatin) and DNA nucleotides. Uncoated capillaries and basic separation buffers were used for the baseline separation of unmodified and modified nucleotides (adducts). The peak pattern was elucidated by CZE–ESI-MS, and monochloro, monoaqua and bifunctional adduct species were detected.

Coupled CZE–ESI-MS was applied to obtain the structural information needed for routine CZE. The mass spectrum of a solution of dGMP after 6 h incubation with cisplatinum shows besides dGMP (m/z ratio 348), three peaks which are related to the monoaqua (m/z ratio 593), the monochloro (m/z ratio 610), and the bifunctional (m/z ratio 920) platinum adduct of dGMP. A comparable result was obtained for dAMP [1]. For these studies, hyphenation was needed to establish the quality of CZE without using MS. It has now become possible to discriminate the platinum species responsible for the desired effects and unwanted side-effects. This may result in an increase in the therapeutic efficacy of already registered and new platinum species.

7. Concluding remarks

Hyphenated techniques have become important tools in cancer research, both in fundamental and clinical studies. Owing to the labile character of the many substances under investigation, LC–MS(–MS) and CE–MS(–MS) seem to be the current analytical tools of major interest. Furthermore, developments in micro-array techniques suggest a markedly increased throughput if combined on-line with mass spectrometry.

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