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Review

Separation methods for anthraquinone related anti-cancer drugs

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Abstract

The quinoid anthracycline-related anti-cancer agents represent an important group of anti-tumour drugs with a wide spectrum of activity. We review here some of the separation techniques used for the analysis of anthracyclines and related compounds. In this review we have covered a range of compounds from the early anthracycline antibiotics such as doxorubicin to the more recent anthracenediones and anthrapyrazoles such as mitoxantrone and losoxantrone, respectively. We also include novel compounds such as AQ4N and C1311, both awaiting clinical trial. Separations of the anthraquinone related anti-cancer agents are predominantly by HPLC. These separation techniques have been used for a variety of applications including drug stability, protein binding and therapeutic drug monitoring as well as detailed pharmacokinetic and metabolic studies. Pharmacokinetics, and therefore drug analysis, plays a central role in both the development of new agents and also leads to a better understanding of clinically established agents in this class. Sample preparation and extraction methods including solid-phase and liquid–liquid extraction have also been highlighted. Many anthraquinone related compounds are highly coloured and fluoresce. They are suitable for a range of detection methods including UV–Vis, electrochemical and fluorescence. The methods described are used for sometimes complex separations that are needed for the evaluation of such compounds in biological samples.

Keywords: Reviews; Anthracyclines; Anthraquinone

Contents

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1. Introduction

The quinoid anti-cancer agents represent an important group of anti-tumour drugs. Quinoids appear widely in nature, the most common of which are the quinones which have oxygen at the terminal positions. Quinones readily undergo addition and substitution reactions with direct activity on cellular nucleophils such as thiols. A classical action of the quinones is the production of a semiquinone radical following one electron reduction. Once formed, a semiquinone radical can be oxidised back to the quinone form by the transfer of the electron to a suitable donor. Important biological molecules that can facilitate this oxidation include oxygen (with the formation of a superoxide anion radical) and cytochrome C. The semiquinone radical has been shown to bind readily to DNA and other biological molecules [1]. The most common method of reduction of simple quinones occurs via cellular flavoenzymes in the presence of suitable electron donors. Some of these enzymes, such as NADPH-cytochrome P450 reductase, catalyse only single electron reduction whereas others (such as DT Diaphorase) catalyse two electron reduction with the production of hydroquinone. In addition, enzymes such as xanthine oxidase catalyse both one and two electron reduction.

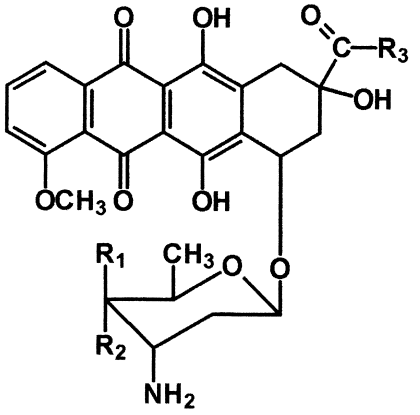
The nature of quinoid agents in the damage of cellular DNA has made them very useful as anticancer agents from some of the earliest therapies (e.g. the anthracycline antibiotics to the more recent anthraquinones and anthrapyrazoles). Many of these compounds, such as doxorubicin (adriamycin), exert their activities through a number of mechanisms such as redox cycling (resulting in generation of reactive oxygen species), direct intercalation into DNA and inhibition of the enzyme topoisomerase II. Indeed, recent structure activity studies with a range of anthraquinone derivatives investigated the influence of redox active groups in these molecules, on the murine leukaemia P388 cell line in vitro [2]. These studies indicated that the major mode of action is through direct DNA affinity rather than via redox activity. The DNA interacting activity of these types of compounds has also recently been investigated with the anthacene-dione class of compounds [3]. Therefore, based on the successes of the anthracyclines in the clinic, such direct DNA damaging cytotoxic agents are still of great interest in terms of future drug development in the treatment of cancer. With such agents, accurate assessment of both circulating concentrations and metabolically transformed compound is of great importance in the clinic. Planar agents, such as the anthraquinones, are known to be substrates of the P450 1A subfamily of metabolising enzymes [4], and in many cases are readily metabolised to both inactive and, perhaps more importantly, active or toxic species. The potential biotransformation of compounds to metabolites, which exert cytotoxic activity in their own right, must be considered with novel agents. Therefore, the requirement of robust analytical methods is paramount during the drug development process. Development of increasingly more potent compounds requires methods to be particularly sensitive in terms of detection, with LC–MS methodologies taking this role on many occasions. Reversed-phase HPLC using MS compatible mobile phases can allow the development of electrospray MS methods using highly specific selected ion monitoring techniques.

In this review, we have tried to summarise the analytical methods used by various groups to study and monitor a number of anthraquinone related compounds both in preclinical and clinical settings, from the historical anthracycline antibiotics to the more recent anthrapyrazoles and imidazoacridinones. The methods used have been varied but, in most cases, the method of choice appears to be the use of high-performance liquid chromatography to achieve the often complex separations desired for the evaluation of compounds in biological samples.

2. Anthracycline antibiotics

2.1. Biological and pharmacological aspects

One of the largest classes of anti-tumour quinoids, and most studied, are the anthracycline glycoside antibiotics which were initially isolated from varied strains of *Streptomyces*, with daunomycin (daunorubicin) (Fig. 1) from *Streptomyces peucetius* being intensively studied in the earliest examinations [5]. The potent anti-leukaemic activity of daunorubicin brought about an extensive screen for other anthracyclines that exhibited anti-tumour activity. This screen resulted in the isolation and identification of doxorubicin [6] (Fig. 1). Doxorubicin has shown a wide range of activity in solid human tumours such as tumours of the breast, lung, ovary, head and bladder, and is the most effective single agent against soft-tissue sarcomas in adults. Toxic effects of the anthracyclines include myelosuppression, cardiomyopathy (which is the dose-limiting toxicity), nausea and vomiting, alopecia and potential tissue necrosis. The most studied potential mechanism for cytotoxicity of the anthracyclines is through action on DNA via intercalation of the non-glycone moiety between adjacent base pairs of the DNA double helix [7]. This results in profound effects on DNA, RNA and protein synthesis, DNA protein associated strand breaks and DNA repair inhibition [8].



	R_1	R_2	R_3
Epirubicin	OH	H	CH_2OH
Doxorubicin	H	OH	CH_2OH
Daunorubicin	H	OH	CH_3

Fig. 1. Chemical structures of some anthracycline antibiotics, epirubicin, doxorubicin and daunorubicin.

The major dose-limiting toxicity of the anthracyclines is cardiotoxicity via the production of free radicals from the quinone moieties. Early observations showed that the anti-tumour activity of the anthracyclines required the anthracycline ring and the basic amino group of the daunosamine sugar whereas cardiotoxicity was dependent on the daunosamine sugar (which enabled uptake by cardiac muscle cells) [9]. These observations were very influential in the development of alternative anthraquinone related compounds such as the bis (substituted aminoalkylamino) anthraquinones.

2.2. Analytical methods

The anthracyclines are best represented by doxorubicin. Analytical methods available for doxorubicin and related anthracyclines are numerous. We have found over 50 reported methods in the literature for doxorubicin alone. The reader is referred to an excellent review of anthracycline separations by De Jong et al. [10]. De Jong not only covers separation of anthracyclines but just as importantly sample handling and sample clean-up, as the extraction procedures have often confounded quantitative measurement. The ‘‘sticky-lipophilic properties’’ of the anthracycline type compounds give them the potential to bind to a variety of materials, especially glass. De Jong et al. [10] suggest that the investigator should avoid glass and polystyrene and use only polypropylene tubes. The stability of the compounds is also discussed with emphasis on pH, as stability decreases with increase in pH. Bosanquet [11] covers the topic of anthracycline stability in more detail. The majority of separation techniques described in the literature use reversed-phase HPLC and utilise the fluorescent properties of doxorubicin. Only minor adjustments are needed to analyse related compounds such as daunorubicin or epirubicin and indeed most investigators use one of these compounds as an internal standard in the analysis of the other.

In our experience one of the best and most comprehensive publications for the analysis of doxorubicin and metabolites was by Nicholls et al. [12]. The authors have used a solvent selectivity triangle to optimise the mobile phase for the separation of doxorubicin epirubicin and six metabolites (Fig. 2). The resulting mobile phase consisted of acetonitrile–0.06 *M* Na_2HPO_4 containing 0.05% (v/v) triethylamine adjusted to pH 3.6 with 0.1 *M* citric acid (67.5:32.5, v/v). This is a method which we have used successfully in our own laboratory. A solid-phase extraction procedure for extracting doxorubicin and metabolites from biological material is also described using C_8 solid-phase cartridges with recoveries of >90% quoted for both doxorubicin and epirubicin. Detection by fluorescence (excitation 254 nm, emission 560 nm) gives a detection limit for doxorubicin in serum of 1 ng/ml. Further detailed discussion of anthracycline separation and reversed-phase column technology is given in a later communication [13]. The use of phosphate–citric acid in the mobile phase, however, makes this method incompatible with the majority of online MS techniques, as volatile buffers are preferred such as those used by Cox et al. [14]. Cox et al. [14] studied tissue levels of adriamycin (doxorubicin) using acetonitrile–ammonium formate as a mobile phase which is preferred by the majority of LC–MS systems. Lachatre et al. [15] achieved full LC–ES-MS (liquid chromatography–electrospray mass spectrometry) analysis of four anthracyclines including epirubicin, daunorubicin and doxorubicin using a similar 5-m*M* ammonium formate buffer (pH 3)–acetonitrile (70:30, v/v) mobile phase. Following solid-phase extraction of the anthracyclines from plasma they were separated using a Symmetry C_{18} column and detected by electrospray MS. Highly sensitive and specific detection was achieved by manipulating the cone voltage (between 60 and 120 V) and by using the selected ion monitoring mode. The resulting method gave limits of detection approaching 1 ng/ml.

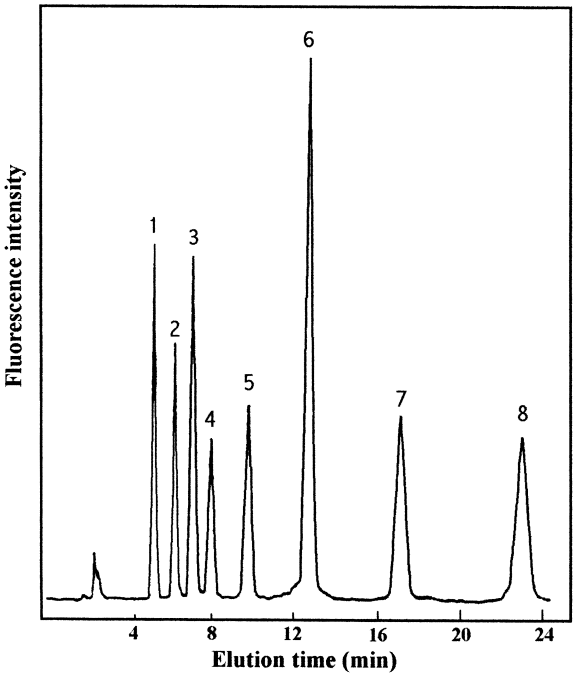


Fig. 2. HPLC chromatogram of extracted serum samples using 250×4.6 I.D. Spherisorb ODS-1 column eluting with acetonitrile–0.06 *M* Na_2HPO_4 containing 0.05% (v/v) triethylamine, adjusted to pH 4.6 with 0.03 *M* citric acid prior to mixing (35:65, v/v). Peaks: 1, doxorubicinol; 2, epirubicinol; 3, 7-hydroxydoxorubicinol; 4, doxorubicin; 5, epirubicin; 6, 7-hydroxydoxorubicin aglycone; 7, daunorubicin; 8, 7-deoxydoxorubicin aglycone. Reprinted from Ref. [12] with permission from Elsevier Science.

Alvarez-Cedron et al. [16] have found solid-phase extraction to be ''involved and laborious'' and therefore developed a single solvent deproteinising step (using methanol–$ZnSO_4$) with a high recovery of doxorubicin (close to 100%). Although the method was tested for pharmacokinetic applicability there was no mention of the separation of metabolites. Murdter et al. [17] also developed an alternative extraction system to solid phase by using $AgNO_3$ to deproteinise the sample. They then removed the excess $AgNO_3$ with strong NaCl (3 *M*). Separation of doxorubicin and its metabolites was achieved on a Lichrospher100 RP18 5-μm column and a mobile phase of 20 m*M* citric acid (with 0.14% triethylamine) (pH 2.4)–acetonitrile–methanol–tetrahydrofuran (100:50:25:5, v/v/v/v). The tetrahydrofuran was added and pH adjusted to enable the difficult separation of doxorubicin aglycone and doxorubicinol desoxyaglycone.

Microbore HPLC is becoming an increasingly popular technique due to the advantages described by Zhao and Dash [18], which include rapid column equilibration and enhanced sensitivity and resolution,

and applicability to LC–MS methodologies. This group carried out their analysis on a 50×1-mm C_{18} Luna column with a flow rate of 0.1 ml/min and a mobile phase of acetonitrile–1% acetic acid (80:20). Fluorescence detection was used (λ_{Ex}=505 nm, λ_{Em}=550 nm) though limits of detection in the region of 1 ng/ml seemed very similar to those on a normal column.

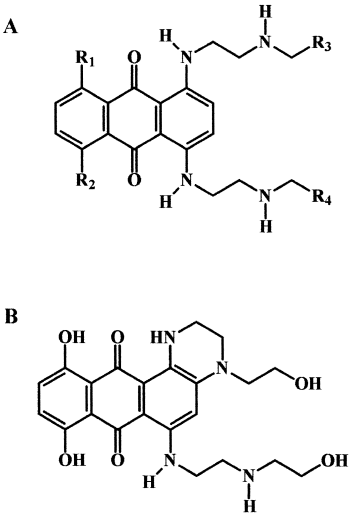
As previously mentioned separation methods for anthracyclines are numerous. Though the analysis of doxorubicin or other anthracyclines alone is relatively straightforward, careful attention needs to be paid, when analysing drugs in biological samples, to the separation of metabolites. Anthracycline metabolism is complex and as some metabolites are active their detection is important. Many reports of anthracycline separation do not adequately cover the separation of metabolites.

3. Anthracenediones

3.1. Biological and pharmacological aspects

As previously indicated, the anthracycline antibiotics doxorubicin and daunorubicin have been shown to exhibit very high anti-tumour activity. Contained within the tetracyclic chromophore portion of the ring structures of these compounds is an anthracenedione ring framework. Mitoxantrone (1,4-dihydroxy-5, 8-bis({2[(2-hydroxyethyl) amino]-ethyl}amino)-9,10-anthracenedione dihydrochloride) (Fig. 3A) is a synthetic representative of a group of compounds termed the anthracenediones. In the 1970s, the bis-substituted anthracenedione ametantrone (Fig. 3A) (originally developed for used as a ballpoint pen ink) was shown to exhibit very good anti-tumour activity [19].

Based on these early studies, and in order to develop more active compounds, two groups (Murdock and colleagues [21,22] and Zee-Cheng and colleagues [23]) assessed a number of structural variations and found mitoxantrone to show the most promising activity [20–23]. Both ametantrone and mitoxantrone showed curative activity versus P388 leukaemia and B16 melanoma although mitoxantrone was shown to have reproducibly better activity versus L1210 leukaemia, colon carcinoma 26 and,



	R_1	R_2	R_3	R_4
Ametantrone	OH	OH	CH_2OH	CH_2OH
Mitoxantrone	H	H	CH_2OH	CH_2OH
Monocarboxylic mitoxantrone metabolite	OH	OH	CH_2OH	COOH
Dicarboxylic mitoxantrone metabolite	OH	OH	COOH	COOH

Fig. 3. (A) Chemical structures of ametantrone, mitoxantrone and the mono and dicarboxylic acid derivatives of mitoxantrone. (B) The phenylenediamine oxidised metabolite of mitoxantrone.

importantly, anthracycline resistant sublines of P388 [19]. Studies on the synthetic variants [19,21] showed a number of factors that appeared to be absolute requirements for activity. These were a protonable nitrogen in the side chain, a $(CH_2)_2$ spacer between the ring and side chain nitrogens and a 1,4 location for the two side chains. In addition, hydroxyl groups at positions 5 and 8 (as in mitoxantrone) gave compounds with higher biological potency and efficacy.

In the clinic, mitoxantrone has shown activity against breast cancer, acute leukaemias and non-Hodgkin's lymphoma with marginal activity observed versus non-small cell lung cancer, Hodgkin's

lymphoma, myeloma and cancer of the liver, prostate, bladder, head and neck [24–26].

3.2. Analytical methods

Several methods for the analysis of mitoxantrone have been evaluated during the history of the compound. These include radioimmunoassay [27], ELISA [28] and HPTLC [29,30]. However, determination of concentrations of mitoxantrone and its metabolites by HPLC is currently the method of choice. Described HPLC methods are very numerous with most utilising a C_{18} stationary phase based system [31–39]. In addition, the various HPLC methodologies showed several extraction procedures and detection systems. Liquid–liquid extraction (using dichloromethane [33] and methylene chloride [35]) and cartridge based solid-phase extraction (using XAD-2 resin [32], PRP-1 resin [38] or C_{18} [31]) were the most common methods used. Many authors have found that the addition of ascorbic acid (0.5%) in the final eluent can stabilise this class of compound by inhibiting oxidative degradation. An interesting pre-column switching system was described by Payet and colleagues [36] and involved direct application of biological samples onto a pre-column (C_{18}) (where protein and salts were washed from the samples) before retained mitoxantrone was back-flushed onto an analytical column. This method is advantageous due to the potential for simple and rapid analysis of urine and plasma samples from treated patients. The limit of detection of this assay was improved by the incorporation of a deproteinisation step prior to application of samples to the pre-column [39]. This enabled larger volumes of sample to be applied and gave a limit of detection of 1 ng/ml.

The incorporation of ion-pairing reagents into the described mobile phases was also variable between methods with 1-pentane sulphonic acid [31] and hexane sulphonic acid [33], examples of commonly used ion-pair formers. Importantly, the use of an internal standard (e.g. ametantrone [33], methylene blue [37] and bisantrene [35]) was not commonplace in the methodologies despite the sometimes complex nature of the sample clean-up procedures. Detection systems were more consistent between described methods with wavelengths between 655 and 665 nm routinely used [31,33,37,39]. Electrochemical detection has also been employed with Choi and coworkers [35] achieving a limit of detection of 0.1 ng/ml for mitoxantrone (using an applied potential of +0.75 V vs. an Ag/AgCl reference electrode) compared to 1–75 ng/ml for the range of UV–Vis detection methods.

During the early investigations of mitoxantrone pharmacokinetics in patients, two metabolites were detected in plasma and urine samples by a number of different groups [32–34,40–42]. UV–Visible (UV–Vis) spectral studies on these metabolites [40] indicated that both contained an intact anthraquinone nucleus. Also, the same group showed that the retention times were not altered by incubation with β-glucuronidase or sulphatase. These two metabolites were observed to co-elute with the mono and dicarboxylic acid derivatives (Fig. 3A) during HPLC analysis [32,43].

A further metabolite of mitoxantrone was identified by Blanz and colleagues [44] using tandem mass spectrometry and UV–Vis spectroscopy with confirmation by comparison with independently synthesised molecules. The metabolite is a product of enzymatic oxidation of the phenylenediamine substrate of mitoxantrone (Fig. 3B) and has been observed in patient urine as well as that from pigs and rats. The nature of the molecule makes covalent binding to intracellular targets a possibility via a two electron oxidation of the phenylenediamine substructure to form a highly electrophilic intermediate.

The related bisantrene (9,10-anthracenedicarboxaldehyde bis [(4,5-dihydro-1*H*-imidazol-2-yl) hydrazone] dihydrochloride, CL 216942; NSC 337766) has been separated on the same system as mitoxantrone by Peng et al. [31], who later carried out detailed metabolic studies on the compound [45], and Choi et al. [35] who used bisantrene as an internal standard when using an electrochemical detector for mitoxantrone analysis. Likewise analytical methods for ametantrone (1,4-bis [[2-[(2-hydroxyethyl)-amino] ethyl]amino]-9,10-anthracenedione) are similar to those of mitoxantrone as shown when ametantrone has been used as an internal standard in the analysis of mitoxantrone [46].

AQ4N(1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]-amino} 5,8-dihydroxyanthracene-9,10-dione) (Fig. 4) is also closely related to mitoxantrone and is a lead

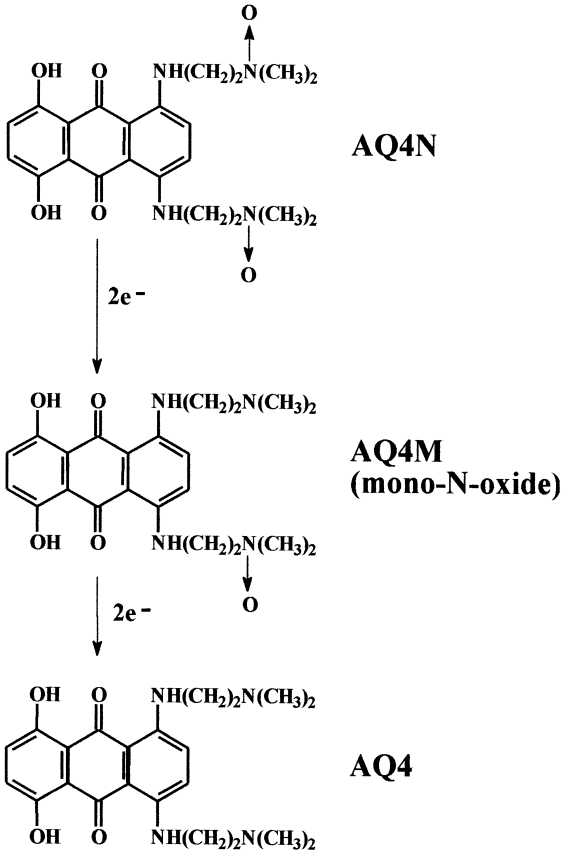


Fig. 4. Chemical structure of AQ4N and its two major metabolites.

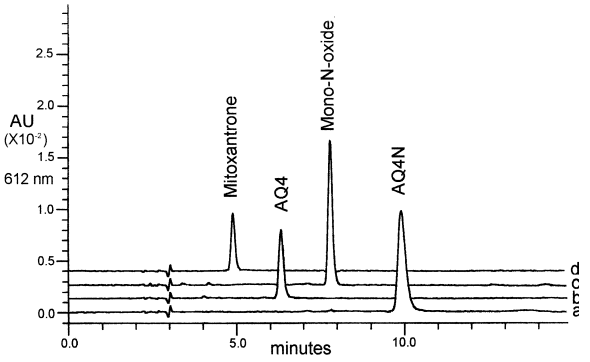


Fig. 5. Chromatographic traces demonstrating the separation achieved with an HIRPB column and a mobile phase consisting of acetonitrile–ammonium formate buffer (0.05 *M*) (22:78, v/v), pH 3.6, and a flow rate of 1.2 ml/min. Traces shown are (a) AQ4N (2.5 μg/ml) retention time 10.0 min, (b) AQ4 (2.0 μg/ml) retention time 6.4 min, (c) mono-*N*-oxide (3.5 μg/ml) retention time 8.0 min, and (d) mitoxantrone, used as internal standard (1.5 μg/ml), retention time of 5.0 min. Reprinted from Ref. [48] with permission from Elsevier Science.

compound from a new series of anti-cancer agents proposed for clinical evaluation in the UK in early 2001 [47]. Swaine et al. [48] have developed a sensitive and reproducible reversed-phase HPLC method for the analysis of AQ4N and its metabolite AQ4 (Fig. 4) in plasma, using mitoxantrone as an internal standard (Fig. 5). The method of Swaine et al. was based originally on a paper by Raleigh et al. [49], and similar methods for the analysis of mitoxantrone [33] and the anthrapyrazole CI-941 [50]. For the analysis of AQ4N Raleigh et al. used a Waters C_8 reversed-phase column with a mobile phase consisting of 80% aqueous ammonium formate (0.5 *M*, pH 4.2)–20% acetonitrile. While for the determination of the structurally related mitoxantrone van Belle et al. [33], employed a C_{18} Bondapak reversed-phase column with a mobile phase consisting of acetonitrile–ammonium formate buffer (0.16 *M*) (30:70, v/v) (pH 2.7) with hexane sulphonic acid added as an ion-pair reagent. However, for the analysis of CI-941, Graham et al. [50] achieved a good separation with a Spherisorb C_6 analytical column and a mobile phase of acetonitrile–methanol–0.25 *M* ammonium formate (adjusted to pH 3 with 98% formic acid) (1:1:8, v/v/v). The use of ammonium formate buffer (pH 3.6) and a Hichrom HIRPB column by Swaine [48] gives excellent peak shape and separation of AQ4N from its metabolites. Also the use of PDA detection in the visible region at 612 nm confers a high degree of selectivity for the blue coloured AQ4N and its metabolites, which simplifies identification when extracting from biological samples. AQ4N can also be visualised using krypton laser (excitation 647 or 514 nm) as AQ4N has intrinsic fluorescence in the far-red spectrum (650–800 nm) [51].

4. Anthrapyrazoles

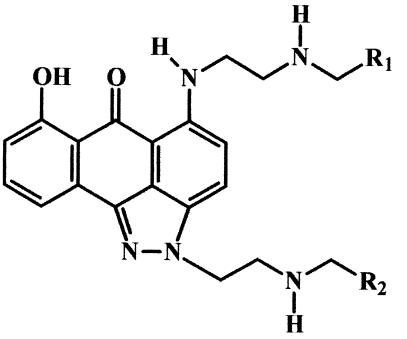
4.1. Biological and pharmacological aspects

As was the case with the development of the aminoanthraquinones, the search for anti-cancer

drugs that retain the spectrum of activity of doxorubicin but exhibit lower cardiotoxic properties has been widespread. In response to this, Warner-Lambert synthesised a group of compounds termed the anthrapyrazoles [52–54]. Structurally, the anthrapyrazoles are similar to the aminoanthraquinones with, in this case, a fourth ring present on the chromophore to form a quinoneamine which is less likely to undergo the redox-cycling reactions observed for the anthracycline antibiotics. Studies by Leopold and colleagues [55], in which more than 80 anthrapyrazoles were studied versus a range of murine tumour models (including L1210 leukaemia, B16 melanoma, M5076 sarcoma and the mammary xenograft MX-1), suggested that both the N-2 and C-5 positions were required for maximum antitumour activity. In addition, compounds hydroxylated at positions 7 or 7 and 10 were most active.

On the basis of their broad spectrum of activity and potential lack of doxorubicin cross-resistance and cardiotoxicity, three compounds were designated as the lead compounds from this group. These were CI-937 (DuP937), CI-941 (DuP-941, biantrazole, losoxantrone, Fig. 6) and CI-942 (oxantrazole, piroxantrone) [55] which were thought to be the true successors to the anthracyclines [56].

In the clinic, CI-941 has shown significant single agent anti-tumour activity in a phase II study versus metastatic breast cancer with an objective response of 63% in 30 patients treated with a dose of 50 mg/m^2 [57]. Preclinical evaluation of the anthrapyrazoles confirmed CI-941 to bind to DNA inducing both single and double strand breaks [52]. In addition, it appears to be a potent inhibitor of DNA synthesis although its effects on RNA synthesis are less striking [53].



	R_1	R_2
CI-941	CH_2OH	CH_2OH
Monocarboxylic acid metabolite 1	**COOH**	CH_2OH
Monocarboxylic acid metabolite 2	CH_2OH	**COOH**
Dicarboxylic acid metabolite	**COOH**	**COOH**

Fig. 6. Chemical structure of the anthrapyrazole CI-941and three of its major metabolites.

4.2. Analytical methods

HPLC is by far the major analytical method for the measurement of CI-941 in biological tissues. A variety of stationary phase systems have been used including C_6 [50] and C_{18} [58]. Methods of extraction of CI-941 samples commonly utilised solid-phase methods with RP-select B, C_2 and C_{18} cartridges employed [50,58,59]. The methods of Blanz and colleagues [59] and Proksch and colleagues [58] involved the inclusion of ion-pair reagents (e.g. 1-pentane sulphonic acid) as a wash step prior to extraction of samples.

Mobile phases used for the separation of CI-941 varied between groups with Graham and colleagues [50] describing an acetonitrile–methanol–ammonium formate mobile phase (pH 3 using formic acid) on an isocratic system. No ion-pair reagent was required due to the excellent peak shape observed through the use of a C_6 stationary phase. UV–Vis detection was performed at 492 nm (λ_{max} for CI-941) with peak identity confirmed using a multiple wavelength ratio method. The limit of detection achieved was 1 ng/ml from a 2-ml aliquot of human plasma. Proksch and colleagues [58] also developed an isocratic system comprising a mobile phase of acetonitrile–water containing 5 m*M* 1-pentane sulphonic acid as an ion-pair reagent. Detection was performed at 491 nm with a limit of detection of 5 ng/ml. An acidic mobile phase was employed by

Blanz and colleagues (trifluoroacetic acid–acetonitrile–dimethyl formide at pH 2.2) [59]. Interestingly, the addition of the buffer salt (ammonium trifluoroacetate) improved the general chromatography, as did addition of the dimethyl formide. In this case, a gradient system was used with the proportion of acetonitrile increasing from 14 to 60% between 20- and 25-min run time. This was performed with to separate potential metabolites of CI-941 in urine and plasma. Also, a volatile solvent system was used due to the requirement for on-line connection with an ion-spray MS system for metabolite identification.

During the previously mentioned phase I study for CI-941 performed by Allan and colleagues [60] two water soluble metabolites were observed in the urine but not plasma. The separation and elucidation of the chemical structures of these metabolites was not performed during this study but modifications in the analytical techniques by Blanz et al. [59] in which a gradient separation system was linked to ion-spray MS equipment enabled their separation. These HPLC studies showed the presence of these two metabolites which were slightly more polar than the parent compound as observed by their retention times. Analysis using HPLC–MS and tandem MS–MS indicated the more polar metabolite (designated metabolite 1) to be a dicarboxyllic acid product of CI-941 with both side chains oxidised at the hydroxymethylene groups (Fig. 6). The second metabolite (metabolite 2) was found to be the analogous mono-oxidation product (Fig. 6) although the side chain on which the carboxylic acid moiety was located could not be identified.

Percentages of parent compound and metabolites in the urine of a treated patient (64 mg i.v. infusion) over a 100-h study period were 0.6, 0.3 and 7.8% for metabolite 1, 2 and parent compound, respectively. Subsequent studies of plasma and urine for the presence of CI-941 metabolites [58] also showed the presence of metabolite 1. In addition, further separation enabled the resolution of both monocarboxylic acid isomers. Both of the monocarboxylic acid isomers and the dicarboxyllic metabolite (Fig. 6) were detected in patient urine as well as a further metabolite (slightly more polar than either of the isomers) which was unidentified. Metabolite 1 and one of the isomers were observed in the plasma. Therefore, although negligible metabolism was observed in preclinical metabolism studies, subsequent modifications in analytical techniques have shown the presence of at least two circulating metabolites of CI-941 albeit at very low concentrations.

CI 942 is closely related to CI-941 and its preclinical pharmacology has been studied by Frank et al. [61]. The method used to study the in vivo characteristics of the drug was based on a reversed-phase system [62] using a dimethylformamide–acetonitrile–ammonium acetate mobile phase at pH 4.5 and a C_2 column; detection limits were reported to be 10 ng/ml. Using a detection wavelength of 514 nm the detection is likely to be highly specific. Extraction was by a solid-phase C_{18} cartridge system from which compounds were eluted at pH 4.0. The pH was found to be important as CI 942 was found to be unstable in neutral and alkaline aqueous solution. CI-942 was also found to be particularly unstable in human plasma ($t_{1/2}$=<5 min), but relatively stable in mouse ($t_{1/2}$=1.75 h), though this disappearance could be inhibited with the use of ascorbic acid suggesting oxidative degradation. Frank et al. [61] also describe the detection of a major metabolite (thought to be a glucuronide) using this system.

Due to the difficulty of reproducibly extracting the anthrapyrazole CI-937 from biological fluids (particularly plasma), Nordblom et al. [63] thought that HPLC was not a feasible option. They therefore developed a radioimmunoassay by conjugating CI-937 to porcine thyroglobulin to a rabbit antibody containing an iodinated label. They report a limit of quantitation of 100 pg/ml with minimal cross reactivity with other anthrapyrazoles or commonly used anti-cancer agents.

5. Imidazoacridinones

5.1. Biological and pharmacological aspects

The structural development of the imidazoacridinones (and also a related group known as triazoloacridinones) was based on the results of studies on the mechanism of action of mitoxantrone [64]. The major features of the imidazoacridinones are a planar, polycyclic nucleus (capable of DNA intercalation) and a polyethylenediamine side chain. In

addition, a pyrazole ring was attached to the chromophore in order to increase the electron density of the π system, making the chromophore more resistant to enzymatic reduction to radical species (as was the rationale behind the development of the anthrapyrazoles). In addition, the presence of the hydroxyl group at position 8 of the acridinone moiety appeared to be very important in the growth inhibiting activity of these compounds [65] with metabolic activation within the cell postulated [66]. Several acridine derivatives have been evaluated for antitumour activity in the past (including amsacrine (mAMSA), Fig. 7) [67], with much work performed on the 1-nitro-9-aminoacridines including nitracrine (marketed in Poland under the name Ledakrin) (Fig. 7). Despite showing anti-tumour activity both in vitro and in vivo, and a lack of myelosuppression, nitracrine was discarded following toxicity in clinical trials [68]. More recently, two of the imidazoacridinone group — C1310 (Fig. 7) and the lead compound C1311 (Fig. 7) — have been extensively studied both in vitro and in vivo, against a range of colon tumours (both murine and human) [69] with C1311 now a strong candidate for clinical evaluation.

Similarities in the structures of the imidazoacridinones, mitoxantrone and the anthrapyrazoles would suggest that all compounds have similar cellular targets.

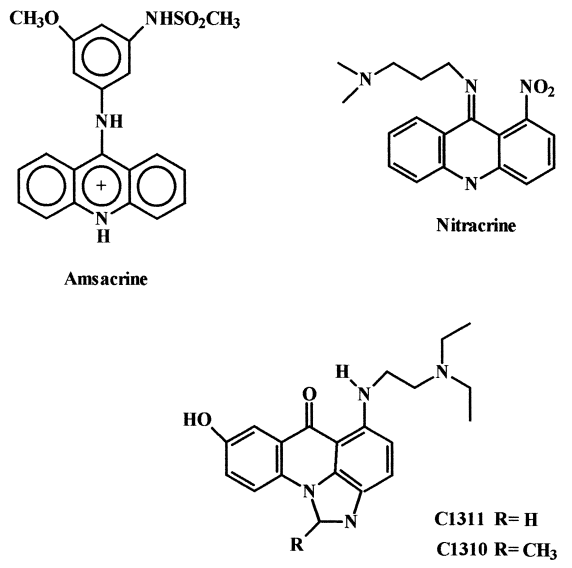


Fig. 7. Chemical structure of the imidazoacridinones C1310 and C1311 and the related compounds amsacrine and nitracrine.

5.2. Analytical methods

Analytical methods available for the analysis of the imidazoacridinones are based on those described for related compounds described earlier. Mazerska et al. [70] have described preliminary electrochemical studies on the oxidation of anti-tumour active imidazoacridinones identifying oxidation products and focusing on the change in spectral characteristics. Analysis by HPLC was also used to monitor reaction products following a relatively straightforward solid-phase extraction of the samples using methanol and water. A methanol–water (45:55) mobile phase was used to separate the compounds and reaction products on a Spherisorb ODS-2 column at a wavelength of 265 nm. This mobile phase was later changed to methanol–0.5 *M* phosphate buffer, pH 2.5 (7:3) and included 0.01% diethylamine [71] when studying the enzymatic oxidation of imidazoacridinone derivatives by horseradish peroxidase.

A further HPLC method has been developed by Calabrese et al. for quantification of C1311 in biological tissues and fluid [72]. Using a mobile phase of phosphate–citrate buffer at pH 4.0 containing 0.07% triethylamine with acetonitrile (50%) and a Spherisorb S5 ODS-1 column, the method was shown to be selective, sensitive (LOD, 1 ng/ml) and reproducible. The sensitivity was achieved by taking advantage of the fluorescent properties of the compound using excitation and emission wavelengths of 420 and 520 nm, respectively. Protein was precipitated from samples using acetonitrile before HPLC analysis. A gradient reversed-phase system was later developed based on the isocratic method to analyse complex urine samples in which at least ten metabolites were separated (Fig. 8) [73]. These methods were later used in further studies to monitor the tissue distribution and metabolism of the drug in mice [74].

Several chromatographic methods are described for the analysis of amsacrine, one by gas chromatography [75]. Brons et al. [76] described a reversed-phase separation on a C_8 column with methanol (40%)–1% triethylamine phosphate (60%) mobile phase. The separation followed extraction of amsac-

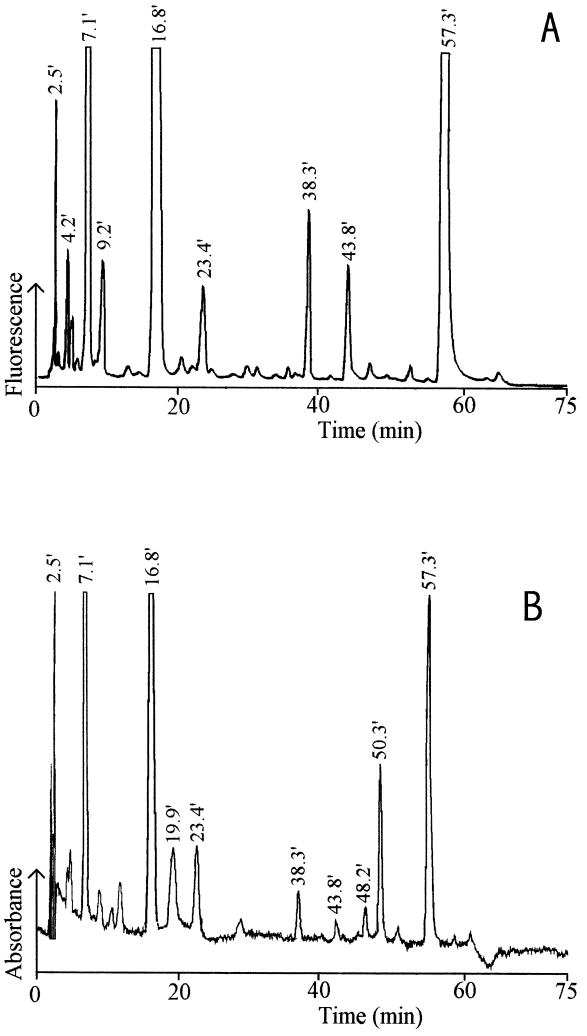


Fig. 8. Fluorescence (A) and $PDA_{420\ nm}$ (B) chromatograms from a gradient separated urine sample, 3 h after treatment of mice with the imidazoacidinone C1311 (50 mg/kg, i.p.) showing the presence of more than ten metabolites. For conditions see Ref. [73]. The imidazoacridinone C1311 elutes at 57.3 min.

rine from blood in ethyl ether. The detection limit with this method was 6 ng/ml at 265 nm

6. Overview

Due to their broad spectrum of activity, anthraquinone related compounds are widely used in cancer chemotherapy with probably the most successful and best known being doxorubicin and mitoxantrone.

We have reviewed here some of the literature covering the separation of anthraquinone related anticancer agents. The review reflects the literature, in that the vast majority of techniques used reversed-phase high-performance liquid chromatography. C_{18} columns either endcapped or with an ion-pair agents are most frequently employed to give very elegant separations of some complex metabolic profiles. A range of detection methods has been discussed including UV–Vis (as many anthraquinones are highly coloured), electrochemical (as many anthraquinones are redox active) and fluorescence (many anthraquinones have excitation wavelengths that overlap those of commonly used lasers, argon in particular at 488 nm). It can also be advantageous to combine detection methods, for example using diode array detection in series with fluorescence, as some fluorescent parent molecules can be metabolised to non-fluorescent species.

Though HPLC has been covered in some detail, other techniques are available but not as widely used. High separation efficiencies and short analysis times make capillary electrophoresis an attractive and very powerful separation technique, though is not commonly cited in the literature for the separation of anti-cancer drugs. As many of the compounds discussed in the review fluoresce, the use of capillary electrophoresis with LIF detection could potentially give low limits of detection. Simeon et al. [77] have indeed shown this with a capillary electrophoresis method to study the daunorubicin concentrations in both tumours and plasma. In one of the few examples of anthracycline separation by CE, Simeon et al. [77] used a fused-silica capillary (42 cm×75 μm) with a separation buffer of acetonitrile–sodium dihydrogen phosphate, pH 4.0 (2:8, v/v) to separate both daunorubicin and doxorubicin (the internal standard). A voltage of 20 kV gave a working electrophoretic current of 61 μA. Useful comparisons are made between the sensitivities of an equivalent HPLC technique using both conventional fluorescence detection and LIF. They conclude that one of the major advantages of CE is the small sample volume required, which allowed pharmacokinetic analysis to be carried out on samples of only a few microlitres. This view is supported by Hempel et al.

[78], who developed a similar electrophoretic separation and used non-invasive blood sampling techniques (from the fingertip) to study the pharmacokinetics of doxorubicin in paediatric patients. Others have used related separations, such as micellar electrokinetic capillary chromatography, to separate anthraquinone derivatives in rhubarb [79] and pressurised capillary electrochromatography to separate doxorubicin [80]. However in our experience HPLC is far more robust than current electrophoretic methodologies and continues to be the more popular technique.

The limit of detection and sensitivity of an assay is always going to be an issue when analysing anti-cancer agents due to the potency of many of them. Steady state levels of some of the compounds discussed (particularly doxorubicin) can be as low as 10 ng/ml and it is therefore desirable to have an analytical technique with a limit of detection approaching 1 ng/ml. The need for this level of sensitivity of course will vary from compound to compound. Methoxymorpholinyldoxorubicin [81] for example, is 100–1000 times more potent in vitro than doxorubicin. The steady state concentrations of such a compound in vivo are likely to be proportionately lower, which could potentially cause analytical problems for such potent compounds.

Sample preparation has also been covered in some detail. With some of the more highly light absorbing anti-cancer agents (e.g. mitoxantrone and AQ4N) a relatively crude protein precipitation will suffice for extraction as specificity is gained by the long detection wavelengths used (550–600 nm). However, great care must be taken with sample preparation and handling of this class of compound as many are known to bind strongly to glass. Lin et al. [37] and Priston and Sewell [46] cover the subject in some detail. Protonated amines are known to react with silanol groups in glass. Lin et al. [37] suggest as much as 95% of loaded mitoxantrone bound to a glass syringe used for sample injection. Polypropylene labware or silylated glassware is generally recommended. Apart from the straightforward protein precipitation, liquid–liquid extraction and solid-phase extraction have also been covered. Solid phase extraction is a very versatile technique that can be highly selective. Many examples have been cited where a range of metabolites can be concentrated and co-elute with the parent compound, but when working with novel anti-cancer drugs the metabolic cascade is not always known, and potential polar metabolites could be inadvertently lost due to this selectivity of the solid-phase matrix.

The separation techniques described in this review have been used for a variety of applications including drug stability, protein binding, metabolic studies and therapeutic drug monitoring or pharmacokinetic analysis. Pharmacokinetics plays a crucial role in a better clinical understanding of current therapies and in the development of new drugs. A sensitive and robust analytical method is of course central to the study of pharmacokinetics. However the recent development of combinatorial approaches to drug development together with high throughput screens has meant that pharmacokinetic evaluation of potential drug candidates has produced a bottleneck in the drug development process. High throughput pharmacokinetics procedures using cassette (multiple-drug) dosing are now common practice [82]. This calls for sophisticated analytical procedures to enable the pharmacokinetic analysis of several compounds simultaneously [83]. This is generally achieved by LC–MS–MS and other sophisticated assays are now used for drug analysis, which include non-invasive techniques such as PET [84] and NMR [85].

7. Conclusions

The vast majority of separation techniques used for the separation of anthraquinone related anti-cancer drugs are currently based on reversed-phase HPLC using UV or fluorescence detection. Tandem mass spectrometry is often used for more complex multi-drug analysis but even so chromatographic separation will continue to play an important role in the understanding of the metabolism and pharmacokinetics of this class of compound.

8. Nomenclature

DT Diaphorase	EC 1.6.99.2; NAD(P)H:quinone oxoreductase, NQO1
LC–MS	Liquid chromatography–mass spectrometry

ELISA	Enzyme linked immunosorbent assay
HPTLC	High-performance thin layer chromatography
LIF	Laser induced fluorescence

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