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Review

High-performance liquid chromatographic methods for the determination of topoisomerase II inhibitors

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Abstract

Various methods for separating eleven different types of topoisomerase II (TOPO-2) inhibitors, including epipodophyllotoxins, anthracyclines, anthracenediones, anthrapyrazoles, anthracenebishydrazones, indole derivatives, aminoacridines, benzisoquinolinediones, isoflavones, bisdioxopiperazines and thiobarbituric acids, are summarized. Proper sample preparation and storage is critical to the successful analysis of some TOPO-2 inhibitors due to difficulties associated with adsorption, instability and complex biological components. Solid-phase and liquid–liquid extractions are widely used to separate TOPO-2 inhibitors from biological samples, although simple deproteinization followed by direct analysis of the supernatant is preferable to extraction based on its speed and simplicity. High-performance liquid chromatography (HPLC) is the favored method for the bioanalysis of TOPO-2 inhibitors. UV or diode array detection is generally employed for early pharmacokinetic studies, while fluorescence or electrochemical detection is used more frequently for analytes with fluorescent or oxidative–reductive properties. For analyses requiring highly sensitive and/or specific detection, electrospray mass spectrometry (ESI-MS or ESI-MS–MS) provides a suitable alternative. A comprehensive compilation of the HPLC techniques currently used to separate TOPO-2 inhibitors will aid the future development of analytical methods for new TOPO-2 inhibitors. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Topoisomerase II inhibitors; Enzymes

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1. Introduction

1.1. Topoisomerase II

The ability of topoisomerase II (TOPO-2) to induce double-strand DNA cleavage is essential for the knotting or unknotting of circular DNA, the introduction or removal of supercoils and formation and resolution of catenanes [1,2]. The two main types of mammalian DNA TOPO-2 (TOPO-2α and TOPO-2β) each consist of a homodimer of a single polypeptide. TOPO-2 is required for proper chromosome structure and segregation and promotes cell survival [3,4]. Thus, TOPO-2 is an excellent molecular target for antitumor agents [5,6].

1.2. Topoisomerase II inhibitors

Drugs targeting TOPO-2 can be divided into three main categories: TOPO-2 poisons that stabilize the cleavable complex, catalytic inhibitors and dual inhibitors [7–13]. The classifications of various TOPO-2 inhibitors are summarized in Table 1 and their structures are shown in Figs. 1–3. Many TOPO-2 inhibitors have been widely used as antineoplastic and antibacterial agents. However, the use of these TOPO-2 inhibitors as antitumor agents is often associated with drug resistance [14–16], occurrence of secondary tumors [17], mutagenicity [18] and ‘‘illegitimate’’ recombination [19–22]. Thus, development of novel, potent TOPO-2 inhibitors is necessary. New methods for the analysis of novel TOPO-2 inhibitors are needed as well, since determination of the levels of novel TOPO-2 inhibitors in biological samples is essential for various pharmacokinetic studies. These studies include assessing bioavailability, identifying metabolites, characterizing drug–drug interactions and measuring free drug concentrations in biological fluids. This review describes the separation techniques and bioanalytical methods used in the analysis of TOPO-2 inhibitors that are currently used as antitumor agents, and also provides guidance for the development of analytical methods for new TOPO-2 inhibitors. Additional information on topoisomerase inhibitors can be obtained from Oguma's review in this volume.

Table 1
Classifications of TOPO-2 inhibitors [6–13]

1. TOPO-2 poisons	
Epipodophyllotoxins	VP-16, VM-26, VP-16 phosphate, TOP-53, NK-611
Anthracyclines	Doxorubicin, Epirubicin, Daunorubicin, Idarubicin, Pirarubicin
Anthracenediones	Mitoxantrone
Anthrapyrazoles	CI-937, CI-941, CI-942
Anthracenebishydrazones	Bisantrene
Indole derivatives	Ellipticine, 9-Hydroxyellipticine, 2-Methyl-9-hydroxyellipticine
Aminoacridines	AMSA, CI-921
Benzisoquinolinediones	Amonafide
Isoflavones	Genistein
2. Catalytic inhibitors of TOPO-2	
Bisdioxopiperazines	ICRF-159, ICRF-186, ICRF-187
Thiobarbituric acids	Merbarone
Others	Fostriecin, Aclarubicin, Novobiocin
3. Dual inhibitors of TOPO-2	
TOPO-2/TOPO-1 inhibitors	Intoplicine, Actinomycin D

2. Analytical approaches to topoisomerase II inhibitors

2.1. Sample preparation and storage

Proper sample preparation and storage is critical to the successful analysis of drugs in biological samples in order to avoid problems associated with adsorption, instability and complex biological components. Some drugs, such as mitoxantrone (MTO), adsorb to glass tubes, requiring the use of siliconized or polypropylene tubes. Addition of anticoagulators to plasma samples is sometimes important. Most TOPO-2 inhibitors are stable in biological samples when stored at temperatures of −20°C and lower. Prevention of degradation or oxidation of analytes is often preventable through the use of additives (e.g., antioxidants).

2.2. Sample pretreatment

Simple deproteinization of biological samples followed by direct analysis of the supernatant is preferred due to its speed and simplicity. However, this process involves dilution and is unsuitable for analysis of samples containing a low concentration of drug or metabolite(s). As an alternative, whole samples can be dried under nitrogen, reconstituted in an appropriate solvent and analyzed by HPLC, although contamination of the HPLC column is likely using this method. With column-switching techniques, simple deproteinization is a time-saving extraction method that yields higher extraction recoveries. When using any extraction method, the biological samples must be protected from light, heat and oxidation.

Liquid–liquid extraction (LLE) is the most frequently used extraction method. The most commonly used extractants are chloroform, 1,2-dichloroethane, ethyl acetate, methylene chloride, diisopropyl ether or some combination of these solvents. In most cases, the biological matrix can be used directly for extraction. In order to yield a higher extraction recovery or to eliminate contaminants, adjusting the pH of the biological matrix prior to LLE is necessary. Such changes to the pH of the matrix must not decrease the stability of the drug or metabolite(s), however. Additionally, extraction with lipophilic solvents may result in the loss of highly polar drugs or metabolites(s) during the extraction. For conjugated metabolite(s), chemical or enzymatic hydrolysis prior to LLE is required in order to isolate and identify the metabolite(s).

Ion-pair extraction is a useful method for the separation of certain drugs. Precautions must be taken to minimize reaction between the ion-pairing agent and the plasma proteins, however. The extraction recovery may be reduced due to retention of

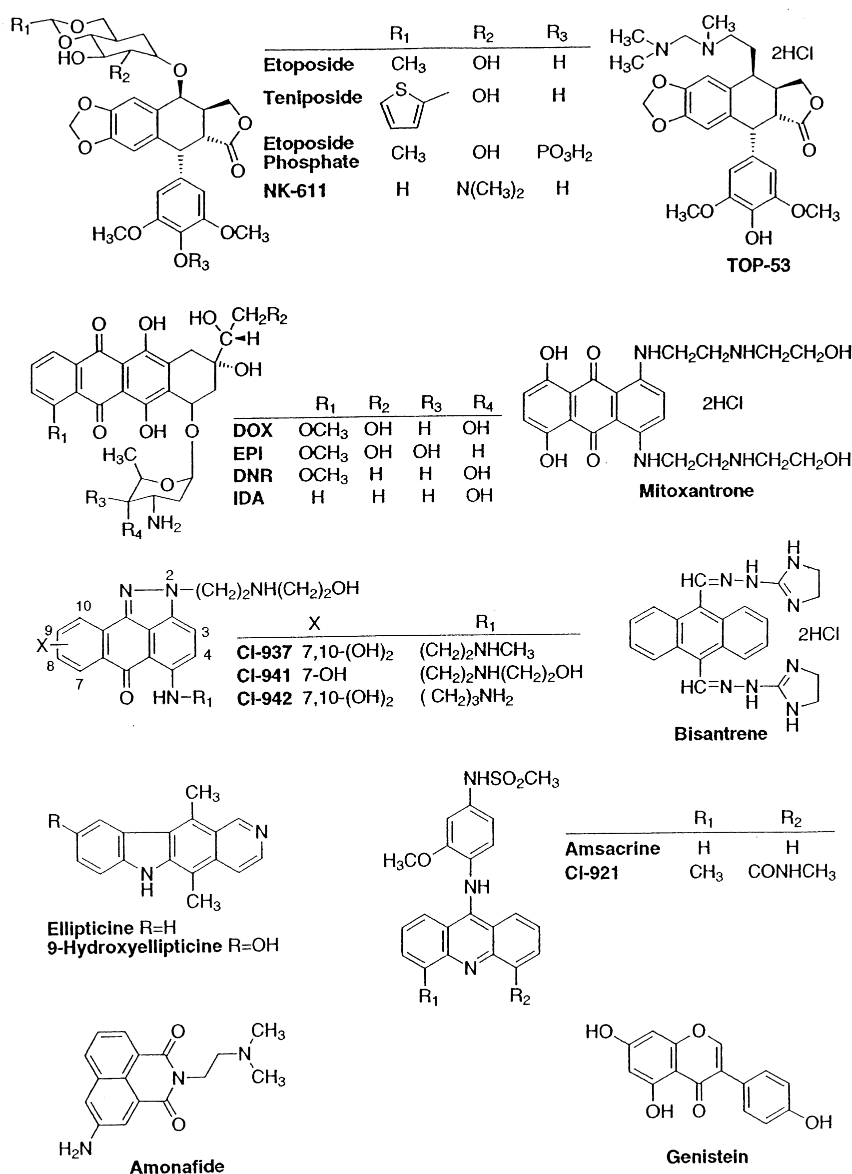

Fig. 1. Structures of the TOPO-2 poisons.

ion pairs of the acid anion and the cationic drug by the organic phase.

Solid-phase extraction (SPE) is a widely used extraction method for therapeutic drug monitoring (TDM). SPE allows rapid handling of large numbers of samples. This method is simple and conducive to automation. While C_{18} sorbents are most commonly used, careful selection of the sorbent material may allow separations more selective than those possible using LLE. Precautions should be taken to minimize unwanted interactions between a basic drug and residual silanol groups on the sorbent. Batch-to-batch variability of the sorbent often results in unpredicted changes in performance and elution characteristics.

Automation is an important way to speed up the analytical process when a large number of samples

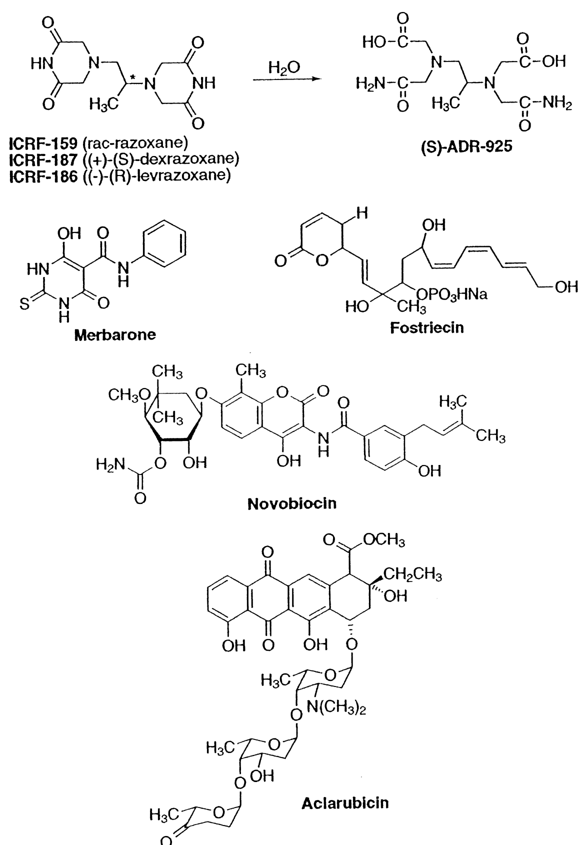

Fig. 2. Structures of the catalytic inhibitors of TOPO-2.

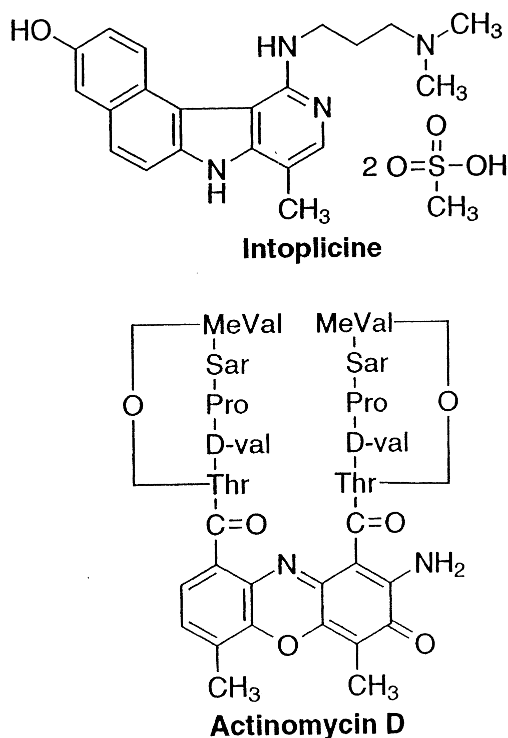

Fig. 3. Structures of the dual inhibitors of TOPO-2 and TOPO-1.

must be analyzed. LLE and SPE are time-consuming and require cumbersome sample preparation, including partitioning between the organic and aqueous phases and careful removal, drying and reconstitution of the organic layers. Further, special care must be taken to ensure that the extraction of strongly protein-bound drugs is quantitative. Simple pretreatments (such as hydrolysis of proteins in blood samples by a proteolytic enzyme), column-switching techniques, and surfactant-mediated sample clean-up can be easily automated, however.

2.3. High-performance liquid chormatographic separation and detection

Several analytical methods, such as gas chromatography and electromigration, are suitable for the determination of TOPO-2 inhibitors. This review focuses on the application of high-performance liquid chromatography (HPLC) to the analysis of TOPO-2-inhibitors, however, as this technique is the most commonly used for the quantitation of these compounds. Reverse-phase HPLC is preferred for analysis of most biological samples and is especially well-suited for the simultaneous analysis of several metabolites. Normal-phase and ion-pair HPLC methods are also used routinely in determining levels of TOPO-2 inhibitors. Chiral HPLC is especially useful for separating diastereoisomers.

HPLC with UV or diode array detection (HPLC–UV and HPLC–DAD, respectively) is suitable for the analysis of most parent compounds and related metabolites due to the stability of these methods. However, interferences from structurally-related endogenous substances cause the detection limit for these methods to be higher than for HPLC with fluorescence or electrochemical detection (HPLC–FLD and HPLC–ECD, respectively). HPLC–ECD is very sensitive, but several TOPO-2 inhibitors are unable to be detected using this approach as they do

not undergo oxidation–reduction at the electrode potentials. Further, the electrodes can easily be contaminated, resulting in a dramatic decrease in sensitivity. HPLC–ECD also requires extensive equilibration. Fluorescence detection (FLD) overcomes several of these drawbacks, as it is nearly as sensitive as ECD and as stable as UV detection. While FLD is generally very specific, however, not all compounds fluoresce. This difficulty can usually be overcome through pre- or post-column derivatization. HPLC with electrospray ionization-mass spectrometric detection (HPLC–ESI-MS) is available in most labs [23], and hence are widely used in the bioanalysis of TOPO-2 inhibitors due to their high specificity and sensitivity. While HPLC with radiodetection is a highly sensitive method, the routine application of this technique for quantitation of TOPO-2 inhibitors is limited by the difficulties in generating radiolabeled drugs and dealing with radioactive waste.

Regardless of the specific separation and detection methods chosen, full validation of the method (including determination of precision, accuracy, sensitivity, specificity, linearity and reproducibility) is required. The stability of the analyte in the matrix in which it is being stored should also be examined [24–27].

3. Topoisomerase II poisons

The action of TOPO-2 poisons is based on the binding of the drug to the enzyme–DNA complex at the strand cleavage stage, thereby forming a ‘‘cleavable complex’’ and leaving the DNA with a permanent strand break that ultimately results in cell death. Different TOPO-2 poisons yield varying DNA cleavage patterns in the genome which in turn result in different cytotoxicities and anticancer activities [9].

3.1. Etoposide

3.1.1. Sample preparation and storage

Etoposide (VP-16), a semisynthetic derivative of podophyllotoxin, is stable in biological samples when stored at low temperatures. Plasma samples spiked with VP-16 and stored for up to 6 months at a temperature of −80°C showed no appreciable degradation [28]. The metabolites of VP-16, such as catechol, are easily oxidized and hence must be protected from oxidation by the addition of ascorbic acid to the samples [29,30].

3.1.2. Sample pretreatment

The most common extraction method for epipodophyllotoxins is LLE using acetonitrile [30], chloroform [31–33,35–37], 1,2-dichloroethane [34, 38–40], ethyl acetate [41], diisopropyl ether [42] or methylene chloride [28] as the solvent. The extractant was evaporated and reconstituted in the mobile phase or another appropriate solution. The extraction efficiency for etoposide is usually over 70%. Use of a methanol–chloroform mixture for extraction improves the recovery of etoposide and catechol to over 90% [29]. Adjustment of the pH of plasma or urine samples to 4.5 prior to chloroform extraction further increases the extraction recovery to 97% [43–45]. The recovery of VP-16 from plasma was over 90% when extracting with 1,2-dichloroethane, dichloromethane, chloroform or ethyl acetate [28,39], although extraction with 1,2-dichloroethane produced the fewest interfering peaks on the chromatogram [34,46]. The hydroxy acid derivatives of VP-16 were not recovered when extracting with these solvents, however. Analysis of these derivatives will likely require the development of ion-pair extraction procedures, since both the hydroxy acid derivatives and the aglycone of VP-16 are protonated at the pH of plasma. LLE with chloroform as the solvent has also been used to separate free etoposide from filtered plasma, cerebrospinal fluid and tissues [33,41,47,48].

While the above extraction methods are suitable for separation of VP-16 from plasma, extraction of VP-16 from whole blood presents a different set of challenges. Solid-phase extraction of VP-16 is impractical due to the high viscosity and degree of clotting of the blood samples. LLE using chlorinated hydrocarbons such as dichloroethane and chloroform results in formation of a clear bottom layer that contains the VP-16, but this layer is difficult to aspirate without contamination by the upper blood layer. Using a dichloroethane–hexane mixture (1:1, v/v) transfers the etoposide-containing layer to the top, which can then be easily aspirated with no risk of contamination [49].

Solid-phase extraction using a reversed-phase stationary phase and simple purging with water and methanol has been used to elute VP-16 [50]. Bond-Elute cartridges have been found to extract VP-16 from plasma [51,52] as efficiently as liquid–liquid extraction. The use of C_{18} sorbent for clean-up of plasma is associated with a low extraction recovery [37].

Werkhoven-Goewie et al. [53] automated the HPLC analysis of VP-16 and its aglycone in serum, plasma and urine samples. The samples were hydrolyzed by a proteolytic enzyme, subtilisin A or proteinase K. The hydrolysis step served both to release the strongly protein-bound drugs and to prevent clogging of the HPLC system. The samples were then concentrated using a small precolumn packed with a microparticulate divinylbenzene–styrene copolymeric sorbent. Chromatographic separation was performed following column switching in a C_{18}–methanol–water system. After a post-column clean-up step involving continuous extraction with dichloroethane in an autoanalyzer system, the analytes were detected using FLD. Recovery of VP-16 from spiked serum and plasma samples was 100%. This method allows the continous, routine analysis of enzymatically-hydrolyzed blood samples.

3.1.3. High performance liquid chromatographic separation and detection

The chromatographic and detection methods used to determine etoposide are summarized in Table 2. The sensitivity of HPLC separation and UV detection of VP-16 is 0.2 μM. The detection wavelengths used for VP-16 are 229 [36], 230 [32], 233 [39], 254 [31,55] and 280 nm [47]. The sensitivity and specificity increases significantly when fluorescence detection is used (LOD=0.1 μM) [28,33,51, 53,56,57]. The use of fluorescence detection also reduces the considerable background noise from endogenous compounds in the plasma and cells that is typical with UV detection. Electrochemical detection further improves the sensitivity (LOD=0.02 μM) [29,30,34,38,41,46,49,52,54,58]. However, ECD is less stable and technically more complicated, requiring extensive equilibration prior to analysis. Each of these methods involve multiple-step extractions, and some even involve enzymatic hydrolysis of proteins, column-switching clean-up or mass spectrometric identification of sample components. El-Yazigi and Martin [37] developed a method employing a simple one-step extraction and radial-compression liquid chromatography with electrochemical detection for analysis of VP-16. This method greatly decreases the analysis time, yields a high chromatographic efficiency and is technically simple.

Etoposide levels can be measured by LC–MS with a ^{252}Cf fission fragment-induced ionization interface [43,44,59]. This interface is not routinely available for mass spectrometers, however [23]. More recently, HPLC–electrospray mass spectrometry using an atmospheric pressure ionization (API) interface was used for the analysis of VP-16 [35]. The sensitivity and specificity of this method for the quantitation of VP-16 surpasses those of all previous methods employed, including HPLC–UV, HPLC–DAD, HPLC–FLD and HPLC–ECD (Fig. 4).

3.1.4. Determination of etoposide metabolites

Several metabolites of etoposide have been identified, including the *cis*-isomer [34,36,41,47,52,57], the hydroxy acid [31,41], the aglycone [32,41,45], the glucuronide [60], the sulfate [60], picro VP-16 [31] and picro VP-16 hydroxy acid [31]. Only some studies reported finding certain metabolites, such as the hydroxy acid and the *cis*-isomer, in plasma and urine samples [31,41]. Further, some of the metabolites may actually be degradation products formed in an acidic or basic environment [41,55]. Currently, only the demethylation of etoposide to form the active catechol metabolite has been fully characterized [61–63]. A quantitative analysis involving HPLC–ECD for simultaneously monitoring etoposide and catechol has been developed (Fig. 5) [29,30].

3.1.5. Determination of free etoposide

Etoposide is lipophilic and almost entirely (94%) binds to the albumin in human plasma [35,64]. To measure levels of free etoposide, radioactive reagents or time-consuming equilibrium dialyses are generally employed [64,65]. However, inexpensive, disposable ultrafiltration devices have recently been developed for the separation of free and bound drugs. The in vivo protein binding of etoposide was measured by using ultrafiltration followed by RP-HPLC equipped

Table 2
HPLC analyses of epipodophyllotoxins

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	References/Notes
VP-16	Injectable formulation	Methyl-*p*-amino-benzoate	Direct injection	μBondapak phenyl (10 μm)	20 m*M* NaOAc buffer (pH 4.0)–ACN (74:26)	UV (254 nm)	ND	Four impurities detected [55]
VP-16	Plasma (1 ml)	VM-26	LLE (chloroform)	μBondapak C_{18} (10 μm)	MeOH–water (60:40)	FLD (288 nm/328 nm)	50 ng/ml	*cis*-Picro and hydroxy acid metabolites detected [56]
VP-16	Plasma (0.1 ml)	None	LLE (dichloroethane)	μBondapak phenyl (10 μm)	MeOH–65 m*M* phosphate buffer (pH 7.0) (40:60)	ECD	2 ng/ml	*cis*-Isomer and the aglycone detected [34]
VP-16	Plasma, urine or CSF (0.5 ml)	VM-26	LLE (ethyl acetate)	μBondapak phenyl (10 μm)	Water–ACN–acetic acid (74:25:1)	ECD	20 ng/ml	Picro-lactone, hydroxy acid and the aglycone detected [41]
VP-16	Plasma (1 ml) or urine (200 μl)	DPH or MPPH	LLE (chloroform)	ODS Hypersil	MeOH–water (51:49)	UV (229 nm)	0.1 μg/ml	[36]
VP-16	Plasma or urine (1 ml)	VM-26	LLE (chloroform)	Radial-Pak C_{18} (10 μm)	MeOH–ACN–water (2:1:1)	MS	1 μg/ml	[44,58]
VP-16	Plasma (1 ml)	VM-26	LLE (dichloroethane)	μBondapak phenyl (5 μm)	MeOH–phosphate buffer (60:40)	ECD	5 ng/ml	[46]
VP-16	Plasma (1 ml)	None	LLE (dichloroethane)	μBondapak CN (10 μm)	ACN–20 m*M* sodium acetate (pH 4.0) (17.7:82.3)	ECD	10 ng/ml	[38]
VP16	Plasma (0.5 ml)	VM-26	LLE (chloroform)	μBondapak phenyl (10 μm)	10 μ*M* ammonium acetate (pH 5.5) in MeOH–water–ACN (50:45:5)	UV (230 nm)	0.4 μg/ml[a]	*cis*-Picro lactone, hydroxy and the aglycone detected [32]
VP-16	Plasma (1 ml)	VM-26	LLE (chloroform)	μBondapak C_{18} (10 μm)	MeOH–0.25 *M* ammonium acetate–acetic acid (54:45:1)	ECD	10 ng/ml	[58]
VP-16	Plasma (1 ml)	VM-26	LLE (chloroform)	Radial-Pak C_{18} (5 μm)	10 m*M* phosphate nuffer (pH 3.0)–MeOH	ECD	10 ng/ml	[37]
VP-16	Plasma (0.5 ml)	Phenacetin	LLE (dichloroethane)	μBondapak phenyl (5 μm)	Water–ACN–acetic acid (70:30:1)	UV (233 nm)	0.05 μg/ml	[39]
VP-16	Plasma (1 ml)	VM-26	LLE (methylene chloride)	Econosil C_{18} (10 μm)	MeOH–ACN–water–acetic acid (39:15:45:1) (pH 3.0)	FLD (230 nm/328 nm)	50 ng/ml	[28]

Table 2. Continued

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	References/Notes
VP-16	Whole blood (0.2 ml)	VM-26	LLE (dichloroethane–hexane)	μBondapak phenyl (10 μm)	0.65 *M* sodium citrate–water–ACN	ECD	0.24 ng	[49]
VP-16	Plasma (0.5 ml) or leukemic cells (50×10^6)	VM-26	LLE (chloroform)	Spherisorb phenyl (5 μm)	MeOH–water–acetic acid (45:54:1)	FLD (230 nm/330 nm)	10 ng/ml plasma, 10 ng/50×10^6 cells	*cis*-Isoform detected [57]
Free VP-16	Plasma (0.5 ml)	VM-26	LLE (chloroform)	Nucleosil phenyl (7 μm)	MeOH–water–acetic acid (60:39:1)	UV (280 nm)	ND	*cis* and *trans*-isomers detected [47]
Free VP-16	Plasma (1 ml)	VM-26	LLE (chloroform)	μBondapak phenyl (10 μm)	ACN–water–acetic acid (35:64:1)	FLD (288 nm/328 nm)	0.05 μg/ml[a]	[33]
VP-16	Plasma (0.15 ml)	None	LLE (acetonitrile)	Hypersil ODS RP-18 (5 μm)	MeOH–0.01 *M* Na_2HPO_4 (pH 6.0) (43:57)	ECD	10 ng/ml	Catechol measured [30]
VP-16	Plasma (0.2 ml)	None	LLE (chloroform–methanol)	μ-Bondapak phenyl (10 μm)	25 m*M* citric acid–50 m*M* sodium phosphate (pH 2.4)–ACN (76.6:23.4)	ECD	1.2 n*M* for VP-16, 0.2 n*M* for catechol	Catechol measured [29]
VP-16	Plasma or serum (0.1–0.2 ml)	None	LLE (chloroform)	Lichrospher RP-18 (5 μm)	ACN–0.1% acetic acid (45:55)	MS (SIM, *m/z* 589)	0.005 μ*M*	[35]
VP-16	Plasma (0.5 ml)	17-β-Estradiol hemihydrate	LLE (dichloroethane)	Zorbax phenyl (5 μm)	ACN–MeOH–water–glacial acetic acid (30:15:54:0.5) with 10 m*M* TEAH[b]	ECD	ND	[50]
VP-16, VM-26	Serum (0.5 ml)	α-Peltatin	SPE (Bond-Elute C_{18})	Hypersil ODS (5 μm)	ACN–0.5 *M* ammonium acetate (pH 6.0) (37.5:62.5) with 100 mg/l of EDTA	ECD	500 pg for VP-16, 750 pg for VM-26	Picro-VP-16 and picro-VM-26 detected [52]
VP-16	Serum (0.5 ml)	Podophyllotoxin	SPE (Bond-Elute PH cartridge)	Bondclone 10 C_{18}	MeOH–40 m*M* KH_2PO_4 (pH 6.9)–0.14 m*M* 1-heptansulfonic acid (40:60:0.6)	FLD (230 nm/330 nm)	0.2 μg/ml	[51]
VP-16, VM-26	Plasma (1–2 ml)	None	Column-switching (protein hydrolysis with subtilisin A)	Nucleosil C_{18} (5 μm)	MeOH–water–acetic acid (46:64:1)	FLD (230 nm/328 nm)	8 ng/ml	[53]
VP-16, VM-26	Plsama (450 μl)	VM-26 or VP-16	Column-switching (Chromsep C_{18} for clean-up)	μBondapak phenyl (10 μm)	10 m*M* phosphate buffer (pH 7.0)–MeOH (45:55)	ECD	20 ng/ml	[54]

Table 2. Continued

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	References/Notes
VP-16 phosphate	Plasma (0.5 ml)	None	SPE (Bond-Elute C_{18})	Deltabond phenyl (5 mm)	ACN–water (12:88) with 10 m*M* TEAH[b] and 20 m*M* $NH_4H_2PO_4$ (pH 3.0)	FLD (200 nm/325 nm)	ND	[50]
VM-26	Plasma (20 μl)	2,6-Dimethoxy-phenol	MLC[c] (Chromsep C_{18} for cleanup)	Chromspher C_{18} (5 μm)	SDS (40 m*M*) and n-propanol (0.5 *M*) in 10 m*M* phosphate buffer (pH 7.0)	ECD	500 ng/ml	[71]
VM-26	Plasma (0.1 ml)	2,6-Dimethoxy -phenol	SDS (Chromsep C_{18} for cleanup)	μBondapak phenyl (10 μm)	MeOH–10 m*M* phosphate buffer (pH 7.0) (55:45)	ECD	10 ng/ml	[71]
VM-26	Plasma (1 ml)	VP-16	LLE (dichloroethane)	Novapak phenyl (10 μm)	MeOH–10 m*M* phosphate buffer (pH 7.0) (55:45)	ECD	10 ng/ml	[71]
VM-26	Plasma (0.5 ml)	Ibuprofen	LLE (ethyl acetate)	μBondapak phenyl (10 μm)	ACN–water–acetic acid (30:68:2)	UV (240 nm)	0.2 μg/ml	[72]
TOP-53	Plasma (0.5 ml) or urine (0.1 ml)	Derivative	LLE (dichloromethane)	Develosil ODS-HG-5 or Inertsil ODS-2	MeOH–0.1 *M* phosphate buffer (pH 7.0) (43:57)	ECD	2 ng/ml plasma, 10 ng/ml (urine)	Glucuronide detected [75]
NK-611	Plasma (0.5 ml)	Derivative	Column-switching	Capcelpak C_{18} SG120	ACN–20 m*M* K_3PO_4 buffer (pH 4.5) (32:68)	UV (288 nm)	0.04 μg/ml	DeNK-611 measured [77]
NK-611	Plasma (0.5 ml)	Pr-NK-611	SPE (C_{18} cartridge)	Reduced-activity C_{18} column	ACN–water–0.1 *M* phosphoric acid (23:76:1)	UV (205 nm)	20 ng/ml	DeNK-611 measured [79]
NK-611	Urine (0.5 ml)	Pr-NK-611	LLE (diisopropyl ether)	Novapak C_{18} (4 μm)	ACN–50 m*M* KH_2PO_4 (pH 6.4) (23:77)	UV (205 nm)	0.1 μg/ml[a]	Picro form and DeNK-611 detected [78]

[a] Limit of quantitation.
[b] TEAH = Tetraethylammonium hydroxide.
[c] MLC = Micellar liquid chromatography.

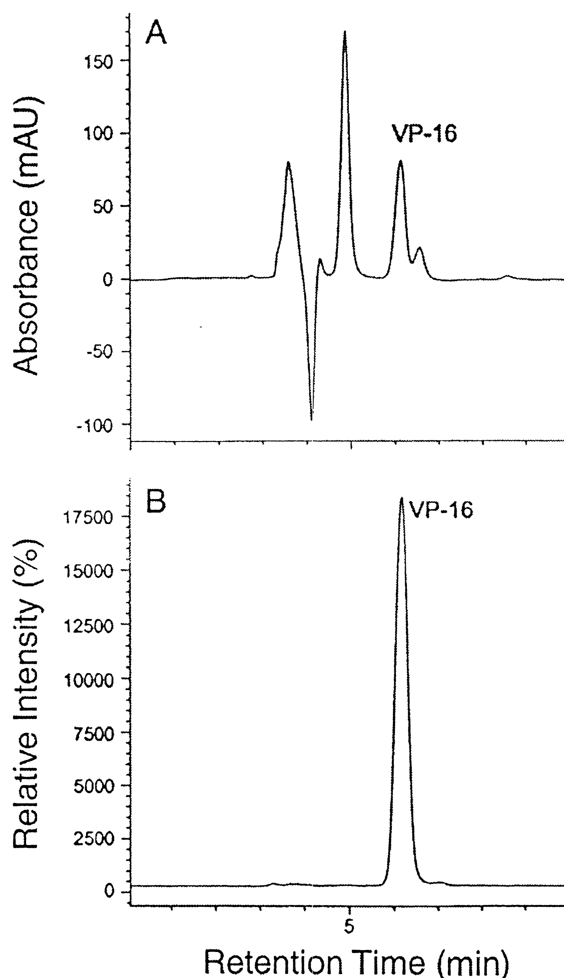

Fig. 4. Representative chromatograms of (A) blank serum (UV detection) and (B) a serum sample from a patient treated with etoposide (MS detection). (Reprinted from Ref. [35] with permission from Elsevier Science.)

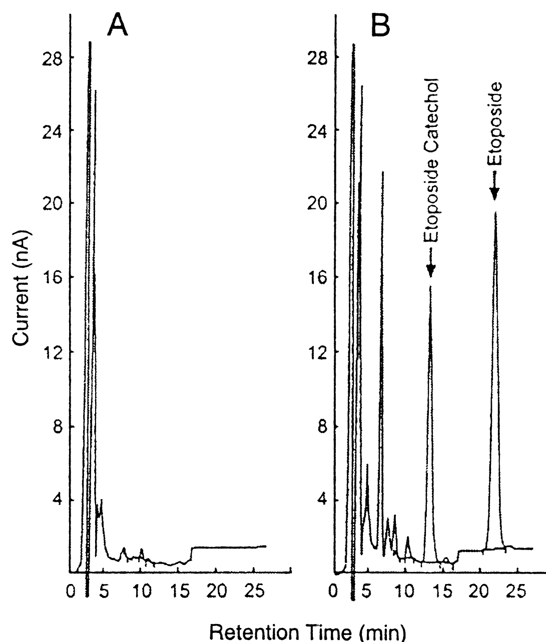

Fig. 5. Representative chromatograms of plasma (A) before infusion and (B) 6 h after 2-h infusion of 300 mg/m^2 etoposide to an acute lymphoblastic leukemia patient. (Reprinted from Ref. [29] with permission from Elsevier Science.)

with a UV [33] and radiodetector [47]. The use of a radiodetector allows more hydrophilic metabolites in ultrafiltrates and dialysates to be identified [47].

3.2. Etoposide phosphate

The aqueous solubility (>100 mg/ml) of etoposide phosphate eliminates the need for organic solvents in the formulation, allowing rapid infusion of the drug to the patients. Since etoposide phosphate is a prodrug of etoposide, the phosphate analog is quickly and extensively converted to etoposide [50,66–70].

To measure levels of etoposide phosphate in the blood, samples should be collected in tubes containing the anticoagulant K_3EDTA to prevent hydrolysis of the etoposide phosphate to etoposide. Plasma samples can be stored at −20°C for at least 8 months [50]. Methods have been developed for the extraction of either etoposide or etoposide phosphate (Table 2) [50], but there is currently no technique available for the simultaneous quantitation of both compounds due to the substantial differences in their physico–chemical properties.

3.3. Teniposide

Teniposide (VM-26), a semisynthetic podophyllotoxin derivative similar to etoposide, is stable in biological samples when stored at low temperatures. The methods described above for the extraction and analysis of etoposide are also suitable

for the determination of VM-26 in biological fluids (Table 2) [28,31–33,37,41,44,46,47,49,52,54,56,57].

VM-26 can be extracted with chloroform or ethyl acetate [41,58,71,72] and detected by UV at 240 [72] or 280 nm [41]. VM-26 is most often analyzed by RP-HPLC with electrochemical detection [31,41,58]. The degradation of VM-26 in an acidic mobile phase is dramatic ($t_{1/2}$=3.55 h) [72].

Online surfactant-mediated sample clean-up, which involves column-switching prior to conventional RP-HPLC, combined with micellar liquid chromatography (MLC) and electrochemical detection has been used to analyze teniposide in plasma [54,71]. The use of a surfactant-mediated technique allows full automation of plasma sample analysis, since protein precipitation is prevented by the addition of the surfactant sodium dodecyl sulfate.

3.4. TOP-53

TOP-53, a novel podophyllotoxin derivative that targets TOPO-2, is active against lung and lung metastatic cancers [73,74]. TOP-53 can be extracted with dichloromethane while its glucuronide metabolite requires enzymatic hydrolysis prior to extraction. The extracted residues are then analyzed by RP-HPLC with electrochemical detection (Table 2) [75].

3.5. NK-611

NK-611 is a novel, water-soluble podophyllotoxin derivative. The antitumor activity of NK-611 is comparable to that of etoposide, but NK-611 has a greater potency and bioavailability in animals than etoposide [76]. An acetate buffer (pH 3.5) containing ascorbic acid has been used to prevent isomerization of NK-611 and to avoid the oxidation of its *O*-demethylated metabolite, DeNK-611 [77,78]. A column-switching HPLC method involving 288-nm UV detection has been employed for the simultaneous analysis of NK-611 and DeNK-611 and has also been used to determine the absolute oral bioavailability of NK-611. The determination of NK-611 and DeNK-611 in cancer patients has been accomplished by solid-phase extraction (SPE) with diisopropyl ether (to remove contaminants) and chloroform using a C_{18} cartridge, followed by HPLC separation and 205-nm UV detection (Table 2) [78,79].

3.6. Doxorubicin

Doxorubicin (also known as DOX or adriamycin), along with epirubicin, idarubicin and daunorubicin, belongs to the anthracycline family of antitumor drugs. These compounds are active against a wide variety of malignancies [80]. Two previous reviews have discussed the methods developed prior to 1990 for separating and analyzing anthracyclines [81,82]. Accordingly, this review will summarize only the methods that have emerged since 1990 (Table 3).

3.6.1. Sample preparation and storage

Doxorubicin is stable in plasma when stored at −20°C or lower [83–86]. Both doxorubicin and its metabolites are reproducibly recovered after up to ten cycles of thawing and refreezing at −70°C [85]; a decrease in plasma doxorubicin concentrations was found after ten cycles, however [87]. Some degradation of metabolites in urine was observed after 8 weeks of storage [84]. Anthracyclines are light sensitive and adsorb to glass containers [88].

3.6.2. Sample pretreatment

Simple precipitation from biological samples can be accomplished using acetonitrile [89], methanol [98], acetonitrile–sodium hydroxide [91], acetonitrile–phosphoric acid [92], acetonitrile–methanol [93,94], ethanol–hydrochloric acid [95], methanol–zinc sulfate [86], water–zinc sulfate heptahydrate [96], acetonitrile–silver nitrate [89,97] or methanol–silver nitrate [89,97]. Sequential addition of zinc sulfate and methanol requires the addition of citrate dextrose or sodium citrate to the sample as an anticoagulant, and this method does not yield complete precipitation for serum samples or for plasma samples containing lithium–heparin or K_3EDTA [85]. Attempts to precipitate plasma proteins using sulfosalicylic acid or perchloric acid were unsuccessful due to poor recoveries at low pH values. Precipitation with a solvent such as acetonitrile or acetone resulted in poor chromatographic separation of the compounds.

The most common method for extracting anthracyclines is liquid–liquid extraction using ethyl acetate–1-propanol (9:1, v/v) [90], chloroform–isopropanol (1:1 or 2:1, v/v) [100,101], chloroform–1-propanol (4:1, v/v) [83,99], chloroform–1-heptanol

Table 3
Recent HPLC analyses of anthracyclines

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
DOX	Plasma (0.2 ml)	None	Simple precipitation ($ZnSO_4$–MeOH)	Supelcosil LC_{18} (3 μm)	0.28 *M* Sodium formate buffer (pH 3.55) –acetone–isopropanol (72.5:25:2.5)	FLD (500 nm/580 nm)	1–2 nM	Five metabolites measured [85]
DOX	Cells	DNR and HMM	Simple deproteinization ($AgNO_3$–ACN)	RP18 Hibar LiChrocart (7 μm)	0.2 *M* KH_2PO_4–0.2% TEA (pH 3.0)–ACN	UV (237 nm)	2–29 pmol	VCR, S9788 and VRP measured [89]
DOX	Plasma (1 ml)	DNR	Simple deproteinization (acetone–zinc sulfate·$7H_2O$)	Inertsil ODS-80A (5 μm)	Water–ACN–THF (pH 2.0) (75:24:0.5) with perchloric acid	FLD (480 nm/560 nm)	<1.0 ng/ml	13-dihydro metabolite measured [96]
DOX	Plasma (150 μl) or tissues	None	Simple deproteinization (MeOH–40% zinc sulfate)	Nucleosil C_{18} (10 μm)	MeOH–10 m*M* phosphate buffer (pH 2.96) (65:35)	FLD (470 nm/555 nm)	1 ng/ml	[86]
HMR-1826, DOX	Lung tissue	EPI	Simple precipitation ($AgNO_3$–ACN–MeOH)	LiChrospher 100 RP-18 (5 μm)	20 m*M* Citric acid (0.14% TEA, pH 2.4) –ACN–MeOH–THF (100:50:25:5)	FLD (490 nm/590 nm)	0.01 μg/g	Five metabolites measured [97]
DOX	Plasma (1 ml)	DNR	LLE (chloroform–1-propanol)	Lichrosorb RP-8 (5 μm)	ACN–THF–phosphate buffer (pH 2.2) (80:0.5:20)	FLD (460 nm/550 nm)	<1 ng/ml	Five metabolites measured [83]
DOX	Plasma (1 ml) or cell suspension	DNR	LLE (chloroform–MeOH)	μBondapak C_{18}	50 m*M* H_3PO_4–ACN–THF–TEA (pH 2.5)	FLD (478 nm/550 nm)	1 ng/ml	[104]
DOX	Plasma (0.5 ml) or formulations	DNR	LLE (chloroform–isopropanol)	μBondapak phenyl (10 μm)	Linear gradient of 85–50% 16 m*M* ammonium formate buffer in THF over 10 min	FLD (500 nm/560 nm)	~1 pmol/0.5 ml plasma	[100]
DOX	Plasma, urine, tissues or feces (200 μl)	DNR	LLE (chloroform–1-propanol)	Lichrosorb RP-8 (5 μm)	Water (pH 2.05)–ACN–THF (80:30:1)	FLD (460 nm/550 nm)	1.8–2.4 n*M*[a]	Three metabolites measured [99]

Table 3. Continued

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
DOX, DNR, EPI, IDA	Plasma (0.5 ml)	None	LLE (two-step; chloroform–1-heptanol; 0.1 *M* phosphoric acid)	Supelcosil LC–CN (5 μm)	50 m*M* Monobasic sodium phosphate–ACN (pH 4.0) (65:35)	FLD (480 nm/560 nm)	0.4 ng/ml[a]	13-dihydro metabolite measured [103]
DOX, EPI	Serum (0.8 ml)	DNR	SPE (C_8 Bond-Elute cartridge)	Spherisorb ODS I (5 μm)	ACN–60 m*M* Na_2HPO_4 containing 0.05% TEA (adjusted to pH 4.6 with citric acid) (35:65)	FLD (254 nm/560 nm)	1 ng/ml	Five metabolites detected [111]
DOX	Plasma or urine (1 ml)	EPI	SPE (ODS–silica sorbent)	Ultrasphere ODS (5 μm)	ACN–20 m*M* phosphate buffer (pH 3.0) with 0.05% TEA (25:75)	FLD (470 nm/550 nm)	18 pg	DOX-ol measured [84]
DOX	Plasma (0.5 ml)	DNR	SPE (spe* octadecyl)	Spherisorb Octyl (5 μm)	ACN–MeOH–phosphoric acid–diethylamine (60:15:0.08:0.08)	FLD (230 nm/550 nm)	6.25 ng/ml	[108]
DOX, DNR, EPI, IDA	Serum (0.5 ml)	Aclarubicin	SPE (Bond-Elute C_{18} cartridge)	Symmetry C_{18} (3.5 μm)	5 m*M* Ammonium formate (pH 3.0)–ACN (70:30)	MS	$<$2.5 ng/ml	13-dihydro metabolite measured [88]
DOX	Plasma (0.5 ml)	DNR	Column-switching (Vydac SC201 RP)	μBondapak phenyl (10 μm)	10 m*M* Phosphoric acid–ACN (20:80)	UV (254 nm)	10 ng/ml	[113]
DOX	Formulations	DNR	None	RP C_{18} Luna (5 μm)	Water–ACN–acetic acid (80:19:1)	FLD (505 nm/550 nm)	0.8 ng/ml	[115]
EPI	Plasma or serum (0.2 ml)	None	Simple precipitation (ACN–orthophosphoric acid)	Spherisorb C_{18} (5 μm)	ACN–60 m*M* Na_2HPO_4 buffer containing 0.05% TEA (pH 4.2) (35:65)	FLD (480 nm/560 nm)	5 ng/ml[a]	Five metabolites measured [118]

Table 3. Continued

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
EPI	Plasma (1 ml)	DNR	SPE (C_{18} Sep Pak)	Hypersil ODS C_{18} (5 μm)	ACN–formate buffer (35:65)	FLD (254 nm/565 nm)	2 ng/ml	Three metabolites measured [121]
EPI	Plasma (1 ml)	DNR	SPE (C_{18} Sep Pak)	μBondapak phenyl	ACN–1% $CH_2O_2NH_3$ buffer (pH 4.0) (34:66)	FLD (480 nm/592 nm)	1 ng/ml	Three metabolites measured [123]
EPI	Plasma (1 ml)	DOX	SPE (C_{18} Sep Pak)	μBondapak phenyl	ACN–1% $CH_2O_2NH_3$ buffer (pH 4.0) (32:68)	FLD (480 nm/592 nm)	1 ng/ml	Three metabolites measured [107,122]
EPI	Plasma (200 μl)	DOX	SPE (polymeric adsorbent column)	Lichrosorb RP-18 (10 μm)	Water–ACN (71:29) with 50 m*M* Na_2HPO_4 and 0.05% v/v TEA (final pH adjusted to 4.6 with citric acid)	ECD	1 ng/ml[a]	13-dihydro metabolite measured [124]
DNR	Plasma (1 ml) or tissues (10 mg)	DOX	SPE (C_{18} Sep Pak	Spherisorb ODS-2 (5 μm)	20 m*M* NaH_2PO_4 (pH 4.0)–ACN (1.35:1)	FLD (480 nm/580 nm)	0.2–0.9 n*M* in plasma, $<$8–30 pmol/g tissues	Five metabolites measured [125]
IDA	Plasma (1 ml)	DNR	SPE (Bond-Elute C_{18} cartridge)	Supelcosil LC–CN (5 μm)	Gradient elution[b]	FLD (470 nm/580 nm)	0.2 ng/ml	Two metabolites detected [127]
PIRA	Plasma (0.5 ml)	DNR	LLE (ACN)	Spherisorb phenyl (5 μm)	ACN–30 m*M* citrate buffer (30:70) (final pH adjusted to 4.0 with formic acid)	FLD (480 nm/590 nm)	0.5 ng/ml for DOX, 1 ng/ml for PIRA	DOX and DOX-nol measured [128]

[a] Limit of quantitation.

[b] 78% KH_2PO_4 (10 m*M*)+22% ACN (A), 30% KH_2PO_4 (10 m*M*)+H_3PO_4 (6 m*M*)]+70% ACN (B); $t=0$ min, A=90%, B=10%; $t=9$ min, A=80%, B=20%.

(1:1 or 9:1, v/v) [102,103] or chloroform–methanol (4:1, v/v)) [104] as the extractant. A high matrix pH (pH≥9) is important for obtaining high recoveries of doxorubicin and its metabolites [83,99,104]. The use of sodium hydroxide or phosphate or borate buffers results in low recoveries and chromatographically detectable degradation products. To avoid such problems, a volatile buffer such as ammonium formate is used instead [105].

Sample clean-up is improved by incorporating an alkaline extraction into chloroform, followed by a back extraction into an acid solution. Final sample clean-up involves shaking the acidic extract with an organic solvent [106].

Solid-phase extractions of doxorubicin have been performed using a C_{18} [107,108], phenyl [109], silica [110], C_8 [111] and ODS–silica [84] sorbents. The recoveries of doxorubicin using LLE or SPE are frequently inefficient, ranging from 50 to 100%. Further, the recoveries vary dramatically for different anthracyclines. A loop column extraction is an ideal way to prevent drug deterioration during sample purification [112,113].

3.6.3. High-performance liquid chromatographic separation and detection

Reversed-phase liquid chromatographic separation is primarily used to analyze anthracyclines (Table 3), since normal-phase HPLC methods are unable to separate the relatively non-polar anthracycline aglycones. Ion-pair chromatography and gradient elution offer no significant advantages over the simpler isocratic RP-HPLC methods [83,114]. Various stationary phases have been employed, including C_{18} [84–86,88,89,96,97,101,104,105,107,115], C_8 [83,99, 102,108], CN [103] and phenyl [100,109,113].

Fluorescence detection is mainly used in the analysis of doxorubicin and its metabolites. The excitation and emission wavelengths employed vary considerably. The reported excitation wavelengths include 230 [108], 254 [111], 460 [83,99], 470 [84,86,102], 475 [98], 478 [104], 480 [96,105,110], 490 [97], 500 [85,100] and 505 nm [115]. The emission wavelengths used include 550 [83,84, 99,104,108,115], 555 [86], 560 [96,100,110,111], 565 [102], 580 [85,98,105] and 590 nm [97]. Comparison of the noise levels at excitation wavelengths of 233, 254 and 480 nm revealed that the noise was significantly less for the 480 nm excitation wavelength [103]. This method exhibits good specificity, as several other commonly used anticancer drugs do not interfere with the detection of doxorubicin. Electrochemical [106,109,116] and UV (237 [89] and 254 nm [113]) detection have also been used to detect doxorubicin and its metabolites.

Four anthracyclines and three metabolites were analyzed by HPLC equipped with electrospray mass spectrometry (Fig. 6) [88]. Selected ion monitoring was used to detect idarubicin and idarubicinol (m/z 291), daunorubicin and daunorubicinol (m/z 321), epirubicin and doxorubicin (m/z 361), doxorubicinol (m/z 363) and the internal standard (m/z 812). While this method is one of the few techniques that allows simultaneous analysis of parent drugs and active metabolites, the limits of detection and quantitation (LOD and LOQ) are low enough to permit application of the method to pharmacokinetic studies in humans.

HMR-1826 is a prodrug of doxorubicin that requires bioactivation by β-glucuronidase to form doxorubicin. In humans, doxorubicin is further metabolized to yield DOX-one, DOX-done, DOX-ol, DOX-ol-one and DOX-ol-done, among others. RP-HPLC with fluorescence detection (490/590 nm) has been used for the simultaneous quantitation of HMR-1826, doxorubicin and its metabolites in lung tissue [97]. For analysis of HMR-1826, 2 m*M* D-saccharic acid-1,4-lactone needs to be added to the lung tissue to inhibit β-glucuronidase activity. Precipitation with silver nitrate is also necessary to liberate intercalated doxorubicin and DOX-ol. The recovery of 86–99% was highly reproducible and was obtained even at low concentrations. This method is one of the few techniques able to achieve separation of DOX-one and DOX-ol-done [97,101].

3.7. Epirubicin

The structure of epirubicin (EPI) differs from that of doxorubicin only in the spatial orientation of the 4′-moiety. Epirubicin is active against a wide variety of tumors [117].

3.7.1. Sample pretreatment

Simple protein precipitation has been accomplished by adding an acetonitrile (80%): orthophos-

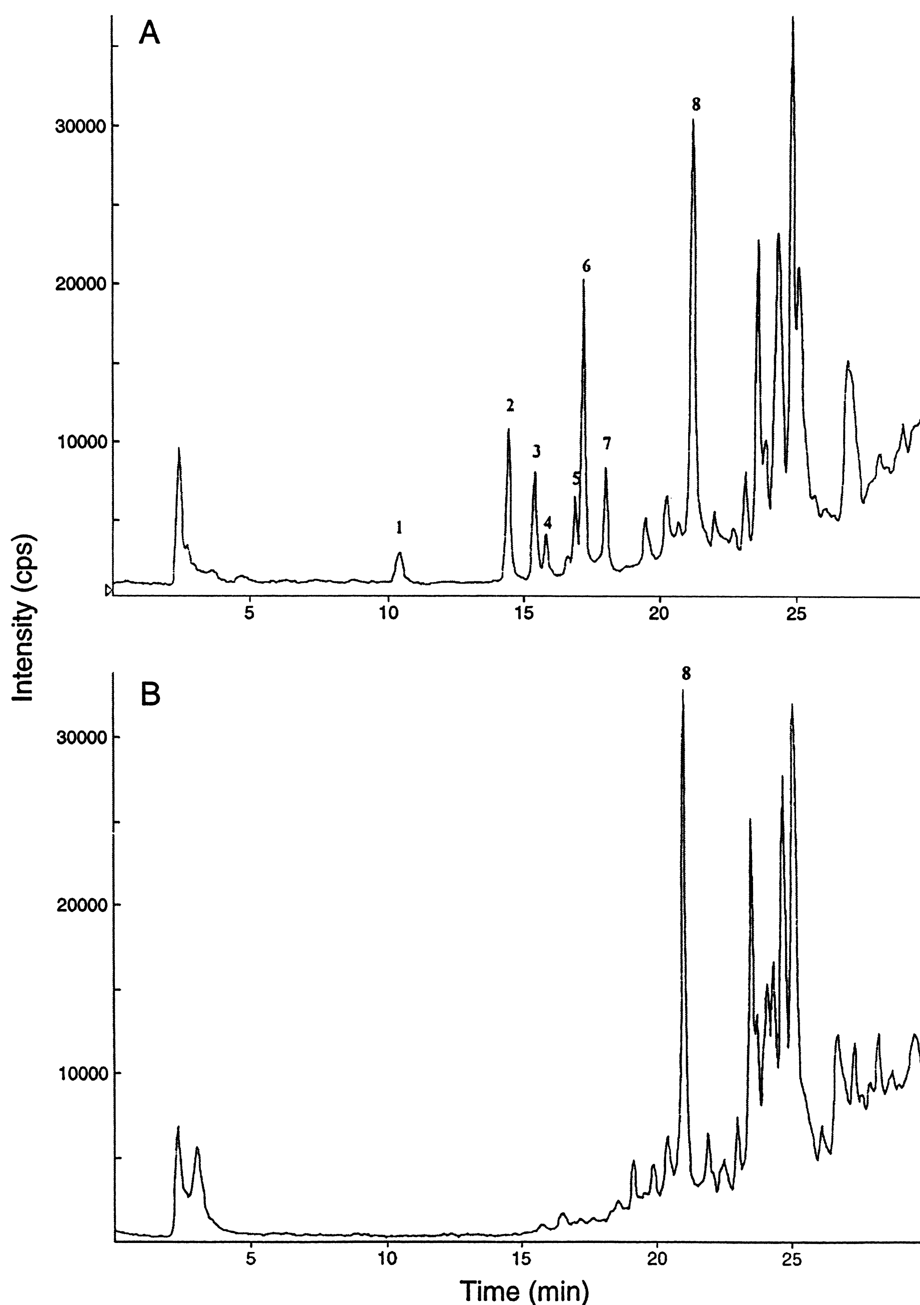

Fig. 6. Total chromatograms of (A) an extract of a serum sample spiked at 50 ng/ml for the parent drugs and 20 ng/ml for the metabolites [(1) = Doxorubicinol, (2) = doxorubicin, (3) = epirubicin, (4) = daunorubicinol, (5) = idarubicinol, (6) = daunorubicin, (7) = idarubicin, (8) = aclarubicin (I.S.)] and (B) an extract of a blank serum sample. (Reprinted Ref. [88] with permission from Elsevier Science).

phoric acid (0.1 *M*, 20%) solution to an equal volume of plasma or serum sample [118]. The recovery of epirubicin and its metabolites using this method was over 94% [118]. Chloroform [106], isopropanol–methylene chloride (1:1, v/v) [119] and isopropanol–chloroform (4:1, v/v) [120] have also

been used to extract epirubicin and its metabolites from biological samples. SPE using C_{18} [107,121–123] or polymeric [124] sorbents is also suitable for extraction of epirubicin and its metabolites.

3.7.2. High-performance liquid chromatographic separation and detection

Reversed-phase separation of epirubicin and its metabolites has been achieved using a C_{18} [118,120,124] or phenyl [107,119,122,123] stationary phase. FLD employing excitation/emission wavelengths of 254/565 [121], 470/580 [120], 470/585 [119], 480/560 [118] or 480/592 nm [107,122,123] has been used to detect epirubicin and its metabolites, as has electrochemical detection [106,124] (Table 3).

3.8. Daunorubicin

Simple deproteinization was achieved using the method described above for epirubicin. Daunorubicin (DNR, Daunomycin) was then separated from the supernatant using RP-HPLC equipped with a C_2 stationary phase and a fluorescence detector (475/557 nm) [92]. Solid-phase extraction using a silica [110] or C_{18} [125] sorbent followed by RP-HPLC separation and fluorescence detection (480/580 nm) has also been used to analyze daunorubicin and its metabolites (Table 3).

3.9. Idarubicin

Idarubicin (IDA) is a novel analog of daunorubicin that exhibits greater biological activity than its predecessor. Idarubicin can be extracted from plasma samples with a recovery of 83% by using organic solvents followed by counter-extraction with phosphoric acid (0.03 M) [126]. Unfortunately, this method results in loss of the idarubicin aglycone. Solid-phase extraction using a C_{18} bonded silica sorbent yields a recovery of over 80% for idarubicin and its metabolites [127]. Detection can be accomplished using either FLD (254/530 or 470/580 nm) [126,127] or photometry (484 nm) [126] with nearly identical sensitivities (Table 3).

3.10. Pirarubicin

Pirarubicin (PIRA), a derivative of doxorubicin, is more effective in inhibiting mouse tumors and exhibits lower cardiac toxicity than doxorubicin [128]. Doxorubicin is also a metabolite of pirarubicin. Extraction of pirarubicin and doxorubicin was achieved with a recovery of over 72% using acetonitrile as the extractant and NaCl to release the anthracyclines in the organic phase [128]. Since pirarubicin can be converted to doxorubicin in the reconstituted solution, immediate analysis by HPLC is necessary.

3.11. Mitoxantrone

Mitoxantrone {1,4-dihydroxy-5,8-bis(2-[2-hydroxyethylamino]ethylamino)-9, 10-anthracenedione dihydrochloride; also known as MTO or Novantrone} is active against lymphoma, breast cancer, acute leukemia and other malignancies [129]. Although MTO is structurally similar to doxorubicin and other DNA-intercalating agents, there are substantial differences in the mechanisms of action of these drugs [129,130].

3.11.1. Sample preparation and storage

MTO is unstable due to facile oxidation. Thus, plasma and homogenized tissue samples should be kept frozen, acidified ($pH < 7.0$) and fortified with an antioxidant such as ascorbic acid or sodium metabisulfite [131–136]. Several studies have reported different stability results for MTO, however. For example, MTO was found to be stable in spiked whole blood for 3–6 h when the samples were kept on ice, regardless of whether heparin or EDTA was used as an anticoagulant. MTO is stable in plasma and deproteinized plasma samples for at least 24 h, irrespective of the incubation temperature [137]. Further, MTO is stable in bone marrow samples at −20°C for 14 days even in the absence of antioxidants [138]. The differences in these stability results may be at least partly due to the use of polypropylene rather than glass tubes, since MTO strongly adsorbs to glass surfaces [139,140]. Siliconized pipette tips and glassware coated with Supelco Sylon CT (5% dimethyldichlorosilane in toluene) should be used to avoid possible MTO adsorption.

3.11.2. Sample pretreatment

Simple deproteinization by 5′-sulfosalicylic acid (SSA) followed by direct HPLC analysis of the supernatant has been used by Slordal et al. [137]. The most common method of extracting MTO is LLE using solvents such as dichloromethane [134–136,138,144–146] and chloroform–carbon tetrachloride [140]. An extraction recovery of 85% was obtained by alkalinizing the plasma sample to pH 11 with NaOH or borate buffer prior to extraction with dichloromethane [134,138]. Other studies reported that a low pH is not responsible for the low recovery rate in tissue homogenates, but is instead accounted for by the distribution of MTO in the tissues and by the strong binding of MTO to proteins [135,136]. Reynolds et al. [131] demonstrated that the extraction efficiency is better when using proton-donating solvents (e.g., chloroform or dichloromethane) than with proton-accepting solvents (e.g., diethyl ether or ethyl acetate). The extraction efficiency can be further improved by adding a second proton-donating species (e.g., 1-pentanol) to the extractant. Two-step liquid–liquid extraction of MTO from biological samples has also been developed [140–143]. The first step involves acidification of the sample with HCl and extraction of impurities using chloroform or a chloroform–methanol mixture (2:1, v/v). In the second step, the aqueous layer is alkalinized with 30% NH_4OH and extracted with dichloromethane or chloroform.

A high extraction recovery for MTO can be obtained using SPE (95% with a XAD-2 or C_{18} sorbent) [131,132,147,148]. SPE using non-bonded silica gel (e.g., Bond-Elute C_{18} sorbent) is advantageous since an internal standard is included in the system [139]. Online SPE of MTO using an advanced automated sample processor (AASP) coupled to an HPLC system has also been developed [153]. In contrast to traditional SPE methods which involve elution, drying and reconstitution of MTO in an appropriate solution, the AASP allows elution of MTO directly onto the HPLC system, thereby significantly reducing the processing time. The MTO recovery using this method was nearly 100%.

An automated SPE method has been reported where the sample is loaded online and the MTO is isolated in a concentration precolumn, followed by a rapid back-flushed elution and direct analysis using a C_{18} column [150]. The sensitivity of this method is 4 and 10 ng/ml for urine and plasma samples, respectively. This method also allows the simultaneous determination of MTO and its mono- and dicarboxylic acid metabolites. A separate assay involves deproteinization of plasma samples using 5-sulfosalicylic acid and addition of an internal standard prior to automated analysis. The sensitivity of this method can be increased by loading large quantities of plasma onto the concentration precolumn; the use of a concentration precolumn will also help ensure a reasonable column lifetime [133]. This technique is considerably faster and involves simpler sample manipulation than conventional extraction procedures.

On-column concentration of MTO involves three steps: (1) injection of a wash fluid for polar equilibration of the C_{18} loop-column, (2) injection of the plasma sample and (3) injection of wash fluid to remove matrix constituents from the sample [151]. This procedure is highly sensitive, as plasma volumes up to 1 ml can be injected. This method can also be adapted for automated, routine HPLC analysis in a clinical setting.

3.11.3. High-performance liquid chromatographic separation and detection

Separation of MTO is most often accomplished using RP-HPLC (Table 4). Increasing the temperature as well as adding a paired-ion such as sodium octanesulfonate or hexane sulfonic acid to the mobile phase improves the resolution of this method [131,144,146,152]. Several different internal standards have been used for the analysis of MTO. Methylene blue and cresyl violet rapidly lose their color due to the ascorbic acid added to the samples and to the air in the mobile phase. The use of bisantrene as an internal standard requires electrochemical detection, while haloperidol absorbs only at the relatively non-specific wavelength of 242 nm. The structure of ametantrone is similar to that of MTO and, hence, can be expected to be the preferred internal standard for MTO determinations [135,136,138].

MTO contains several highly conjugated bonds, causing it to strongly absorb UV radiation at wavelengths in the 600–700 nm range. Since there are no endogenous substances and only a few other drugs

that exhibit significant UV absorption at wavelengths greater than 600 nm, UV detection is frequently used in the analysis of MTO (Table 4). The UV wavelengths employed are 242 [140], 254 [131,142], 546 [141], 600 [146], 655 [150], 656 [151], 658 [132,135–137,139,143–145,147,153], 660 [149] and 665 nm [133]. The detection limit using this approach ranged from 1 to 75 ng/ml. Electrochemical detection is ten-fold more sensitive than UV detection, however, with a detection limit of only 0.1 ng/ml [134,138,148].

3.12. CI-937

CI-937 is a member of the anthrapyrazole class of anticancer agents that also includes CI-941 and CI-942. While the structures of the anthrapyrazoles and the anthracenediones are closely related, the anthrapyrazoles are significantly less cardiotoxic [154–156].

3.12.1. Sample preparation and storage

Blood and urine samples were collected in plastic tubes containing a citrate–ascorbate mixture. Laboratory glassware was not used in order to prevent non-specific binding of CI-937. The samples were kept frozen at −20°C prior to analysis [157].

3.12.2. High-performance liquid chromatographic separation and detection

Since CI-937 cannot be extracted reproducibly from biological fluids due to oxidation, HPLC is not a feasible analytical method for this compound. Therefore, nonchromatographic methods such as radioimmunoassays have been used instead for the determination of CI-937 concentrations in plasma and urine [157,158].

3.13. CI-941

3.13.1. Sample preparation and extraction

CI-941 (also known as Biantrazole, Dup-941 or Losoxantrone) was extracted from plasma and urine using a sensitive SPE technique that involves elution of CI-941 from a C_2 Bond Elute cartridge followed by concentration of the analyte. The extraction recoveries were 90 and 82–91% from plasma and urine, respectively [159]. A different SPE method used C_{18} sorbent to isolate CI-941 and four of its metabolites from 1 to 5 ml of serum or urine with an extraction recovery of over 95% [160].

3.13.2. High-performance liquid chromatographic separation and detection

A RP-HPLC method developed by Graham et al. [159,161] for the analysis of CI-941 was found to be unable to separate the metabolites of CI-941 from the parent drug. A separate method for the elucidation of CI-941 metabolites that involves LC–ESI-MS and a gradient HPLC system was established by Blanz and coworkers [162], but this procedure has not been validated for quantitation purposes. Ion-pair chromatography employing a C_{18} stationary phase, 491-nm UV detection and an acetonitrile–water mobile phase (19:81, v/v) containing 5 m*M* 1-pentanesulfonic acid yielded a detection limit of 5 ng/ml for CI-941. This method also allowed the dicarboxylic acid derivative and isomers of the monocarboxylic acid derivatives to be separated [160] (Table 5).

3.14. CI-942

3.14.1. Sample preparation and storage

CI-942 (also known as Dup-942, Piroxantrone, Pirozantrone or Oxantrazole) undergoes a pH-dependent oxidative degradation with an in vitro half-life of 5 min in human plasma. The degradation process can be inhibited by acids and antioxidants such as ascorbic acid [163]; thus, ascorbic acid is routinely added to plasma samples. Acidification of the samples also diminishes the adsorption of CI-942 to glass and plastic containers.

3.14.2. Sample pretreatment

Simple deproteinization with perchloric acid has been used to treat blood and tissue samples [164], which resulted in recoveries of 70 and 50–103% for the blood and tissue samples, respectively. Extractions using a C_{18} disposable column and a methanol–sodium acetate (0.02 *M*)–glacial acetic acid mixture (pH 4.0, 12:3:1) for elution afforded recoveries of 80–90% [163].

3.14.3. High-performance liquid chromatographic separation and detection

RP-HPLC using a C_2 or C_8 stationary phase and

Table 4
HPLC analyses of mitoxantrone

Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Plasma (0.25 ml)	None	Simple deproteinization (SSA)[a]	ODS C_{18} (3 μm) (ODS)	Triethylammonium formate (pH 3.5)–acetone (75:25)	UV (658 nm)	2.5 nM	[137]
Plasma or serum (1 ml)	Cresyl violet	LLE (two-step; chloroform –MeOH; chloroform)	μBondapak C_{18} (10 μm)	4.4 *M* Ammonium formate (pH 4.3)–ACN–water (50:25:25)	UV (546 nm)	10 ng	[141]
Blood, pleural fluid or tissues	Anthracenedione diacetate	LLE (two-step; chloroform; dichloromethane)	μBondapak C_{18}	53% MeOH–47% ion-pair reagent (PIC B-5 and PIC B-7)[b]	UV (254 nm)	$<$10 ng/ml	[142]
Plasma (1 ml), serum (1 ml) or urine	Ametantrone	LLE (dichloromethane)	μBondapak C_{18}	ACN–0.16 *M* ammonium formate buffer (pH 2.7) (30:70) with 25 m*M* hexanesulfonic acid	UV (658 nm)	1 ng/ml	[144,145]
Plasma (1 ml)	Bisantrene	LLE (dichloromethane)	μBondapak C_{18}	ACN–80 m*M* sodium formate buffer (pH 3.0) (28:72)	ECD	0.1 ng/ml	[134]
Tissues (0.5 g)	Ametantrone	LLE (two-step; chloroform –methanol; chloroform)	μBondapak C_{18}	0.4 *M* Ammonium formate (pH 4.0)–ACN (75:25)	UV (658 nm)	2 ng/g tissue	[143]
Bone marrow (1 ml; 20×10^{6} cells)	Ametantrone	LLE (dichloromethane)	μBondapak C_{18} (10 μm)	0.16 *M* Ammonium formate (pH 2.7)–ACN (70:30) containing 25 m*M* hexanesulfonic acid, 2 m*M* NaCl and 1.34 m*M* EDTA	ECD	1 ng/ml for 20×10^{6} cells	[138]
Plasma (2 ml)	Haloperidol	LLE ($CHCl_3$–CCl_4)	μBondapak C_{18} (10 μm)	MeOH–10 m*M* KH_2PO_4 buffer (pH 3.0) (50:50) containing 0.09% pentanesulfonic acid	UV (242 nm)	1 ng	[140]
Plasma (1 ml)	Ametantrone	LLE (dichloromethane)	μBondpak C_{18}	Gradient elution[c]	UV (600 nm)	ND	[146]
Blood (1 ml) or tissues (50 mg)	Ametantrone	LLE (dichloromethane)	Nucleosil C_{18}	ACN–0.16 *M* ammonium formate buffer (pH 2.7) (33:67)	UV (658 nm)	2 ng/ml	[135,136]

Table 4. Continued

Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Plasma (5 ml)	None	SPE (XAD-2 beads)	Octadecylsilane column	MeOH–50 m*M* ammonium phosphate buffer (pH 2.7) (45:55) in 6 m*M* sodium octanesulfonate	UV (254 nm)	75 ng/ml	[131]
Plasma (1–2 ml)	None	SPE (Bond-Elute C_{18} cartridge)	μBondapak C_{18} (10 μm)	ACN–0.2 *M* ammonium acetate (pH 4.0) (25:75)	UV (658 nm)	1 ng/ml	[132]
Urine (5 ml)	None	SPE (ODS Sep Pak)	Octadecylsilane column (10 μm)	2.9 *M* Ammonium formate (pH 4.3)–ACN (75:25)	ECD	2 ng	[148]
Serum (1–10 ml) or urine (10–20 ml)	None	SPE (XAD-2 beads)	μBondapak C_{18}	ACN–water (25:75) containing 5 m*M* 1-pentanesulfonic acid	UV (658 nm)	1 ng/ml serum, 0.2 ng/ml urine	Mono-and dicarboxylic metabolites measured [147]
Plasma (1 ml)	Methylene blue	SPE (C_{18} Sep Pak)	CN-Resolve Radial Pak cartridge	50 m*M* NH_4PO_4 phosphate–ACN–MeOH (60:35:5)	UV (660 nm)	ND	[149]
Plasma (1 ml)	Methylene blue	SPE (non-bonded silica gel, 40 μm)	Novapak ODS (4 μm)	1 *M* TEAP (pH 3.0)–THF–MeOH –1 *M* $N(CH_3)_4Cl$–water (10:1:2:30:57)	UV (658 nm)	1 ng/ml[d]	[139]
Serum (1–2 ml)	None	SPE (C_{18} cartridge)	μBondapak C_{18} (10 μm)	ACN–0.2 *M* ammonium acetate (pH 4.0) (25:75)	UV (658 nm)	<1 ng/ml	[132,153]
Plasma (0.2 ml) or urine (0.5 ml)	None	Column-switching	Nucleosil C_{18} (10 μm)	1.6 *M* Ammonium formate buffer (pH 4.0)–ACN–water (50:20:30)	UV (655 nm)	10 ng/ml plasma, 4 ng/ml urine	Mono- and dicarboxylic metabolites measured [150]
Plasma (1 ml)	None	On-line loop column (Spherisorb ODS)	LiChrosorb RP-18 (5 μm)	MeOH–water (40:70) containing 5 mmol *n*-heptanesulfonic acid disodium	UV (656 nm)	2 ng/ml	[151]
Plasma	CL 236 143	Column-switching	Nucleosil C_{18} (10 μm)	Gradient elution of 45–55% ACN–water (40:60) in 1.6 *M* ammonium formate buffer (pH 4.3) over 30 min	UV (665 nm)	1 ng/ml[d]	[133]

[a] SSA = 5-Sulfosalicylic acid dihydrate.
[b] PIC B-5 = Pentanesulfonic acid, PIC B-7 = heptanesulfonic acid.
[c] 62–72% Solution B where B contains 99.5% water (2 m*M* hexanesulfonic acid) and 0.05% TFA.
[d] Limit of quantitation.

Table 5
HPLC analyses of anthrapyrazoles

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
CI-941	Plasma (1–2 ml) or urine (1 ml)	None	SPE (C_2 Bond-Elute cartridge)	Spherisorb C6 (5 μm)	ACN–MeOH–0.25 *M* ammonium formate (pH 3.0) (10:10:80)	UV (492 nm)	ND	[159]
CI-941	Serum or urine (1 ml)	None	SPE (LiChrolut RP-select B)	μBondapak C_{18} (10 μm)	ACN–water (19:81) with 5 m*M* 1-pentanesulfonic acid	UV (491 nm)	5 ng/ml	Four metabolites observed [160]
CI-942	Plasma (0.1–1 ml)	CI-942 derivative[a]	SPE (C_{18} cartridge)	Hibar RP-2 (10 mm)	DMF–ACN–0.2 *M* ammonium acetate (pH 4.5) (20:5:75)	UV (514 nm)	10 ng/ml	[163]
CI-942	Whole blood (0.2 ml) or urine	CI-942 derivative[a]	Simple deproteinization (perchloric acid)	Zorbax RX-C_8 (5 μm)	Glacial acetic acid–ACN–water (8:13:79) with 0.16% sodium acetate trihydrate and 0.05% L-ascorbic acid (pH 2.7)	UV (514 nm)	<50 ng/ml	[164]
CI-942	Tissues	CI-942 derivative[a]	Simple deproteinization (perchloric acid)	Zorbax RX-C8 (5 μm)	Glacial acetic acid–ACN–water (8:12:80) with 0.16% sodium acetate trihydrate and 0.05% L-ascorbic acid (pH 2.7)	UV (514 nm)	25–50 ng/g[b]	[164]

[a] 5-[(4-Aminobutyl)amino]-7,10-dihydroxy-2-[2-(2-hydroxyethyl)amino]ethyl]anthra[1,9-cd]-pyrazol-6(2H)one.
[b] Limit of quantitation.

514-nm UV detection has been used for the analysis of CI-942 [163,164] (Table 5).

3.15. Bisantrene

Bisantrene is active against various types of cancer, including refractory breast carcinoma, metastatic renal carcinoma, metastatic malignant melanoma, myeloma, refractory leukemia and lymphoma [165].

3.15.1. Sample preparation and storage

Bisantrene is stable in plasma and urine for up to 2 months at −70°C [166]. However, bisantrene degrades in solution when exposed to light, as indicated by the separation of two additional components from the parent bisantrene upon HPLC analysis [167].

3.15.2. Sample pretreatment

Bisantrene can be separated from alkalinized (pH ≥9) plasma, serum, urine or bile samples using ethyl acetate [167] or dichloromethane [168]. A two-step LLE developed by Kuhn et al. [166] involves extraction of bisantrene from plasma or urine samples using a chloroform–HCl (6 *M*)–methanol (83:1:16) mixture followed by the addition of ammonium hydroxide to the aqueous supernatant and reextraction with chloroform. SPE is also suitable for extraction of bisantrene from biological fluids, with an average recovery of 98% [169].

A column-switching procedure for extracting bisantrene from plasma or urine samples was developed by Pratt et al. [170]. The sample injector was modified such that a prepacked phenyl guard column replaced the standard sample loop. The plasma and urine samples were spiked with an internal standard, injected into the precolumn and washed with water, thereby selectively concentrating bisantrene and the internal standard in the precolumn. The manual injector was then turned to sample injection, resulting in elution of bisantrene and the internal standard onto the separation column. The extraction recovery of this combination method ranged from 96 to 99.5%.

3.15.3. High-performance liquid chromatographic separation and detection

RP-HPLC is the most common method for the quantification of bisantrene in biological samples. Electrochemical [170] and UV (260 [166], 430 [167] or 436 nm [169]) detectors are both suitable for the determination of bisantrene (Table 6).

3.16. Ellipticine and ellipticine derivatives

The potent anticancer activities of ellipticine and its derivatives 9-hydroxyellipticine (9-OH-E) and *N*-methyl-9-hydroxyellipticine (9-OH-NME) are due to the partial inhibition of TOPO-2 [171,172].

3.16.1. Sample preparation and storage

9-Hydroxyellipticine easily decomposes in solution due to oxidation by air dissolved in the solvent. This decomposition can be prevented by storing the solution under an inert atmosphere or by adding a reducing agent such as ascorbic acid to the solvent [175].

3.16.2. Sample pretreatment

Ellipticine can be extracted from blood and homogenized tissue samples using a water-saturated ethyl acetate solution. The overall extraction efficiency was 93% (except from mouse liver tissues) [173].

3.16.3. High-performance liquid chromatographic separation and detection

RP-HPLC–FLD is an excellent method for the quantitation of ellipticine due to the high natural fluorescence of this compound (depending on the pH of the solution; Table 7). Bykadi et al. [173] used this technique for the simultaneous determination of ellipticine and 9-hydroxyellipticine in blood and plasma samples (Table 7). Muzard et al. [174] used a C_{18} stationary phase and either UV (254 or 313 nm) or fluorescence (305/470 nm) detection to analyze ellipticine and five ellipticine derivatives in non-biological samples. 9-Hydroxyellipticine and *N*-methyl-9-hydroxyellipticine have been separated by RP-HPLC using a C_{18} stationary phase and quantitated by fluorescence (360/455 nm) or electrochemical detection (the sensitivities for the two detection methods are 5 and 25 ng/ml, respectively; Table 7) [173,176].

3.17. Amsacrine

The acridine derivative amsacrine (AMSA) is an intercalating agent used to treat various types of tumors [177].

3.17.1. Sample storage

Amsacrine is stable in plasma for 1 month when stored at −20°C [178,179]. Amsacrine levels were reduced in plasma obtained from haemolyzed blood samples, however, possibly due to formation of covalent adducts between amsacrine and protein thio groups [178].

3.17.2. Sample pretreatment

Extraction of amsacrine from plasma requires several steps. The sample is acidified (pH 3–4) and extracted with *n*-hexane. The supernatant is then

Table 6
HPLC analyses of bisantrene

Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Plasma, urine or bile (<2 ml)	Alkyl AAD[a]	LLE (ethyl acetate)	C_2-bonded LiChrosorb RP-2 (5 μm)	Linear gradient of 5–100% MeOH in 0.5 *M* sodium perchlorate (pH 5.3) over 10 min	UV (430 nm)	25 ng/ml	[167]
Serum or plasma (1 ml)	CL 238 985	LLE (dichloromethane)	Bondapak C_{18} (10 μm)	ACN–water–ammonium formate (pH 4.3) (35:60:5)	UV (260 nm)	1 ng/ml	[168]
Plasma (1 ml)	CL 238 985	LLE (two-step, chloroform –methanol; chloroform)	Bondapak C_{18} (10 μm)	ACN–water–ammonium formate (30:60:5)	UV (260 nm)	1 ng/ml	[166]
Plasma (1–2 ml)	None	SPE (Bond-Elute cartridge)	μBondapak C_{18} (10 μm)	ACN–0.2 *M* ammonium acetate (pH 4.0) (27:73)	UV (436 nm)	1 ng/ml	[169]
Plasma or urine	CL 238 985	Column-switching	μBondapak phenyl	100 m*M* Sodium acetate (pH 4.5) –ACN (70:30)	ECD	2 ng/ml	[170]

[a] Alkyl AAD = 1-[2-(2-Hydroxyethyl-1-amino)ethylamino]-4-hydroxy-9,10-anthracenedione.

Table 7
HPLC analyses of indole derivatives

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Ellipticine	Blood (0.5 ml) or tissues	11-Demethyl-ellipticine	LLE (ethyl acetate)	μBondapak C_{18} (10 μm)	ACN–10 m*M* sodium phosphate (pH 3.5) (36:64)	FLD (360 nm/455 nm)	5 ng/ml	[173]
9-OH-E	Blood or tissues	11-Demethyl-ellipticine	LLE (ethyl acetate)	μBondapak C_{18} (10 μm)	ACN–10 m*M* sodium phosphate (pH 3.5) (25:75)	FLD (360 nm/455 nm)	5 ng/ml	[173]
9-OH-NME	Plasma (0.2 ml)	9-OH-NPE	LLE (ethyl acetate)	μBondapak C_{18} (10 μm)	MeOH–water (60:40) with 0.1 *M* ammonium acetate (pH 6.0)	ECD	25 ng/ml	[176]

discarded and the pH of the remaining plasma is adjusted to 9.0 using saturated sodium tetraborate, after which the mixture is reextracted with ethyl acetate or diethyl ether [178,179]. The extract residue is then dissolved in methanol and analyzed by RP-HPLC. The mean recovery ranged from 104 to 115%.

3.17.3. High-performance liquid chromatographic separation and detection

RP-HPLC with UV detection (254 [178] or 265 nm [180,181]) is generally suitable for the determination of amsacrine in whole blood, plasma or urine (Table 8). This method has also been used to measure levels of unbound amsacrine following equilibrium dialysis or ultrafiltration [182].

3.18. CI-921

3.18.1. Sample pretreatment

CI-921, an analog of amsacrine that exhibits potent anticancer activity [183], is stable in plasma for at least 3 months when stored at −20°C. Similar to amsacrine, CI-921 can be separated from plasma by acidifying the sample with HCl (pH 3–4), extracting with hexanes, adjusting the pH to 9.0 with saturated sodium tetraborate and reextracting with diethyl ether [178,184]. A related procedure where the hexanes extraction step was omitted was used to

Table 8
HPLC analyses of aminoacridines

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
AMSA	Plasma (0.5 ml)	4′-(3-Methyl-9-acridinylamino) methane–sulfonanilide)	LLE (two-step; hexane; diethyl ether)	Radial-Pak C_{18} (10 μm)	ACN–water (pH 3.8) (40:60) containing 10 m*M* TEAP	UV (254 nm)	50 nM	9-Aminoacridine metabolite observed [178]
AMSA	Plasma (0.5 ml)	BC-3672	LLE (two-step; hexane; ethyl acetate)	Waters C_{18} (10 μm)	20 m*M* Ammonium phosphate (pH 7.4)–MeOH (36:64)	UV (254 nm)	50 ng/ml	[179]
AMSA	Plasma, whole blood or white blood cells	CI-921	LLE (two-step; hexane; diethyl ether)	Cp-Spher C_8 (8 μm)	ACN–water–TEAP[a] (39.6:59.4:1)	UV (265 nm)	6 ng/ml	[180,181]
CI-921	Plasma (0.5 ml)	CI-921 ethylsulfon-anilide derivative	LLE (diethyl ether)	Radial-Pak C_{18} (10 μm)	ACN–water (43:57) with 10 m*M* TEAP	UV (254 nm)	0.1 μ*M*	[184]
CI-921	Plasma (0.1–0.25 ml) or tissues	CI-921 ethylsulfon-anilide derivative	LLE (diethyl ether)	Novapak C_{18} (4 μm)	1 *M* Ammonium acetate (pH 5.0) –ACN–MeOH (3:2:2)	ECD or UV (254 nm)	0.05 μ*M*[b]	MSA detected [185]
CI-921	Bile or urine	None	Dilute with mobile phase	Radial-Pak C_{18} (10 μm)	ACN (21%) in 10 m*M* TEAP (pH 3.0)	UV (265 nm)	ND	C9-GSH measured [186]

[a] TEAP = Triethylamine phosphate.
[b] Limit of quantitation.

extract CI-921 from urine and liver homogenates [184,185]. Isolation of CI-921 and its metabolites from an in vitro microsome system was carried out by sequential extractions with water-saturated ethyl acetate and a hexane–carbon tetrachloride (90:10, v/v) mixture [186]. The aqueous phase was then analyzed by RP-HPLC [186].

3.18.2. High-performance liquid chromatographic separation and detection

RP-HPLC employing a C_{18} [184] stationary phase has been used to separate the parent CI-921, its C9-GSH metabolite and the 4-amino-3-methoxy-methanesulfonanilide cleavage product (MSA) [185,186]. UV detection (254 or 265 nm) [184,186] can be used in the analysis of CI-921 and its metabolites, although electrochemical detection is more sensitive (LOQ=5 n*M*; Table 8) [185,187].

3.19. Amonafide

Amonafide (also referred to as Nafidimide), a member of the benzoisoquinolinedione family, is a novel synthetic imide derivative of naphthalic acid. The antitumor activity of amonafide has been demonstrated both in vivo and in vitro in experimental test systems and in clinical trials for leukemia as well as prostate and breast cancers [188,189].

3.19.1. Sample pretreatment

Amonafide can be separated from plasma by liquid–liquid extraction with ethyl acetate followed by addition of a saturated sodium borate solution to the plasma sample [189]. SPE using an ODS sorbent has been used to isolate amonafide from tissues, urine and plasma with recoveries greater than 75% [190,191].

3.19.2. High-performance liquid chromatographic separation and detection

RP-HPLC employing a C_{18} [189,190] or C_2 [192] stationary phase has been used to separate amonafide and its metabolites from various biological fluids. FLD is more sensitive than UV detection for the analysis of amonafide, although the latter is generally better suited for determination of amonafide levels in solution or formulations (Table 9). As additional amonafide metabolites are identified, HPLC method development will be required in order to permit quantification of amonafide and its metabolites in biological fluids [191].

3.20. Genistein

Genistein is an isoflavone that inhibits the tyrosine-specific protein kinase [193] in addition to TOPO-2 [194,195]. Genistein suppresses tumor growth [196] and is particularly effective in cancer treatments when conjugated to CD19 or EGF [197,198]. Several techniques have been developed specifically for the determination of genistein levels in biological fluids.

3.20.1. Sample storage

Genistein is stable in plasma or urine for 9 h when stored at 37.1°C. Decreasing the storage temperature to −17°C or −69°C increases the length of time for which genistein is stable to at least 1 month [199].

Table 9
HPLC analyses of amonafide

Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Plasma (0.1–0.2 ml)	Acridone	LLE (ethyl acetate)	ODS (5 μm)	0.4 *M* ammonium acetate –MeOH (60:40)	FLD (245 nm/550 nm)	5 ng/ml	AA and NA metabolites observed [189]
Plasma, urine or tissues	None	SPE (C_{18} Sep Pak)	μBondapak C_{18}	20 m*M* acetate buffer (pH 4.0) –ACN (85:15)	FLD (350 nm/550 nm)	30 ng/ml	*N*-Oxide and *N*-demethylated metabolite observed [190]
Solution or formulations	None	None	Spherisorb ODS-2	Acetate buffer (pH 4.6) –ACN (30:70)	UV (254 nm)	0.1 ng/ml	[192]

3.20.2. Sample pretreatment

Genistein has been separated from plasma and urine with recoveries greater than 88% by extracting the samples with *tert*.-butylmethyl ether [199]. Genistein and its metabolites have also been extracted from a microsome system by using ethyl acetate [200]. SPE of genistein and eight of its metabolites from bile and urine was accomplished using a Sephadex LH-20 cartridge; the recoveries of the parent drug and three of the major metabolites were greater than 86% [201].

3.20.3. High-performance liquid chromatographic separation and detection

HPLC–UV employing a reversed-phase ODS column has been used to separate genistein and eight of its metabolites, although only the parent drug and three of the major metabolites could be quantitated [201]. A similar method using a C_{18} stationary phase and 260-nm UV detection was used to isolate genistein and five of its metabolites [200]. The use of a 4-μm Nova-Pak C_8 column and 260-nm UV detection resulted in a detection limit of 20 ng/ml for genistein in plasma and urine samples [199]. LC–MS analysis using thermospray ionization and selected ion monitoring at m/z 271 for positive ions or m/z 269 for negative ions decreased the genistein detection limit to 5 ng/ml [199].

4. Catalytic inhibitors of topoisomerase II

Catalytic inhibitors of TOPO-2, including ICRF-159, ICRF-187, merbarone, fostriecin, aclarubicin and novobiocin, prevent the catalytic activity of the enzyme but lack the ability to stabilize the cleavable complex. These compounds can enhance the efficacy of TOPO-2 poisons and have also been shown to be antagonists against TOPO-2 poisons, a trait which is useful for detoxification of TOPO-2 poisons [10].

4.1. ICRF-159

ICRF-159 (also known as Razoxane or NSC-129943), a bisdioxopiperazine derivative, is under clinical investigation as a chemotherapeutic agent against various neoplasms [202–205].

4.1.1. Sample storage

ICRF-159 is stable in plasma, serum and cerebrospinal fluid for up to 12 weeks when stored at −70°C [206]. Addition of phosphoric acid (43%, w/v) to plasma samples is used to prevent hydrolysis of ICRF-159 [207].

4.1.2. Sample pretreatment

ICRF-159 is highly polar and hence difficult to extract using LLE techniques. Both SPE using a C_{18} sorbent [208] and ultrafiltration using Chemlab Model C10 cells [206] have been employed successfully for the extraction of ICRF-159 prior to HPLC analysis. The recovery of ICRF-159 by SPE was greater than 72% [208].

4.1.3. High-performance liquid chromatographic seperation and detection

Both RP- and chiral-HPLC have also been used to quantitate ICRF-159 in biological samples (Table 10). RP-HPLC with 206-nm UV detection yields an ICRF-159 sensitivity of 0.1 μg/ml [206]. This method is incapable of separating the two enantiomers of ICRF-159, however. Accordingly, Hasinoff et al. [208] used chiral chromatography to study the stereoselective metabolism of ICRF-159 in rats (Fig. 7). This technique separates the (+)-(*S*)- and (−)-(*R*)-enantiomers of ICRF-159, although isolation of the (−)-(*R*)-isomer (ICRF-186, also known as levrazoxane) was incomplete since traces (<0.5%) of the (+)-(*S*)-isomer (ICRF-187, also known as dexrazoxane) were still detectable. No other pharmacokinetic studies of ICRF-159 have measured the relative amounts of each enantiomer [206].

4.2. ICRF-187

ICRF-187, the (+)-(*S*)-enantiomer of ICRF-159, was selected for further development since it is more water-soluble than the racemic mixture by a factor of five [220]. ICRF-187 was originally developed as an antitumor agent and has since been found to possess potent cardioprotective activity in animals [209,210].

4.2.1. Sample preparation and storage

ICRF-187 readily decomposes in aqueous solution. HPLC separation of ICRF-187 that had been stored in aqueous solution at a pH and temperature

Table 10
HPLC analyses of bisdioxopiperazines

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
ICRF-159	Plasma, serum or CSF	None	Ultrafiltration	C_{18} RP-bonded on 10 μm silica gel	20% MeOH in 10 m*M* phosphate buffer (pH 7.1)	UV (206 nm)	0.1 μg/ml	[206]
ICRF-159	Plasma (500 μl)	None	SPE (Sep Pak Plus C_{18} cartridge)	Chiracel OD chiral cellulose tris(3,5-dimethylphenyl-carbamate) (10 μm)	HPLC-grade alcohol–HPLC-grade hexane (85:15)	UV (207 nm)	~2 μ*M*[a]	[208]
ICRF-187	Plasma or urine (2 ml)	ICRF-192	LLE (chloroform–2-methyl-1-propanol)	C_{18}-bonded on 10 μm silica gel	18–22% MeOH in 10 m*M* phosphate buffer (pH 6.1)	UV (208 nm)	50 ng/ml[a]	[220]
ICRF-187	Plasma, urine or pleural fluid	None	Ultrafiltration	5 μm Ultra-sphere ODS column	10% ACN in 10 m*M* phosphate buffer (pH 6.1)	UV (208 nm)	0.2 ng/ml	[210]
ICRF-187	Aqueous solution	None	none	μBondapak phenyl or LKB spherisorb ODS2 (3 μm)	0.15 *M* ammonium formate (pH 7.4)–MeOH (t=0 min, 0% MeOH; t=10 min, 8% MeOH; t=20 min, 80% MeOH)	UV (205 nm)	ND	Three hydrolysis products detected [211]
ICRF-187	Plasma or urine (1 ml)	None	LLE (chloroform–2-methyl-2-propanol)	μBondapak phenyl	10 m*M* potassium phosphate (pH 4.7)–MeOH (80:20)	UV (208 nm)	0.1 μg/ml plasma, 10 μg/ml urine[a]	[218]
ICRF-187	Plasma or urine	ICRF-192	SPE (C_{18} and C_8 cartridges)	Column-switching; Ultrasphere silica columns (5 μm)	ACN–water–phosphoric acid (90:10:20, v/v/m*M*)	ECD	5 ng/ml plasma[a] 2 μg/ml urine[a]	[217]
ICRF-187	Aqueous solution	None	None	μBondapak C_{18} (10 μm)	MeOH–0.5 m*M* EDTA (pH 4.5) (t=0 min, 0% MeOH; t=10 min, 8% MeOH; t=20 min, 80% MeOH)	UV (215 nm) (205 nm for hydrolysis products)	ND	One and two-ring open products measured [215]
ADR-925	Plasma	None	Precolumn reaction	μBondapak C_{18} (10 μm)	MeOH–4 m*M* aqueous 1-heptanesulfonate (50:50)	FLD (200 nm/544 nm)	25 pmol	[207]

[a] Limit of quantitation.

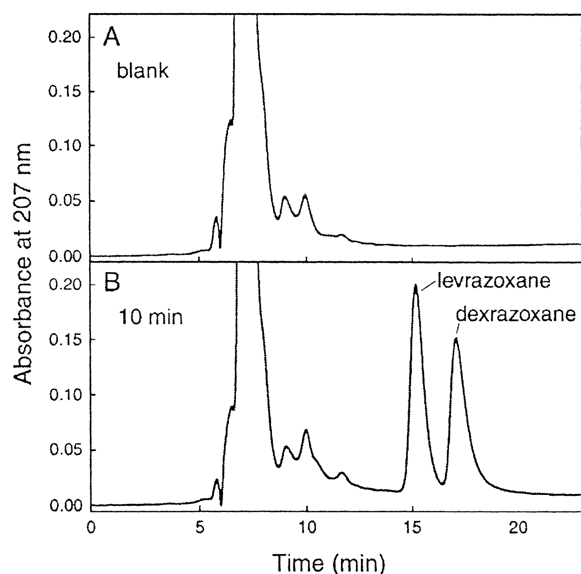

Fig. 7. The enantiomers dexrazoxane (ICRF-187) and levrazoxane (ICRF-186) were separated on a chiral HPLC. Representative chromatograms of (A) blank plasma and (B) samples obtained 10 min after dosing the rat with 20 mg/kg of razoxane (ICRF-159) are shown. (Reprinted from Ref. [208] with permission from Wiley-Liss, a subsidiary of Wiley.)

approximating physiological conditions and subsequent analysis by fast atom bombardment mass spectrometry revealed the presence of at least three degradation products [211,212]. Full hydrolysis of ICRF-187 produced ADR-925 [203,208,212–216]. The hydrolysis of ICRF-187 was considerably slower under acidic conditions, however [215,217]. Thus, acidification of pharmacokinetic samples containing ICRF-187 prior to storage is recommended [215]. The ICRF-187 in urine samples is stable for 24 h when stored at 4–8°C [218]. Decreasing the storage temperature to −30°C increases the length of stability to at least 4 months.

4.2.2. Sample pretreatment

Due to the low protein-binding properties of ICRF-187, Vogel et al. [210] developed a rapid, simple extraction method that uses an Amicon Centrifree micropartition system to separate ICRF-187 from biological samples. The recoveries of ICRF-187 from plasma and urine were 97 and 114%, respectively. Extraction of ICRF-187 from plasma or urine has also been accomplished using a chloroform–isobutyl alcohol or chloroform–*tert.*-butyl alcohol mixture (90:10, v/v) as the solvent [218,220]. Lewis et al. [217] developed a two-column SPE technique for the separation of ICRF-187 from plasma and urine samples. The samples were initially deproteinated and desalted on C_{18} sorbent. Further sample clean-up was achieved in the cation-exchange mode via silanol interaction using C_8 sorbent. The recovery of ICRF-187 was greater than 95%.

4.2.3. High-performance liquid chromatographic separation and detection

The polar nature of ICRF-187 complicates both the extraction of this drug from plasma and the RP-HPLC separation of this compound. Nonetheless, RP-HPLC separation of ICRF-187 has been achieved using C_{18} [206,207,210,215,220] and phenyl [211, 218] sorbents (Table 10). The phenyl sorbent is superior to the others, as it yields a longer ICRF-187 retention time and a better separation of ICRF-187 from endogenous compounds [218]. ICRF-187 can be detected by UV at 205–208 nm [206,208,210, 211,218,220].

The addition of EDTA (or another ion-pairing reagent, such as 1-heptanesulfonate) to the mobile phase is critical to the separation of ICRF-187. If EDTA is absent, the metal ion-complexing hydrolysis product ADR-925 scavenges iron from the flow system, interfering with the determination of this and other hydrolysis products [207]. Hasinoff [207] developed a fluorescence detection method used to follow the hydrolysis of ICRF-187 to ADR-925 in buffer or plasma. The plasma proteins were first precipitated using HCl. The mixture was then neutralized and terbium(III) was added to the solution, yielding the terbium(III)–ADR-925 complex. Fluorescence detection (220/544 nm) of the metal complex was more sensitive (LOD=25 pmol) than UV detection (LOD=500 pmol).

The poor chromophoric properties of ICRF-187 (i.e., no UV maxima >210 nm) severely limit the use of spectrophotometric methods (UV, FLD, etc.) for the selective detection of ICRF-187. HPLC–ECD has been used to quantitate ICRF-187 with a limit of quantitation (LOQ) of 5 ng/ml in plasma and 2 μg/ml in urine [217]. This method has been also used to study the pharmacokinetics of ICRF-187

Table 11
HPLC analyses of merbarone

Sample matrix	Internal standard	Extraction	Mobile phase[a]	Detection	LOD	Refs./notes
Plasma (50 μl)	Thiopental sodium	Simple precipitation (MeOH–DMSO)	Gradient elution[b]	UV (300 nm)	0.5 μg/ml	[223]
Plasma (50 μl)	3′-Fluoro-merbarone	Simple precipitation (MeOH–DMSO)	MeOH–0.1 *M* ammonium acetate –0.4 *M* acetic acid–2.08 *M* $MgSO_4 \cdot 7H_2O$ (30:60:8:2) containing 1 m*M* SDS	UV (306 nm)	0.050 μg/ml	2-oxo-DTMB metabolite observed [224]
Urine	None	SPE (XAD-2 column)	MeOH–water (25:75) with 67 m*M* ammonium acetate, 33 m*M* acetic acid, 40 m*M* magnesium sulfate, and 1 m*M* SDS	UV (293 nm)	ND	4-OH-MB, 4′-OH-2-oxo-DTMB and 2-oxo-DTMB metabolites observed [225]

[a] A C_{18} Nova-Pak (4 mm) stationary phase was used in each case.
[b] Gradient elution of 40% MeOH (in 300 m*M* ammonium acetate: 60 m*M* $MgCl_2$:7.2 m*M* TEA) to 80% MeOH (in 0.1 *M* ammonium acetate: 60 m*M* $MgCl_2$:7.2 m*M* TEA) over 15 min. MB, merbarone. DTMB, desthiomerbarone.

administered in escalating doses combined with a fixed dose of doxorubicin [219].

4.3. Merbarone

The thiobarbituric acid derivative merbarone is a catalytic inhibitor of TOPO-2 that is currently in clinical trials as an anticancer agent [221].

4.3.1. Sample pretreatment

The development of an analytical method for merbarone posed significant challenges, as this compound (1) has a limited solubility in many solvents, including chloroform, methylene chloride, benzene, methanol, ethanol, acetonitrile, trifluoroacetic acid, water and acetone, (2) adsorbs onto the surfaces of glass or plastic containers from aqueous solutions [222] and (3) is adsorbed strongly by all stationary phase packings. Thus, a method involving direct injection of plasma samples following simple protein precipitation was explored. The plasma was mixed with a methanol–DMSO solution (85:15, v/v) to precipitate the plasma proteins. The resulting supernatant was then diluted with water and analyzed by HPLC [223,224]. The merbarone recovery using this method was greater than 94%. SPE using a polystyrene resin has been used to isolate several merbarone metabolites in urine [225]. The sorbent was eluted with a dioxane–methanol solution and the solvents were evaporated from the residue prior to HPLC analysis. Isolation and purification of merbarone and its metabolites can also be achieved by SPE using a C_2 sorbent [225].

4.3.2. High-performance liquid chromatograhpic separation and detection

The primary method used to determine merbarone levels in biological fluids is RP-HPLC–UV (Table 11). The high adsorptivity of merbarone to most reversed-phase stationary phases, including C_{18}, C_8, CN and phenyl packings, results in pronounced peak tailing for both merbarone and the internal standard (3′-fluoromerbarone) when a mobile phase composed of methanol or acetonitrile and a phosphate or acetate buffer (pH 4–7) is used. The adsorption can be slightly reduced by adding concentrated ammonium acetate to the mobile phase. Addition of magnesium sulfate to a methanol–ammonium acetate mobile phase substantially decreased the adsorption of merbarone, resulting in minimal peak tailing and a greatly improved peak shape [224]. Incorporation of triethylamine into the mobile phase has also been found to improve peak definition [223].

A method for the determination of urinary metabolites of merbarone involving precolumn derivatization was developed by Malspeis et al. [224] and modified by Dimaggio and coworkers [222]. Urinary samples were spiked with 3′-fluoromerbarone as an internal standard, alkalinized with potassium carbonate (to a final concentration of 0.1 *M*) and derivatized with 8% (v/v) acetic anhydride for 10 min. The derivatized samples were then diluted with the eluent

and analyzed by HPLC–UV (293 nm). Variations of this method have been used for the simultaneous separation of several metabolites from urine and from an in vitro microsome system [224–226].

4.4. Fostriecin

The novel antitumor antibiotic fostriecin (also known as CI-920), which inhibits the catalytic activity of TOPO-2 [227], is stable in solution or in spiked plasma samples for 12 days when stored at −20°C. Decreasing the storage temperature to −70°C or −80°C increases the length of time for which fostriecin is stable to 7 weeks and 4 months, respectively [227]. Extraction of fostriecin from plasma was accomplished at an efficiency of 70% by using dichloromethane as the solvent. The extracts were analyzed by HPLC–UV (268 nm), using sulfaquinoxaline as the internal standard (Table 12) [228].

4.5. Aclarubicin

While most anthracyclines are potent TOPO-2 poisons, the anthracycline aclarubicin instead inhibits the catalytic activity of TOPO-2. Aclarubicin can be extracted from plasma and whole blood using chloroform. Subsequent detection by FLD (435/505 nm) reveals a limit of quantitation (LOQ) of 20 ng/ml (Table 12) [229].

4.6. Novobiocin

The antibiotic novobiocin is used in the treatment of infectious diseases due to its inhibition of bacterial DNA gyrase. This compound also modulates the cytotoxicity of various anticancer drugs to tumor cells by inhibiting human TOPO-2. Several different techniques have been used to quantitate novobiocin. The HPLC method developed by Moats et al. [230] is very sensitive, but the extraction protocol is too time-consuming to find routine application. Novobiocin has been extracted from plasma or serum with a recovery of greater than 97% by using a simple deproteinization with methanol [231] or acetonitrile [232]. The supernatant was then analyzed by HPLC with a gradient elution (Table 12) [231,232].

5. Dual inhibitors of topoisomerases I and II

5.1. Intoplicine

Intoplicine (also known as RP-60475) is a new antitumor agent that inhibits both TOPO-1 and TOPO-2 by trapping cleavable complexes [233,234]. This compound exhibits anticancer activity against a wide variety of tumors [235,236].

5.1.1. Sample storage

Intoplicine is stable for at least 7 days in infusion fluid and for at least 2 months in plasma, whole blood or isopropanol when stored at −30°C [237].

5.1.2. Sample pretreatment

Precipitation of proteins using isopropanol followed by direct HPLC analysis of the supernatant yielded an intoplicine recovery greater than 97% for plasma samples. The same procedure only yielded a recovery of approximately 60% for whole blood samples, however, due to the high degree of intoplicine binding to blood cells [238]. Separation of intoplicine from faece samples was accomplished using *n*-hexane to clean-up the samples followed by LLE with dichloromethane [239].

5.1.3. High-performance liquid chromatographic separation and detection

RP-HPLC using a C_{18}, C_8 or phenyl stationary phase is unsuitable for the analysis of intoplicine as the basicity of the tertiary amine in the compound causes both peak broadening and peak tailing. Normal-phase HPLC using a silica column is an acceptable alternative (Table 13) [237,239].

5.2. Actinomycin D

Actinomycin D (also known as Dactinomycin) is a naturally-occurring antitumor antibiotic. Actinomycin D can be extracted from plasma at an efficiency of greater than 90% by acidifying the sample with HCl and extracting with water-saturated ethyl acetate. Normal-phase HPLC–UV (254 nm or 445 nm) has been used to separate mixtures of actinomycins, although this method was not used to quantify the actinomycin D in biological samples [240]. RP-HPLC–UV (436 nm) has been successfully

Table 12
HPLC analyses of other catalytical inhibitors of TOPO-2

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Fostriecin	Plasma (0.2 ml)	Sulfaquinoxaline	LLE (dichloromethane)	CSC-spherisorb-ODS-2 (3 μm)	ACN–67 m*M* phosphate buffer (pH 7.1) (7.5:92.5)	UV (268 nm)	100 ng/ml	[228]
Aclarubicin	Plasma (1 ml) or blood cells	Aclacinomycin	LLE (chloroform)	μBondapak phenyl	ACN–40 m*M* ammonium formate buffer (pH 5.0) (50:50)	FLD (435 nm/505 nm)	20 ng/ml[a]	M1, L1, S1 and D1 metabolites detected [229]
Novobiocin	Plasma (0.5–1 ml)	Prednisone	Simple deproteinization (methanol)	C_8 column (5 μm, APEX octyl EC)	Linear gradient elution of acidic water (pH 3.0)–MeOH	UV (254 nm)	5 μ*M* in 0.5 ml of plasma	[231]
Novobiocin	Serum (0.1 ml)	Mitomycin C	Simple deproteinization (ACN)	Brownlee Spheri-5 RP18 (5 μm)	ACN–10 m*M* phosphoric acid (80:20)	UV (340 nm)	1 μg/ml[a]	[232]

[a] Limit of quantitation.

Table 13
HPLC analyses of dual inhibitors of TOPO-2 and TOPO-1

Drug	Sample matrix	Internal standard	Extraction	Stationary phase	Mobile phase	Detection	LOD	Refs./notes
Intoplicine	Whole blood, plasma or feces	None	LLE (isopropanol or dichloromethane)	ChromSpher silica (5 μm)	Dichloromethane–isopropanol–ammonia (40:40:1)	FLD (375 nm/425 nm)	0.5 ng/ml blood, 10 ng/g feces	[237,239]
Actinomycin D	Plasma (1 ml)	None	LLE (ethyl acetate)	μBondapak C_{18} (10 μm)	ACN–30 m*M* acetate buffer (pH 4.6) (65:35)	UV (436 nm)	40 ng/ml	[241]

employed for the quantification of actinomycin D in plasma samples with a LOD of 40 ng/ml (Table 13) [241].

6. Conclusions

This review summarizes various separation methods for at least eleven catagories of TOPO-2 inhibitors, including epipodophyllotoxins, anthracyclines, anthracenediones, anthrapyrazoles, anthracenebishydrazones, indole derivatives, aminoacridines, benzisoquinolinediones, isoflavones, bisdioxopiperazines and thiobarbituric acid derivatives. Depending on the physico–chemical properties of the TOPO-2 inhibitor, proper sample preparation and storage may be critical to the successful analysis of the TOPO-2 inhibitor in biological samples in order to avoid problems associated with adsorption, instability and complex biological components. While simple deproteinization of biological samples followed by direct analysis of the supernatant is preferred due to its speed and simplicity, liquid–liquid and solid-phase extractions are the most widely used methods of sample pretreatment for TOPO-2 inhibitors. HPLC is the favored method for the analysis of TOPO-2 inhibitors. HPLC–UV is generally suitable for early pharmacokinetic studies. HPLC–FLD and HPLC–ECD are typically used for analytes with fluorescent or oxidative–reductive properties. LC–ESI-MS and LC–ESI-MS–MS are often used when a highly sensitive or specific analysis is needed. With an emphasis on recently developed detection methods, this review will be useful for the future development of separation and analytical methods for new TOPO-2 inhibitors.

7. Nomenclature

ACN Acetonitrile
CD Cluster of differentiation
CSF Cerebrospinal fluid
DAD Diode array detector
DMF Dimethylformamide
DPH Diphenyl hydanton
ECD Electrochemical detection
EGF Epidermal growth factor
ESI Electrospray ionization
FLD Fluorescence detection
GC Gas chromatography
HMM Hexamethylmelamine
HPLC High-performance liquid chromatography
ICRF Imperial Cancer Research Fund
IS Internal standard
LLE Liquid–liquid extraction
LOD Limit of detection
LOQ Limit of quantitation
MeOH Methanol
MLC Micellar liquid chromatography
MPPH Methylphenytoin
MTO Mitoxantrone
MS Mass spectrometry
RIA Radioimmunoassay
RP Reversed-phase
SDS Sodium dodecyl sulfate
SIM Selected-ion monitoring
SPE Solid-phase extraction
TDM Therapeutic drug monitoring
TEA Triethylamine
TEAS Triethlammonium sulfate
TEAP Triethylammonium phosphate
TFA Trifluoroacetic acid
TOPO Topoisomerase

TSP Thermospray ionization
VP-16 Etoposide
VM-26 Teniposide

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