Journal of Chromatography B, 879 (2011) 2471–2486

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

Quantitative liquid chromatographic analysis of anthracyclines in biological fluids

Kristof E. Maudens[a,1], Christophe P. Stove[b,1], Willy E. Lambert[b,*]

[a] Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk-Antwerp, Belgium
[b] Ghent University, Faculty of Pharmaceutical Sciences, Department of Bioanalysis, Laboratory of Toxicology, Harelbekestraat 72, 9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 22 April 2011
Accepted 7 July 2011
Available online 18 July 2011

Keywords:
Anthracyclines
Biological fluids
Sample preparation
Chromatography
Fluorescence detection
Tandem mass spectrometry
Occupational exposure

ABSTRACT

Anthracyclines are amongst the most widely used drugs in oncology, being part of the treatment regimen in most patients receiving systemic chemotherapy. This review provides a comprehensive summary of the sample preparation techniques and chromatographic methods that have been developed during the last two decades for the analysis of the 4 most administered anthracyclines, doxorubicin, epirubicin, daunorubicin and idarubicin in plasma, serum, saliva or urine, within the context of clinical and pharmacokinetic studies or for assessing occupational exposure. Following deproteinization, liquid–liquid extraction, solid phase extraction or a combination of these techniques, the vast majority of methods utilizes reversed-phase C18 stationary phases for liquid chromatographic separation, followed by fluorescence detection, or, more recently, tandem mass spectrometric detection. Some pros and cons of the different techniques are addressed, in addition to potential pitfalls that may be encountered in the analysis of this class of compounds.

© 2011 Elsevier B.V. All rights reserved.

Contents

1. Introduction . . . 2472
2. Stability of anthracyclines . . . 2473
   2.1. Stock solutions . . . 2473
   2.2. Stability in biological fluids . . . 2473
3. Determination for clinical and pharmacokinetic studies . . . 2473
   3.1. Analytes and concentrations of interest . . . 2473
   3.2. Sample preparation . . . 2473
      3.2.1. Deproteinization . . . 2482
      3.2.2. Liquid–liquid extraction . . . 2482
      3.2.3. Solid phase extraction . . . 2482
   3.3. Chromatographic analysis . . . 2482
      3.3.1. Liquid chromatography coupled to fluorescence detection . . . 2482
      3.3.2. Liquid chromatography coupled to mass spectrometry . . . 2483
      3.3.3. Liquid chromatography coupled to tandem mass spectrometry . . . 2483
      3.3.4. Other chromatographic techniques . . . 2484
      3.3.5. Non-chromatographic techniques . . . 2485
4. Determination for assessing occupational exposure . . . 2485
5. Conclusion . . . 2485
   References . . . 2485

* Corresponding author. Tel.: +32 9 264 81 35; fax: +32 9 264 81 83.
E-mail address: willy.lambert@UGent.be (W.E. Lambert).
[1] Both authors are equally contributed to this work.

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.07.010

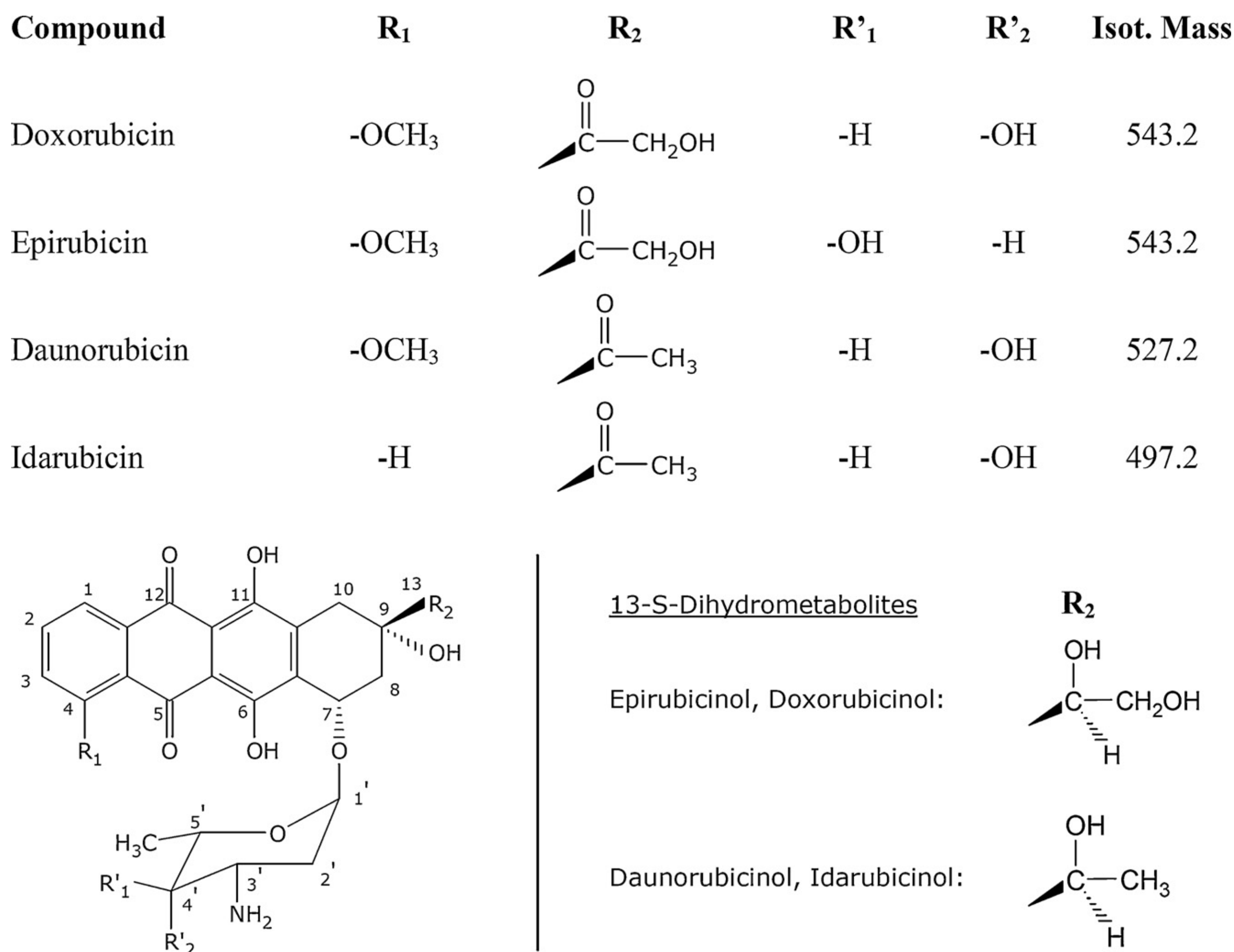

Fig. 1. Chemical structures and monoisotopic mass (amu) of doxorubicin, epirubicin, daunorubicin and idarubicin. For the 13-S-dihydrometabolites the reduced R2 side chain has been depicted.

1. Introduction

In the early 1960s the first identified anthracyclines, daunorubicin (synonym: daunomycin) and doxorubicin (synonym: adriamycin), were isolated from pigment producing *Streptomyces* spp. [1]. These anthracyclines, together with their semi-synthetic derivatives idarubicin and epirubicin (synonym: epiadriamycin), are by far the most frequently administered in clinical practice today. Doxorubicin has indications in the treatment of a wide variety of adult solid tumours (breast, ovarian, gastric cancer, ...), as well as in the treatment of childhood and haematological malignancies. Epirubicin is primarily used in the treatment of adult solid tumours (especially breast cancer), whereas daunorubicin and idarubicin are primarily used for treating both adult and paediatric leukaemia. In fact, an anthracycline is part of the regimen for most patients receiving systemic chemotherapy at some time during treatment [2].

Chemically, all anthracyclines consist of an aglycone ring coupled to an amino sugar (Fig. 1). The aminosugar has basic properties (p*K*a about 7.5), while the two hydroquinone groups are acidic (p*K*a's about 9.5 and 10) [3,4].

Idarubicin is the only anthracycline that can be administered both orally and intravenously. Bioavailability is about 30%, but varies widely between patients [5]. All other anthracyclines are only administered intravenously, predominantly as bolus injection [6].

After bolus administration, plasma anthracycline levels undergo a decay, which can generally be best fitted by a triexponential model, although also biexponential models – the intermediate phase not always being apparent – have been described [6–8]. Despite the fact that a considerable heterogeneity in the pharmacokinetic parameters of anthracyclines has been observed, both within and between studies, the plasma-concentration–time curve after short intravenous infusion can be roughly characterized by (i) a rapid initial (α) distribution phase, lasting up to 1 h, with half-lives in the range of minutes, (ii) an intermediate (β) phase, with half-lives in the range of a few hours, and (iii) a much slower (γ) terminal elimination phase, apparently established after 12–24 h, with half-lives in the order of days [6,9]. Anthracyclines are bound to plasma proteins to an extent of about 70–85% [6,10]. When measured in various organs and in tumours, anthracycline concentrations always exceed plasma concentrations, reflecting the high distribution volume of these drugs [6].

The stereospecific reduction of anthracyclines by cytoplasmic aldo-keto reductases of the carbonyl function at C_{13} in the aglycone moiety yields pharmacologically active 13-S-dihydro metabolites, which are generally denoted by the suffix "-ol" (so doxorubicinol, epirubicinol, daunorubicinol and idarubicinol) (Fig. 1). Generally, daunorubicin and idarubicin are converted more extensively than doxorubicin and epirubicin. Inactive aglycones are formed by deglycosylation of the anthracyclines, and are generally denoted by the suffix "one". Since doxorubicin and epirubicin only differ by their

sugar-moiety, they have identical aglycone metabolites. Hydrolase-type activity yields aglycones that possess a hydroxyl function at position C_7. The 7-deoxy aglycones are present in biological fluids in only some patients, transiently, and at very low concentrations [11]. Epirubicin is characterized by a unique metabolic pathway present only in humans: in contrast to other anthracyclines, the hydroxyl function at $C_{4'}$ in the sugar moiety is positioned equatorially, opening the possibility of glucuronic acid conjugation [12]. Peak plasma concentrations of epirubicin and epirubicinol glucuronides are found 1–2 h after administration of epirubicin, and their plasma concentrations generally exceed those of the parent drug. Glucuronides are devoid of any cytotoxic activity [6]. Formation of epirubicin and epirubicinol from their respective glucuronides by means of enterohepatic recycling has not been described. However, we found the pharmacokinetic profiles from epirubicin-treated patients to contain a slight increase 4 h post-I.V. infusion, which may be indicative that enterohepatic recycling may exist [13].

The relevance of the analytical determination of chemotherapeutics, and of anthracyclines in particular, lies in the fact that there is a marked inter-individual variation in the occurrence of unwanted toxicity. When aiming at maximizing therapeutic efficiency while reducing toxic side effects, validated analytical methods are needed to establish the pharmacokinetics of these compounds. Rather than considering a therapeutic interval, parameters taken into consideration include area under the plasma-concentration–time curve (AUC), plasma concentration 2 h post-dose and/or terminal half-life [14].

More than a decade has past since the publication of the last comprehensive reviews covering determination of anthracyclines [15–17]. Given the new developments in the field – amongst which the use of tandem mass spectrometry – the aim of the current review is to bundle chromatographic strategies, new insights and developments for the detection of anthracyclines in biological matrices. First some aspects concerning the stability of anthracyclines in stock solutions and biological fluids will be discussed. Subsequently, the analytical aspects for clinical and pharmacokinetic studies, as well as for assessing occupational exposure will be reviewed.

2. Stability of anthracyclines

2.1. Stock solutions

Anthracyclines require great care in handling. Firstly, they adsorb to a variety of materials such as glass and polystyrene [3,18]. Polypropylene is recommended [19]. Secondly, anthracyclines are photolabile [20]. Stock solutions in alcohols are stable at −20 °C, but the stability reduces in aqueous solutions, especially with increasing pH, but also in an acidic environment [21,22].

2.2. Stability in biological fluids

In order to avoid misinterpretations of the bioanalytical results certain precautions concerning the handling of biological samples are inevitable. First of all, blood cells need to be removed immediately after collection of a blood sample, since they rapidly concentrate anthracyclines, which then become a substrate for the cytoplasmic aldo-keto reductase enzymes [23]. Although no instability in serum has been reported, plasma is by far the most utilized matrix for anthracycline analysis [15]. Anthracyclines are reported to be stable in plasma when stored at −20 °C or lower. They have been recovered reproducibly after up to ten cycles of thawing and refreezing at −70 °C [24]. However, the choice of anticoagulant can be highly relevant. It has been demonstrated that heparin may directly interact with anthracyclines, interfering with their analysis, especially when starting from aqueous solutions or from plasma samples with high anthracycline concentrations. Therefore EDTA tubes are recommended [19,25,26]. Although data are scarce, no significant instability was reported in saliva and oral fluid. However, repeated freeze–thawing cycles have been noted to exhibit a detrimental effect [27].

It has been recommended to acidify urine samples upon storage to prevent degradation [28]. However, it should be evaluated whether this does not lead to hydrolysis of epirubicin(ol) glucuronide.

3. Determination for clinical and pharmacokinetic studies

Doxorubicin, epirubicin, daunorubicin and idarubicin are by far the most frequently administered anthracyclines. In an attempt to overcome their toxicity or drug resistance, prodrugs and special pharmaceutical formulations have been developed. Since these changes often require a different analytical approach, the interested reader is referred to the individual methods regarding the analysis of peptide-conjugated [29–31] or polymer-bound [32] prodrugs and micellar [33], pegylated liposomal [33–35], liposomal [36,37] or embolizing [38–40] formulations. Here we present an overview of 35 original methods published since 1990 for the determination of doxorubicin, epirubicin, daunorubicin, idarubicin and metabolites in biological fluids. The individual methods are summarized in Table 1.

3.1. Analytes and concentrations of interest

It is important to determine not only the main compounds, but also their respective 13-S-dihydro metabolites, which are not only pharmacologically active, but also have been linked to anthracycline's cardiotoxic side effects [14,41]. Aglycones and glucuronides (in the case of epirubicin) can be measured, but are not considered to be toxicologically relevant [6]. Nevertheless, great care should be taken that these metabolites do not interfere in the determination of the main compounds or their reduced metabolites. From the 35 publications included in this overview, 12 quantify only one main compound [26,42–52]. Others determine a main compound and its reduced metabolite [27,53–59], sometimes together with additional metabolites [24,60–67]. One method could be applied to each of the four pairs (main compound and respective reduced metabolite) individually [68]. Four methods were developed that could determine simultaneously two or more main compounds alone [69] or together with their reduced metabolites [13,70–72].

If the alpha-phase after intravenous bolus administration has to be included in the assay and undiluted samples are to be measured, it should be kept in mind that plasma concentrations for the main compounds up to 10,000 ng/mL are possible. If not, plasma concentrations are 1000 ng/mL or lower. Lower limits of quantification (LLOQ) in the low ng/mL range (<10 ng/mL) should guarantee detection up to 24 h or more after administration. For the reduced metabolites, a similar LLOQ and an upper limit of quantification (ULOQ) of 250 ng/mL in plasma is advisable [6–8,73]. In addition to the determination of the LOQ (and LOD, limit of detection), the validation of analytical methods for the pharmacokinetic determination of anthracyclines in (pre)clinical studies requires that parameters such as precision and accuracy meet pre-set acceptance criteria and parameters such as selectivity, stability, linearity and recovery are evaluated [74–76].

3.2. Sample preparation

Three major strategies are described for sample preparation, i.e. deproteinization, liquid–liquid extraction and solid phase extrac-

Table 1
Chromatographic methods for clinical and pharmacokinetic studies.

Ref.	Compounds quantified	ISTD	Matrix (species) (study[a])	Extraction	Stationary phase	Mobile phase	Run time (min)	Detection	Calibration range (LLOQ) (ng/mL)
Maudens et al. [13]	DOX	EPIDAUN	Plasma (human)(+)	Deproteinization + LLE:	Purospher Star C18e	Gradient elution:	26	Fluorescence	DOX: 2.5–2500 (2.5)
	EPI			(1) 400 μL plasma + 1200 μL ethanol	(150 × 4.6 mm)	Solvent A: 0.1% formic acid in water		(480/555 nm)	EPI: 2.5–2500 (2.5)
	DAUN			(2) 1350 μL supernatant + 2.8 mL dichloromethane + 200 μL 1 M phosphate buffer pH 8.5	5 μm particles	Solvent B: 0.1% formic acid in acetonitrile			DAUN: 2.5–2500 (2.5)
	IDA								IDA: 1–1000 (1)
	DOXol								DOXol: 2.5–1000 (2.5)
	EPIol								EPIol: 2.5–1000 (2.5)
	DAUNol								DAUNol: 2.5–1000 (2.5)
	IDAol								IDAol: 1–400 (1)
Andersen et al. [24]	DOX	None	Plasma (human)(+)	Deproteinization:	Supelcosil LC18	Isocratic elution:	≥20	Fluorescence	DOX: 2.7–550
	DOXol			200 μL plasma + 20 μL 40% zinc sulphate + 200 μL methanol	(150 × 4.6 mm)	0.28 M formate buffer (pH 3.55):acetone:isopropanol (72.5:25:2.5)		(500/580 nm)	DOXol: 2.7–550
	DOXone				3 μm particles				DOXone: 2–400
	DOXolone								DOXolone: 2–400
	7d-DOXone								7d-DOXone: 2–400
	7d-DOXolone								7d-DOXolone: 2–400
Kümmerle et al. [26]	DOX	DAUN	Serum plasma (pig, rat)(+)	Deproteinization:	Nucleosil 100 C18 AB	Gradient elution:	26	Fluorescence	2–1000 (2)
				500 μL plasma + 100 μL water + 250 acetone + 50 μL 70% zinc sulphate	(125 × 4 mm)	Solvent A: 0.2% 1-heptanesulphonic acid (pH 4.0)		(480/550 nm)	
					5 μm particles	Solvent B: acetonitrile			
Dodde et al. [27]	EPI	DOX	Plasma saliva (human)(+)	LLE + LLE (method for plasma):	Nucleosil 100S C18	Isocratic elution:	≥15	Fluorescence	EPI: 5–1000 (5)
	EPIol			(1) 500 μL plasma + 100 μL methanol + 100 μL 0.2 M calcium dichloride + 500 μL 43 mM borax buffer (pH 9.0) + 7 mL chloroform:isopropanol (6:1)	(150 × 4.6 mm)	Water:0.1 M phosphoric acid:triethylamine: acetonitrile		(474/551 nm)	EPIol: 2–400 (2)
				(2) Organic phase + 200 μL 0.1 M phosphoric acid	5 μm particles	(70:3:0.07:27)			
Wall et al. [42]	EPI	DAUN	Serum (human) (+)	LLE:	Prodigy ODS (3) 100 Å	Isocratic elution:	>14	APCI-MSMS:	2.5–2000 (2.5)
				500 μL serum + 500 μL 200 mM ammonium formate buffer (pH 8.5) + 700 μL isopropanol + 1400 μL chloroform	(150 × 2.1 mm)	0.1% formic acid in water:acetonitrile (72:28)		SRM for EPI: 544/397	
					5 μm particles			SRM for DAUN: 528/363	

Yang et al. [43]	DAUN	DOXol	Plasma (rat) (−)	Deproteinization: 100 μL plasma + 20 μL methanol:water (1:1) + 50 μL 70% zinc sulphate + 1 mL methanol:acetone (1:1)	BetaBasic Phenyl (50 × 2.1 mm) 3 μm particles	Gradient elution: Solvent A: 0.1% formic acid in water:acetonitrile (75:25) Solvent B: 0.1% formic acid in water:acetonitrile (10:90)	3	ESI-MSMS: SRM for DAUN: 528.5/321.4 SRM for DOXol: 546.3/363.1	0.25–100 (0.25)
Buehler et al. [44]	DOX	DAUN	Plasma (human) (−)	SPE (Isolute C2(EC) 10 mL 200 mg): Condition: 1 mL acetone:isopropanol (8:2) + 1 mL water + 1 mL 0.28 M formate buffer (pH 3.55) Load: 1 mL plasma diluted with 100 μL saline solution Wash: 0.28 M formate buffer (pH 3.55) Elution: 1 mL 0.28 M formate buffer (pH 3.55):acetone:isopropanol (60:32:8)	Prodigy ODS (250 × 4.6 mm) 5 μm particles	Isocratic elution: 0.28 M formate buffer (pH 3.55):acetone:isopropanol (60:32:8)	≥8	Fluorescence (500/580 nm)	1–100 (1)
Alvarez-Cedron et al. [45]	DOX	None	Plasma (rat)(+)	Deproteinization: 150 μL plasma + 200 μL methanol:40% zinc sulphate (1:1)	Nucleosil C18 (250 × 4 mm) 10 μm particles	Isocratic elution: 10 mM phophate buffer (pH 2.96):methanol (35:65)	<10	Fluorescence (470/555 nm)	DOX: 5–75 (5) DOX: 50–600 DOX: 500–5000
Mou et al. [46]	DOX	DAUN	Plasma (human) (+)	SPE (Bakerbond spe octadecyl 3 mL): Condition: 3 mL methanol + 3 mL water:methanol (3:1) + 3 mL 50 mM phosphate buffer (pH 8.5) Load: 500 μL plasma Wash: 2 mL water:methanol (9:1) + 2 mL hexane Elution: 3 times 1 mL chloroform:methanol (2:1)	Spherisorb Octyl (150 × 4.6 mm) 5 μm particles	Isocratic elution: Water containing 0.08% phosphoric acid and 0.08% diethylamine:acetonitrile: methanol (25:60:15)	≥15	Fluorescence (230/550 nm)	10–2000 (6.25)
Cox et al. [47]	DOX	DAUN	Plasma (dog)(+)	SPE (C18 Sep-pak): Condition: 3 mL methanol + 3 mL methanol:water (1:1) + 10 mL 50 mM phosphate buffer (pH 7.0) Load: 1 mL plasma diluted with 25 μL methanol Wash: 3 mL 50 mM phosphate buffer (pH 7.0) Elution: 3 mL methanol	μBondapak-phenyl (100 × 8 mm) 10 μm particles	Gradient elution: Solvent A: 100 mM formate buffer (pH 4.0) Solvent B: acetonitrile	≥24	Fluorescence (480/550 nm)	25–1000
Li et al. [48]	EPI	EPIDAUN	Plasma (human) (−)	SPE (Oasis HLB 1 mL 30 mg):	Kromasil KR100-5SIL	Isocratic elution:	≥19	UV (254 nm)	50–2500 (50)

Table 1 (*Continued*)

Ref.	Compounds quantified	ISTD	Matrix (species) (study[a])	Extraction	Stationary phase	Mobile phase	Run time (min)	Detection	Calibration range (LLOQ) (ng/mL)
				Condition: 1 mL methanol + 1 mL water Load: 200 μL plasma diluted with 50 μL methanol:water (1:1) Wash 1: 1 mL 5% methanol Wash 2: 1 mL 40% methanol containing 2% ammonia Elution: 500 μL 0.5% formic acid in methanol	(250 × 4.6 mm) 5 μm particles	40 mM ammonium formate buffer (pH 2.9):acetonitrile (10:90)			
Li et al. [49]	EPI	EPIDAUN	Plasma (human)(+)	SPE (Oasis HLB 1 mL 30 mg): Condition: 1 mL methanol + 1 mL water Load: 200 μL plasma diluted with 50 μL methanol:water (1:1) Wash 1: 1 mL 5% methanol Wash 2: 1 mL 40% methanol containing 2% ammonia Elution: 500 μL 0.5% formic acid in methanol	AcQuity BEH C18 (50 × 1 mm) 1.7 μm particles	Gradient elution: Solvent A: 0.1% formic acid in water Solvent B: acetonitrile	4	ESI-MSMS: SRM 1 for EPI: 544/130 SRM 2 for EPI: 544/397 SRM 1 for EPIDAUN: 528/321 SRM 2 for EPIDAUN: 528/363	0.5–100 (0.5)
Krogh-Madsen et al. [50]	DAUN etoposide Ara-C	None	Plasma (human) (+)	SPE (Oasis MCX 3 mL 60 mg): Condition: 2 mL MeOH + 2 mL 50 mM HCl Load: 500 μL plasma diluted with 500 μL 50 mM HCl Wash: 1 mL 50 mM HCl + 1 mL 30% MeOH Elution: 2 times 1 mL NH_4OH:MeOH:acetonitrile (10:95:95)	Acclaim Polar Advantage II C18 (150 × 4.6 mm) 3 μm particles)	Gradient elution: Solvent A: phosphate buffer pH 2.0 Solvent B: acetonitrile	15.5	Fluorescence (490/555 nm)	DAUN: 15–1000 (15)
Urva et al. [51]	DOX	DAUN	Plasma (mouse) (+)	Deproteinization: 20 μL plasma + 2 μL 35% perchloric acid + 25 μL mobile phase	Zorbax 300SB C18 (250 × 4.6 mm) 5 μm particles	Isocratic elution: Water:acetonitrile: triethylamine (adjusted to pH 3 with phosphoric acid) (75:25:0.1)	≥16	Fluorescence (480/560 nm)	5–1000 (5)
Al-Abd et al. [52]	DOX	DAUN	Plasma (mouse) (+)	Deproteinization: 100 μL plasma + 250 μL acetone + 100 μL saturated zinc sulphate	Luna C8 (150 × 4.6 mm) 5 μm particles	Isocratic elution (flow-rate gradient): 0.2% heptanesulphonic acid pH 4:acetonitrile (75:25)	30	Fluorescence (482/550 nm)	25–2000 (25)
Gilbert et al. [53]	DOX	DAUN	Plasma (parrot) (+)	Deproteinization + LLE + LLE:	Luna Phenyl Hexyl	Isocratic elution:	20	Fluorescence	DOX: 20–400 (25)

	DOXol			(1) 100 μL plasma + 200 μL acetonitrile (2) supernatant + 2 mL ethyl acetate (3) Supernatant + 100 μL 50 mM hydrochloric acid solution	(100 × 4.6 mm) 5 μm particles	10 mM phosphoric acid:acetonitrile (83:17)		(235/550 nm)	DOXol: 20–400 (25)
Arnold et al. [54]	DOX DOXol	DAUN	Plasma (rat) (−)	Deproteinization: 100 μL plasma + 400 μL 5 mM ammonium acetate buffer (pH 3.5):acetonitrile (2:3)	Zorbax Extend RR C18 (50 × 4.6 mm) 3.5 μm particles	Isocratic elution: 5 mM ammonium acetate buffer (pH 3.5):acetonitrile (60:40)	5	ESI-MSMS: SRM for DOX: 544/361 SRM for DOXol: 546/363 SRM for DAUN: 528/321	DOX: 0.2–5430 (0.2) DOXol: 0.5–5450 (0.4)
de Bruijn et al. [55]	DOX DOXol	DAUN	Plasma (human)(+)	Deproteinization: 1 mL plasma + 600 μL acetone + 100 μL 70% zinc sulphate	Inertsil ODS-80A (150 × 4.6 mm) 5 μm particles	Isocratic elution: Water:acetonitrile: tetrahydrofuran (adjusted to pH 2.0 with perchloric acid) (76:24:0.5)	45	Fluorescence (480/560 nm)	DOX: 1–100 (1) DOXol: 0.5–50 (0.5)
Rossi et al. [56]	DOX DOXol	EPI	Plasma urine (human) (+)	SPE (Sep-pak ODS 500 mg) (method for plasma): Condition: 2 mL methanol + 2 mL water + 2 mL 10 mM phosphate buffer (pH 8.0):methanol (3:1) Load: 1 mL plasma diluted with 50 μL 10 mM phosphoric acid Wash: 1 mL water + 2.5 mL water:methanol (3:1) Elution: 2 mL 26 mM methanolic phosphoric acid	Ultrasphere ODS (250 × 2 mm) 5 μm particles	Isocratic elution: 20 mM phosphate buffer containing 0.05% triethylamine (pH 3.0):acetonitrile (75:25)	≥20	Fluorescence (470/550 nm)	DOX: 0.3–100 (0.3) DOXol: 0.6–100 (0.6)
Di Francesco et al. [57]	DOX DOXol cyclophos.	DAUN	Plasma (human)(+)	SPE (Oasis HLB 1 mL 30 mg): Condition: methanol + water Load: 1 mL of supernatant obtained after vortexing and centrifugation of 400 μL plasma + 80 μL methanol + 800 μL 0.1 N hydrochloric acid solution Wash: 5% methanol Elution: 2× 1 mL methanol	Symmetry C18 (30 × 2.1 mm) 3.5 μm particles	Gradient elution: Solvent A: 5 mM acetate buffer (pH 3.5):methanol (95:5) Solvent B: 5 mM acetate buffer (pH 3.5):methanol (5:95)	11	ESI-MSMS: MRM for DOX: 544.4/321.2 MRM for DOXol: 546.2/363.2 MRM for DAUN: 528.5/321.0	DOX: 7.2–984 (7.2) DOXol: 3.04–104 (3.6)

Table 1 (*Continued*)

Ref.	Compounds quantified	ISTD	Matrix (species) (study[a])	Extraction	Stationary phase	Mobile phase	Run time (min)	Detection	Calibration range (LLOQ) (ng/mL)
Kuhlmann et al. [58]	IDA	None	Plasma (rat) (−)	Deproteinization:	Lichrospher 100 RP-18	Isocratic elution:	10	Fluorescence	IDA: 0.5–500
	IDAol			100 μL plasma + 100 μL acetonitrile	(250 × 4 mm)	Water:acetonitrile: tetrahydrofuran:phosphoric acid:triethylamine (adjusted to pH 2.2 with hydrochloric acid) (624:330:40:2:4)		(485/542 nm)	IDAol: 0.5–500
					5 μm particles				
Ahmed et al. [59]	DOX	None	Plasma (rat) (+)	Deproteinization:	Cosmosil 5C18-AR-II	Isocratic elution:	≥15	Chemiluminescence after post-column photosensitization reaction	DOX: 1.1–543
	DOXol			50 μL plasma + 150 μL methanol	(150 × 2 mm)	50 mM imidazole-trifluoroacetic acid buffer (pH 6.8):acetonitrile:ethanol (55:35:10) containing 20 mM sodium dodecyl sulphate			DOXol: 1.1–545
					5 μm particles				
De Jong et al. [60]	DAUN	DOX	Plasma (human, mouse) (+)	SPE (C18 Sep-pak):	Microspher C18	Isocratic elution:	≥20	Fluorescence	DAUN: 0.5–130
	DAUNol			Condition: 5 mL methanol + 5 mL water + 5 mL 20 mM phosphate buffer (pH 4):acetonitrile (9:1)	(200 × 4.6 mm)	20 mM phosphate buffer (pH 4.0):acetonitrile (27:20)		480/580 nm)	DAUNol: 0.5–130
	DAUNone			Load: 1 mL plasma	3 μm particles				DAUNone: 0.4–100
	DAUNolone			Wash: 2 mL 20 mM phosphate buffer (pH 4):acetonitrile (9:1)					DAUNolone: 0.4–100
	7d-DAUNone			Elution: 4 mL methanol: tetrahydrofuran (3:1)					7d-DAUNone: 0.4–95
	7d-DAUNolone								7d-DAUNolone: 0.4–95
van Asperen et al. [61]	DOX	DAUN	Plasma urine (mouse) (+)	LLE (plasma):	Lichrosorb RP-8	Isocratic elution:	22	Fluorescence	DOX: 1.2–1170 (1.2)
	DOXol			200 μL plasma + 200 μL 6% borax buffer (pH 9.5) + 100 μL acidified water (pH 2.05) + 1 mL chloroform:n-propanol (4:1)	(100 × 3 mm)	Water:acetonitrile: tetrahydrofuran (adjusted to pH 2.05 with perchloric acid) (80:30:1)		(460/550 nm)	DOXol: 1–990 (1)
	7d-DOXone				7 μm particles				7d-DOXone: 1–955 (1)
	7d-DOXolone								7d-DOXolone: 0.75–475 (0.75)
Zhou et al. [62]	DOX	DAUN	Serum (rat) (+)	Deproteinization:	Xterra C18	Isocratic elution:	≥18.5	Fluorescence	DOX: 10–2500 (10)
	DOXol			50 μL serum + 150 μL methanol	(150 × 4.6 mm)	50 mM phosphate buffer (pH 2.0):acetonitrile:n-propanol (65:25:2)		(480/560 nm)	DOXol: 5–1250 (5)

	DOXone				5 μm particles				DOXone: 5–1250 (5)
	DOXolone								DOXolone: 5–1250 (5)
Beijnen et al. [63]	DOX	DAUN	Plasma (human) (+)	LLE:	Lichrosorb RP8	Isocratic elution:	≥25	Fluorescence	DOX: 1–1000
	DOXol			1 mL plasma + 1 mL 6% borax buffer (pH 9.3) + 300 μL acidified water (pH 2.0) + 5 mL chloroform:n-propanol (4:1)	(125 × 4 mm)	Water (adjusted to pH 2.2 with phosphoric acid):acetonitrile: tetrahydrofuran (80:20:0.5)		(480/560 nm)	DOXol: 1–1000
	DOXone DOXolone 7d-DOXone 7d-DOXolone				5 μm particles				DOXone: 1–100 DOXolone: 1–100 7d-DOXone: 1–100 7d-DOXolone: 1–100
Barker et al. [64]	EPI	None	Plasma serum (human) (+)	Deproteinization:	Spherisorb C18	Isocratic elution:	≥20	Fluorescence	EPI: 5–100
	EPIol			200 μL plasma or serum + 200 μL 100 mM orthophosphoric acid:acetonitrile (1:4)	(250 × 4.6 mm)	60 mM phosphate buffer containing 0.05% triethylamine (pH 4.2):acetonitrile (65:35)		(480/560 nm)	
	7d-DOXone 7d-DOXolone				5 μm particles				
Dobbs et al. [65]	EPI	DAUN	Plasma (human) (+)	SPE (C2):	Apex II ODS	Isocratic elution:	25	Fluorescence	EPI: 1–2000
	EPIol			Condition: 1 mL methanol + 500 μL water + 500 μL 19 mM phosphate buffer (pH 4.0):acetonitrile (9:1)	(100 × 5 mm)	19 mM phosphate buffer (pH 4.0):acetonitrile (9:4)		(480/580 nm)	EPIol: 1–250
	DOXolone			Load: 1 mL plasma diluted with 500 μL water	5 μm particles				DOXolone: 1–250
	7d-DOXone			Wash: 500 μL 19 mM phosphate buffer (pH 4.0):acetonitrile (9:1)					7d-DOXone: 1–250
	7d-DOXolone			Elution: online with mobile phase					7d-DOXolone: 1–250
	EPI-glu EPIol-glu								EPI-glu: 1–500 EPIol-glu: 1–250
Camaggi et al. [66]	IDA	DAUN	Plasma (human) (+)	SPE (Bond Elut C18 6 mL 1 g):	Supelcosil LC-CN	Gradient elution:	≥20	Fluorescence	IDA: 0.3–300
	IDAol			Condition: 3 mL methanol + 3 mL 10 mM phosphate buffer (pH 8):methanol (2:1)	(250 × 4.6 mm)	Solvent A: 10 mM dihydrogen phosphate:acetonitrile (78:22)		(470/580 nm)	IDAol: 0.3–300
	IDAone			Load: 1 mL plasma diluted with 1 mL 10 mM phosphate buffer (pH 8) and 1 mL methanol	5 μm particles	Solvent B: 10 mM dihydrogen phosphate + 6 mM phosphoric acid:acetonitrile (30:70)			IDAone: 0.3–100
				Wash: 4 mL water:methanol (3:1) Elution: 3 mL 30 mM methanolic phosphoric acid					
Dine et al. [67]	EPI	DAUN	Plasma (human) (+)	SPE (C18 Sep-pak):	Hypersil ODS C18	Isocratic elution:	≥15	Fluorescence	EPI: 2.5–1250

Table 1 (*Continued*)

Ref.	Compounds quantified	ISTD	Matrix (species) (study[a])	Extraction	Stationary phase	Mobile phase	Run time (min)	Detection	Calibration range (LLOQ) (ng/mL)
	EPIol			Condition: 3 mL methanol + 3 times 3 mL 50 mM phosphate buffer (pH 7.0)	(100 × 4.6 mm)	formate buffer:acetonitrile (65:35)		(254/565 nm)	EPIol: 7.3–937.5
	EPI-glu			Load: 1 mL plasma diluted with 50 μL water	5 μm particles				
	EPIol-glu			Wash: 2 times 3 mL 50 mM phosphate buffer (pH 7.0) Elution: 3 mL methanol					
Fogli et al. [68]	DOX	None	Plasma (human) (−)	LLE + LLE:	Supelcosil LC-CN	Isocratic elution:	15	Fluorescence	DOX: 0.4–10,000 (0.4)
	EPI			(1) 500 μL plasma + 500 μL 0.2 M disodium hydrogen phosphate (pH 8.4) + 4 mL chloroform: 1-heptanol (9:1)	(250 × 4.6 mm)	50 mM phosphate buffer (pH 4.0):acetonitrile (65:35)		(480/560 nm)	EPI: 0.4–10,000 (0.4)
	DAUN			(2) Organic phase + 250 μL 0.1 M phosphoric acid	5 μm particles				DAUN: 0.4–10,000 (0.4)
	IDA								IDA: 0.4–10,000 (0.4)
	DOXol								DOXol: 0.4–10,000 (0.4)
	EPIol								EPIol: 0.4–10,000 (0.4)
	DAUNol								DAUNol: 0.4–10,000 (0.4)
	IDAol								IDAol: 0.4–10,000 (0.4)
Bermingham et al. [69]	DOX	None	Serum (human) (+)	Online SPE (Biotrap 500 MS):	Zorbax XDB C18	Gradient elution:	±25	UV (254 nm)	500–25,000 (500)
	EPI			Load: 100 μL serum; mobile phase solvent A:B (85:15)	(150 × 4.6 mm)	Solvent A: formate buffer pH 3.5:acetonitrile (90:10)			
	DAUN			Wash: 30 mM ammonium formate buffer pH 6.8:acetonitrile (98:2)	5 μm particles	Solvent B: 0.1% formic acid in water:acetonitrile (10:90)			
	Docetaxel			Elution: gradient elution by mobile phase solvent A & B					
	Paclitaxel								
Lachâtre et al. [70]	DOX	ACLA	Serum (human) (+)	SPE (Bond Elut C18 3 mL 200 mg):	Symmetry C18	Isocratic elution:	≥25	ESI-MS:	DOX: 2.5–2000 (2.5)
	EPI			Condition: 3 mL methanol + 6 mL 50 mM disodium hydrogen phosphate buffer (pH 7.5) + 6 mL water	(150 × 1 mm)	5 mM ammonium formate buffer (pH 3.0):acetonitrile (70:30)		DOX: *m*/*z* 363 (397, 321)	EPI: 2.5–2000 (2.5)
	DAUN			Load: 500 μL serum diluted with 75 μL 5 mM formate buffer (pH 4.5)	3.5 μm particles			EPI: *m*/*z* 361 (397, 321)	DAUN: 5–2000 (5)
	IDA			Wash: 6 mL water				DAUN: *m*/*z* 321 (528)	IDA: 5–2000 (5)
	DOXol			Elution: 1 mL chloroform: isopropanol (4:1)				IDA: *m*/*z* 291 (333)	DOXol: 5–200 (5)
	DAUNol							DOXol: *m*/*z* 363 (399)	DAUNol: 2.5–200 (2.5)

	IDAol							DAUnol: m/z 321 (383, 530) IDAol: m/z 291 (500) ACLA: m/z 812	IDAol: 5–200 (5)
Ricciarello et al. [71]	DOX	None	Plasma (human) (+)	SPE (Oasis HLB):	Lichrosorb RP-18	Isocratic elution:	25	Electrochemical:	DOX: 1–500 (1)
	EPI			Condition: 1 mL mobile phase:water (1:3)	(200 × 4.6 mm)	Water:acetonitrile (71:29), containing 50 mM disodium hydrogen phosphate and 0.05% triethylamine (adjusted to pH 4.6 with citric acid)		First electrode: + 400 mV	EPI: 1–500 (1)
	DOXol			Load: 200 μL plasma diluted with 600 μL mobile phase:water (1:4)	10 μm particles			Second electrode: −300 mV	DOXol: 1–500 (1)
	EPIol			Wash: 1 mL mobile phase:water (1:3) Elution: 600 μL mobile phase:acetonitrile (1:1)					EPIol: 1–500 (1)
Nicholls et al. [72]	DOX	DAUN	Serum (horse) (−)	SPE (Bond Elut C8):	Spherisorb ODS1	Isocratic elution:	≥24	Fluorescence	DOX: 50–800
	EPI			Condition: not specified	(250 × 4.6 mm)	60 mM disodium hydrogen phosphate containing 0.05% triethylamine (adjusted to pH 4.6 with 30 mM citric acid):acetonitrile (65:35)		(254/560 nm)	EPI: 50–800
	DOXol			Load: 1 mL aliquot of solution obtained after mixing 800 μL serum with 1.2 mL 20 mM phosphate buffer (pH 4.0)	5 μm particles				DOXol: 50–800
	EPIol			Wash: 3 times 1 mL water + 3 times 1 mL 20 mM phosphate buffer (pH 4.0)					EPIol: 50–800
	DOXone			Elution: 500 μL 200 mM disodium hydrogen phosphate (containing 0.05% triethylamine and adjusted to pH 3.6 with 0.1 M citric acid):acetonitrile (32.5:67.5)					DOXone: 50–800
	DOXolone 7d-DOXone								DOXolone: 50–800 7d-DOXone: 50–800

Abbreviations: ISTD: internal standard; LLOQ: lower limit of quantification; DOX: doxorubicin; DOXol: doxorubicinol; DOXone: doxorubicinone; DOXolone: doxorubicinolone; 7d-DOXone: 7-deoxydoxorubicinone; 7d-DOXolone: 7-deoxydoxorubicinolone; EPI: epirubicin; EPIol: epirubicinol; EPI-glu: epirubicin glucuronide; EPIol-glu: epirubicinol glucuronide; DAUN: daunorubicin; DAUNol: daunorubicinol; DAUNone: daunorubicinone; DAUNolone: daunorubicinolone; 7d-DAUNone: 7-deoxydaunorubicinone; 7d-DAUNolone: 7-deoxydaunorubicinolone; IDA: idarubicin; IDAol: idarubicinol; EPIDAUN: epidaunorubicin; ACLA: aclarubicin; cyclophos.: cyclophosphamide; Ara-C: cytosine arabinose; LLE: liquid–liquid extraction; SPE: solid phase extraction; ESI: Electro Spray Ionization; APCI: Atmospheric Pressure Chemical Ionization; MS: mass spectrometry; MSMS: tandem mass spectrometry; SRM: selected reaction monitoring.

[a] Study: (+) resp. (−) indicate whether the method has been applied on real (pre)clinical patient or animal samples.

tion (SPE). A combination of these approaches has also been applied.

3.2.1. Deproteinization

Of the four existing protein precipitation techniques (organic solvents, metal ion, acid and salt) [77], only the former three have been applied.

Organic solvent precipitants decrease hydrophobic interactions between proteins, while facilitating electrostatic interactions, resulting in protein aggregation. As organic solvent, acetonitrile (pure or in combination with an acidic buffer or acid) [53,54,58,64], methanol [59,62] and ethanol [13] have been used.

Zinc sulphate, always in the presence of methanol [24,45], acetone [26,52,55] or both [43] has also been applied for protein precipitation. Zinc, a positively charged metal ion, will interact with proteins, reducing a protein's solubility by altering its iso-electric point and by displacing protons, resulting in a lowering of the solution's pH.

Lastly, insoluble salt formation via application of acidic reagents has been applied. Both the use of aqueous perchloric acid and hydrochloric acid solutions, always in the presence of a small amount of organic solvent, has been described [51,57]. Use of the former does not pose a danger as long as no heating and/or solvent evaporation is involved.

Although protein precipitation is mostly combined with an extraction step (see following paragraphs), its use as a single sample pre-treatment step offers great advantages in terms of speed and simplicity, though, possibly at the expense of the quantification of low concentrations. Moreover, matrix effects should be extensively evaluated in the case of mass spectrometric (MS) detection [77]. In addition, when zinc sulphate precipitation is to be followed directly by MS detection, non-volatile salt build-up in the mass spectrometer's interface should be prevented. This can be achieved by including a solvent like acetone in the precipitation step, preventing water and zinc sulphate to move into the supernatant, and/or by applying a solvent divert to waste [43,77]. Additionally, the effect of zinc sulphate has been reported to be dependent on the type of anticoagulant [24].

It is hard to list the protein precipitants of choice to be used for anthracycline analysis in plasma or serum. Important factors to consider are: (i) the effectiveness (with e.g. zinc sulphate and acetonitrile being described as very effective precipitants) [77], (ii) possible co-precipitation, (iii) the limitations imposed by a subsequent additional liquid–liquid or solid phase extraction and (iv) the detection method to be used, with (tandem) mass spectrometry sometimes being more prone to matrix-associated effects than e.g. fluorescence-based detection.

3.2.2. Liquid–liquid extraction

Two major strategies have been followed: (1) an extraction followed by an evaporation step [13,42,61,63] or (2) an extraction followed by a back-extraction [27,53,68]. Since the first step is in both cases similar, this will be discussed together.

3.2.2.1. Extraction into an organic solvent (mixture). Ethylacetate [53], dichloromethane [13], and mixtures of chloroform with n-propanol [61,63] isopropanol [27,42] or 1-heptanol [68] have been used as extractants, mostly after addition of a mild alkaline buffer (buffer pH-range 8.5–9.5) to obtain high recoveries.

Although there is a declining trend in the use of chloroform as an extractant because of environmental and health issues, if it is used, attention should be paid to the stabilizer. More specifically, ethanol-stabilized chloroform should be preferred over non- or amylene-stabilized chloroform because phosgene formation in these latter may impair anthracycline extraction and lead to artefacts [78]. Extraction under neutral or mildly alkaline conditions results in a partial recovery of glucuronic acid metabolites. Since the analytes are diluted by transfer into the organic phase, an evaporation step or back-extraction is necessary.

3.2.2.2. Back-extraction into an aqueous solution. A small volume of diluted phosphoric [27,68] or hydrochloric [53] acid has been applied to perform an efficient back-extraction of the organic phase. Incorporation of this step leads to an improved sample clean-up, but is more time-consuming. All aglycones are almost completely lost during this step. As these are not considered to be toxicologically relevant, this is not a problem in the vast majority of cases; however, it is relevant when a complete metabolite profile is to be made. In addition, when LC–MS/MS is to be performed, one may opt to use a volatile acid for the back-extraction or to include a solvent divert to waste prior to entrance of the compounds in the mass spectrometer.

3.2.3. Solid phase extraction

Solid phase extraction is widely used to extract anthracyclines. Besides silica based reversed-phase C18 [46,47,56,60,66,67,70], C8 [72] and C2 [44,65] sorbents, also polymeric Oasis HLB [48,49,57,71] and MCX [50] sorbents have been employed. A Biotrap 500 MS online SPE column has also been used [69].

To avoid losses due to protein binding, samples have sometimes been diluted [50,65,66,71,72] or have been subjected to a protein precipitation step [57] prior to loading on the sorbent.

Mild washing conditions, usually consisting of water, a neutral or slightly acidic buffer, sometimes in the presence of a small percentage of organic solvent (up to 10% methanol or acetonitrile), have been applied [44,46–50,56,57,60,65,67,69–72]. Occasionally, a stronger (additional) wash-step was included: 25 or 30% methanol at neutral pH [50,56,66], 40% methanol at alkaline conditions [48,49] or hexane [46]. Mild washing conditions improve recoveries, especially if the reduced metabolites or glucuronic acid conjugates have to be included in the assay.

Methanol (pure or in combination with an acid or tetrahydrofuran) [47–49,56,57,60,66,67], acetonitrile–acidic buffer mixtures [71,72] and chloroform–alcohol combinations [46,70] have been applied to elute the C18, C8 and Oasis HLB sorbents. The C2 sorbents and Biotrap 500 MS column were eluted either online with the mobile phase [65,69] or with a 0.28 M formate buffer (pH 3.55):acetone:isopropanol (60:32:8, v/v/v) mixture [44], while the Oasis MCX sorbent was eluted with an alkaline methanol–acetonitrile mixture [50].

Solid phase extraction offers a good alternative for liquid–liquid extraction, in which all but one of the methods make use of halogenated solvents. No consistent differences can be seen between SPE and liquid–liquid extraction with respect to reported recoveries (in most cases around or above 80%) or sensitivities (LLOQ in the low ng/mL range) (Table 1). Mostly applied SPE sorbents are C18 and polymeric sorbents, both of which have proven their utility for the determination of both the main compounds and their metabolites. As is common in SPE, the choice of the stringency of the washing solvent is a compromise; given the same retention of the main compound, more stringent washing steps may lead to cleaner extracts, but with less retention of several metabolites.

3.3. Chromatographic analysis

3.3.1. Liquid chromatography coupled to fluorescence detection

HPLC coupled to fluorescence detection has been the method of choice for many years. Reversed-phase C18 stationary phases have been widely used [13,24,26,27,44,45,50,51,55,56,58,60,62,64,65,67,72], but also C8 [46,52,61,63], cyano [66,68], phenyl [47] and phenyl–hexyl [53] stationary phases have been chosen.

Isocratic water–acetonitrile mobile phases, containing a diluted acid [27,51,53,55,58,61,63] or an acidic buffer [52,56,60,64,65,67,68,72], sometimes in the presence of an additional organic modifier such as tetrahydrofuran [55,61,63,65] or triethylamine [27,51,56,58,64,72] have been employed frequently. Other isocratic mobile phases have been used occasionally [24,44–46,62]. Gradient elution, consisting of a water–acetonitrile system containing a diluted acid [13,26] or an acidic buffer [47,50,66], has also been applied. The pH in the methods that have been applied is typically in the range 2–4 (Table 1). The choice between isocratic and gradient elution primarily depends on the nature and number of anthracycline(s) (metabolites) to be determined and on the run-time. As most isocratic methods require only a moderate percentage of organic solvent, column contamination may build up over time. Acetonitrile has been demonstrated to achieve higher resolution for anthracyclines than alcohols, and is therefore the organic solvent of choice [21]. A method enabling the simultaneous determination of the four anthracyclines, together with their respective reduced metabolites, was developed by our own research group [13,79]. This approach offers the advantage that clinical samples of patients treated with any of these compounds can be quantified in a single sequence, using a single set of calibrators and QC samples.

Various excitation and emission wavelengths have been employed to detect the anthracyclines. Excitation wavelengths are often between 470 and 480 nm [13,26,27,45,47,51,55,56,60,62–66,68], although lower [46,53,61,67,72] and higher [24,44,50,52,58] wavelengths have been reported. Emission wavelengths frequently vary between 550 and 560 nm [13,26,27,45–47,50–53,55,56,61–64,68,72], although lower [58] and higher [24,44,60,65–67] wavelengths have been reported. Comparison of the signal-to-noise levels of plasma extracts at excitation wavelengths of 233, 254 and 480 nm concluded that 480 nm was the excitation wavelength of choice [68]. Idarubicin and its metabolites have, due to the absence of the methoxy group at C_4, slightly different excitation and emission spectra, when compared with the other anthracyclines.

Liquid chromatography coupled to fluorescence detection has two distinct advantages: the cost of analysis is low (as compared to tandem mass spectrometry) and the technique allows sensitive detection of all compounds and major metabolites. Selectivity is a double-edged parameter: on the one hand chances are relatively small that endogenous compounds or co-medication interfere in the analysis, due to the high wavelengths of the fluorophore. On the other hand, anthracyclines undergo a complex metabolism yielding many fluorescent metabolites. Great care should be taken in investigating such interferences. This can be problematic, since many metabolites are not or no longer commercially available. This is nicely exemplified by e.g. epirubicin glucuronide, for which no commercially available standards exist and which may be strongly retained by C18 columns, resulting in unexpectedly late elution, near or even later than epirubicin. Therefore, we highly recommend this glucuronide to be included during method optimization, either patient-derived or *in vitro* generated, utilizing insect-cell derived microsomes in which the glucuronidating enzyme UGT2B7 is expressed [80,81].

3.3.2. Liquid chromatography coupled to mass spectrometry

In 2000, Lachâtre and coworkers [70] developed a pioneering method for the simultaneous determination of the four anthracyclines and the respective reduced metabolites of three of these in serum. The compounds were eluted from the C18 column with an isocratic mobile phase consisting of water:acetonitrile containing a 5 mM ammonium formate buffer (pH 3.0). Analytes were detected in a single quadrupole mass spectrometer after electrospray ionization with in-source fragmentation. Good sensitivity and selectivity were obtained, and the method is applicable over a broad concentration range. However, nowadays there is a trend towards using tandem mass spectrometric detection, which has become more widely available since then.

3.3.3. Liquid chromatography coupled to tandem mass spectrometry

During the last years, a number of liquid chromatographic–tandem mass spectrometric (LC–MS/MS) methods have been developed. Compounds were separated on a C18 [42,49,54,57] or phenyl [43] stationary phase by application of an isocratic [42,54] or gradient [43,49,57] water:acetonitrile mobile phase containing 0.1% formic acid [42,43,49] or 5 mM ammonium acetate buffer pH 3.5 [54,57].

Analytes are usually detected after electrospray ionization (ESI) in the positive mode [43,49,54,57], although also atmospheric pressure chemical ionization (APCI) [42] has been described. Both ionization techniques have been claimed to be preferable to the other, based on sensitivity criteria in preliminary infusion studies [42,54]. In our opinion other criteria, such as the extent of matrix effect and adduct formation, are also highly relevant in the choice of ionization technique, and these phenomena should be evaluated more thoroughly in future research. The aglycone metabolites are reported to have low ionization efficiencies with ESI [54].

Sleno et al. [82] have written an excellent paper about the fragmentation of anthracyclines following ESI. An example of the fragmentation of doxorubicin is displayed in Fig. 2. Fragment ions of other anthracyclines can be predicted, *mutatis mutandis*, by this scheme.

The same authors also investigated the intensity of selected reaction monitoring (SRM) transitions from the protonated anthracyclines to important fragments in function of the applied collision energy.

Although in mass spectrometry, baseline separation of compounds of interest is sometimes considered as being less important, owing to its high, mass-based, selectivity, this presumption may lead to potential pitfalls in the identification and quantification of anthracyclines and their metabolites, because of: (1) the existence of epimers, (2) the "mass + 2" metabolism and (3) the potential late elution of glucuronide conjugates.

Doxorubicin and epirubicin, as well as their reduced metabolites doxorubicinol and epirubicinol, differ chemically only by the orientation of the hydroxylgroup at position 4′ in the daunosamine sugar (axial *vs.* equatorial). Both epimers break down in identical mass fragments under comparable conditions. Only at low collision energies can a small difference in the intensity of some mass fragments occur: an initial water loss (yielding m/z 526) is more pronounced for epirubicin, whereas an initial cleavage of the glycosidic bond (yielding m/z 415 and 397) is strongly dominant for doxorubicin [42,82]. Although this cross-interference is not expected to take place in patient samples (patients are only administered a single anthracycline), it is relevant when setting up MS-based methods capable of measuring both epi- and doxorubicin, using a single set of calibrators.

The metabolism of the carbonyl function in the main compounds to an alcohol group in the reduced metabolites involves the addition of only 2 mass units. Therefore, the isotope distribution of the molecular ions of the main compounds overlaps about 6.7% with that of these metabolites. The overlap with commonly chosen SRM-transitions still accounts for more than 6% [35]. As a result, baseline separation between the main compounds and their reduced metabolites is a prerequisite for unambiguous identification and quantification of the latter ones. This aspect has not always been taken into account, as demonstrated by Fig. 3 in which the SRM transition of doxorubicinol is expected to be influenced by the one from doxorubicin.

Fig. 2. Fragmentation scheme for doxorubicin.
Adapted from [82].

Glucuronide conjugates are known to be prone to conversion to their parent compounds in the source/interface of the mass spectrometer [83]. Especially when co-elution of the parent compound and its glucuronide may occur (as may be the case for epirubicin and its glucuronide) or has not been investigated, it cannot be excluded that the latter may contribute to the signal, thus compromising correct quantification.

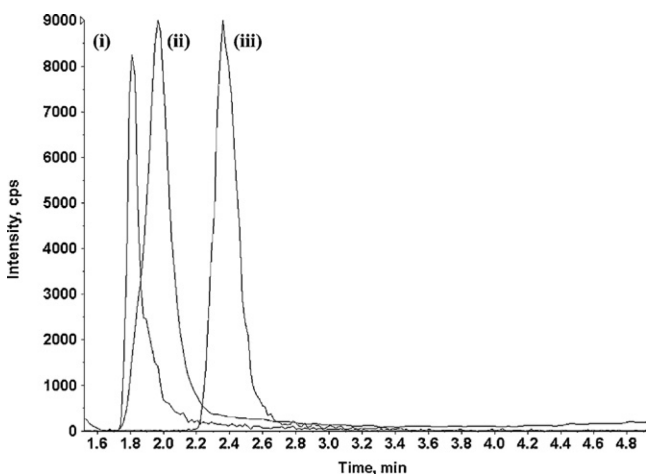

Fig. 3. Combined SRM transitions of doxorubicinol (i, *m*/*z* 546–363), doxorubicin (ii, *m*/*z* 544–361) and daunorubicin (iii, *m*/*z* 528–321).
Adapted from [54].

Owing to its high sensitivity and selectivity, tandem mass spectrometry has increasingly been applied for detecting anthracyclines in plasma during the last decade. However, its application may not result in a neglect of sample preparation or chromatographic separation, since phenomena such as matrix effects, adduct formation, isotope distribution and fragmentation need to be carefully evaluated and controlled. Thus, we do not consider anthracycline analysis to be a very good candidate for a "dilute-and-shoot" approach, in which sample preparation is omitted and the sample is immediately introduced in the mass spectrometer. In addition, although LC–MS/MS has become relatively widely available, its associated cost, both in terms of acquisition and maintenance, remains an important drawback. To our opinion, the application of LC–MS/MS for quantification of anthracyclines in pharmacokinetic studies cannot provide a cost-effective alternative for LC with fluorescence-based detection. The latter has proven to be sufficiently sensitive for detecting the pharmacologically active compounds, even when there is limited sample availability.

3.3.4. Other chromatographic techniques

Two liquid chromatographic methods coupled to UV detection at a wavelength of 254 nm have been described. The first one uses a HILIC-type stationary phase and an isocratic acetonitrile:water mobile phase containing an ammonium formate buffer pH 2.9 [48]. Selectivity with regard to metabolites was not demonstrated and sensitivity was poor. The second one uses gradient elution, but sensitivity is so poor that the anthracyclines can only be monitored for a few minutes after infusion [69].

Liquid chromatography coupled to electrochemical detection [71] or chemiluminescence detection after a post-column photosensitization reaction [59] has been described but, although achieving good sensitivity, has only rarely been applied.

3.3.5. Non-chromatographic techniques

Although the focus of this review is on chromatographic techniques, it needs to be mentioned that also capillary electrophoresis with UV [84], amperometric [85] and laser-induced fluorescence detection [86–92] and other techniques [93,94] have been described. The majority of these methods have been reviewed elsewhere [16,17].

4. Determination for assessing occupational exposure

Although anthracyclines undergo mainly biliary excretion, urine is used as matrix for assessing occupational exposure because of its ease of collection. A small fraction of unchanged drug (5–20%) is recovered in urine, and metabolite concentrations are even expected to be lower [14,95,96].

Based on current knowledge, it is virtually impossible to set a level of exposure that, beyond doubt, can cause no adverse effects [96]. Based on a German study of more than 1000 urine samples of hospital personnel, the highest concentrations reported for doxorubicin and epirubicin are 127 and 182 pg/mL, respectively [97]. Recently, much higher concentrations (up to 33,900 and 84,100 pg/mL for doxorubicin and epirubicin, respectively) were reported in a small Italian study [98]. Until now, daunorubicin and idarubicin have never been detected in urine of hospital personnel. In urine of 2 technicians working in a drug-manufacturing plant, epirubicin concentrations were determined to be 800 and 1200 pg/mL, respectively [99].

The analytical aspects of the trace analysis of doxorubicin, epirubicin, daunorubicin and idarubicin in urine have primarily been described by Sottani and coworkers, who developed and validated two tandem mass spectrometric methods with minor differences [99,100]. Since larger starting volumes are used, solid phase extraction is preferred. Typically, a 5-mL urine sample adjusted to pH 7.0 with 2 mL phosphate buffer was loaded to a previously conditioned silica based reversed-phase C18 sorbent (500 mg). After rinsing the cartridge with phosphate buffer and drying, compounds were eluted with 3 mL of a methylene chloride–isopropanol (50:50, v/v) or methylene chloride–isopropanol–methanol (50:35:15, v/v/v) mixture. After evaporation, the residue was reconstituted in the mobile phase starting conditions. The compounds were separated on a C8 stationary phase by applying a gradient mixture of 0.1% formic acid in water and acetonitrile. Tandem mass spectrometric detection after electrospray ionization enabled detection limits and lower limits of quantification of 40, 40, 10 and 10 pg/mL and 100, 100, 30 and 30 pg/mL for doxorubicin, epirubicin, daunorubicin and idarubicin, respectively [100].

Another method was developed by Pieri and coworkers [98]. A 3-mL acidified urine sample was loaded to a previously conditioned polymeric based reversed-phase sorbent (60 mg). After rinsing the cartridge with a 50-mM formic acid solution and drying, compounds were eluted with 2 times 3 mL of a dichloromethane–isopropanol (50:50, v/v) mixture. After evaporation the compounds were reconstituted in 50 mM formic acid. The compounds were separated on a C8 stationary phase by applying a gradient mixture of 0.1% formic acid in water and acetonitrile. Fluorimetric detection resulted in detection limits of 600 and 1200 pg/mL for doxorubicin and epirubicin, respectively, which are much higher than those obtained by Sottani et al. The fact that at present it is not possible to put forward an exposure level that is certainly devoid of any adverse effects (any detectable level is considered to be a hazard), has as a consequence that it is also not possible to propose a required LOD or LLOQ, below one can assume that exposure can be considered as “safe”. Therefore, because of its high sensitivity/selectivity (pushing down the LOD and LLOQ), the method of choice for workplace testing of urine samples is undoubtedly LC–MS/MS.

5. Conclusion

A multitude of methods are available for the chromatographic separation and detection of the anthracyclines doxorubicin, epirubicin, idarubicin and daunorubicin in biological matrices. Following sample preparation utilizing (a combination of) deproteinization and liquid–liquid or solid phase extraction, anthracyclines (and their metabolites) are separated in the majority of cases utilizing C18 stationary phases. Most methods have opted for fluorescence detection, which is relatively cheap and in most cases provides sufficient sensitivity. For the last few years, several methods with mass spectrometric detection have been developed as well. The method of choice – chromatographic separation and preferred detector – primarily depends on the number of anthracyclines (and metabolites) to be separated and on the aim of the detection (follow-up of patients or workplace monitoring). Whatever method is to be used, great care should be taken in achieving good chromatographic separation and in evaluating possible interferences, such as co-eluting metabolites (e.g. unexpectedly late eluting epirubicin glucuronide) and, when using mass spectrometric detection, matrix effects and adduct formation.

References

[1] A. Dimarco, M. Gaetani, L. Dorigotti, M. Soldati, O. Bellini, Cancer Chemother. Rep. (1964) 31.
[2] V.T. DeVita, T.S. Lawrence, S.A. Rosenberg, R.A. DePinho, R.A. Weinberg, DeVita, Hellman, and Rosenberg's Cancer: Principles and Practice of Oncology, 9th ed., Lippincott Williams & Wilkins, 2011.
[3] J. Bouma, J.H. Beijnen, A. Bult, W.J.M. Underberg, Pharm. Weekbl. [Sci] 8 (1986) 109.
[4] G. Razzano, V. Rizzo, A. Vigevani, Farmaco 45 (1990) 215.
[5] L.M. Hollingshead, D. Faulds, Drugs 42 (1991) 690.
[6] J. Robert, L. Gianni, Cancer Surv. 17 (1993) 219.
[7] J. Robert, Clin. Pharmacokinet. 24 (1993) 275.
[8] J. Robert, Clin. Pharmacokinet. 26 (1994) 428.
[9] J.H.M. Schellens, H.L. McLeod, D.R. Newell, Cancer Clinical Pharmacology, Oxford University Press, 2005.
[10] O. Chassany, S. Urien, P. Claudepierre, G. Bastian, J.P. Tillement, Cancer Chemother. Pharmacol. 38 (1996) 571.
[11] J. Cummings, R. Milstead, D. Cunningham, S. Kaye, Eur. J. Cancer Clin. Oncol. 22 (1986) 991.
[12] H. Weenen, J.M.S. van Maanen, M.M. de Planque, J.G. McVie, H.M. Pinedo, Eur. J. Cancer Clin. Oncol. 20 (1984) 919.
[13] K.E. Maudens, C.P. Stove, V.F.J. Cocquyt, H. Denys, W.E. Lambert, J. Chromatogr. B 877 (2009) 3907.
[14] R. Danesi, S. Fogli, A. Gennari, P. Conte, M. Del Tacca, Clin. Pharmacokinet. 41 (2002) 431.
[15] C.L. Chen, K.K. Thoen, F.M. Uckun, J. Chromatogr. B 764 (2001) 81.
[16] G. Zagotto, B. Gatto, S. Moro, C. Sissi, M. Palumbo, J. Chromatogr. B 764 (2001) 161.
[17] P.M. Loadman, C.R. Calabrese, J. Chromatogr. B 764 (2001) 193.
[18] E. Tomlinson, L. Malspeis, J. Pharm. Sci. 71 (1982) 1121.
[19] J.H. Beijnen, W.J.M. Underberg, J. Pharm. Biomed. Anal. 6 (1988) 677.
[20] P.J. Gray, D.R. Phillips, Photochem. Photobiol. 33 (1981) 297.
[21] S. Eksborg, H. Ehrsson, J. Chromatogr. 340 (1985) 31.
[22] J.H. Beijnen, G. Wiese, W.J.M. Underberg, Pharm. Weekbl. [Sci] 7 (1985) 109.
[23] S. Eksborg, H. Ehrsson, I. Wallin, A. Lindfors, Acta Pharm. Suec. 18 (1981) 215.
[24] A. Andersen, D.J. Warren, L. Slordal, Ther. Drug Monit. 15 (1993) 455.
[25] J. Cummings, J.F.B. Stuart, K.C. Calman, J. Chromatogr. 311 (1984) 125.
[26] A. Kummerle, T. Krueger, M. Dusmet, C. Vallet, Y. Pan, H.B. Ris, L.A. Decosterd, J. Pharm. Biomed. Anal. 33 (2003) 475.
[27] W.I.W. Dodde, J.G. Maring, G. Hendriks, F.M. Wachters, H.J.M. Groen, E.G.E. de Vries, D.R.A. Uges, Ther. Drug Monit. 25 (2003) 433.
[28] P.B. Johansen, S.E. Jensen, S.N. Rasmussen, M. Dalmark, Cancer Chemother. Pharmacol. 13 (1984) 5.
[29] J. Dejong, C.N. Munniksma, W.S. Guerand, A. Bast, W.J.F. Vandervijgh, J. Chromatogr. 574 (1992) 273.

[30] C. Mazuel, J. Grove, G. Gerin, K.P. Keenan, J. Pharm. Biomed. Anal. 33 (2003) 1093.
[31] M.S. Schwartz, B.K. Matuszewski, J. Chromatogr. B 780 (2002) 171.
[32] D. Fraier, E. Frigerio, E. Pianezzola, M. Strolin Benedetti, J. Cassidy, P. Vasey, J. Pharm. Biomed. Anal. 13 (1995) 625.
[33] G.L. Wei, S.H. Xiao, D.Y. Si, C.X. Liu, Biomed. Chromatogr. 22 (2008) 1252.
[34] D.L. Chin, B.L. Lum, B.I. Sikic, J. Chromatogr. B 779 (2002) 259.
[35] Y.H. Liu, Y.H. Yang, X.T. Liu, T. Jiang, Talanta 74 (2008) 887.
[36] R. Bellott, P. Pouna, J. Robert, J. Chromatogr. B 757 (2001) 257.
[37] L. Embree, K.A. Gelmon, A. Lohr, L.D. Mayer, A.J. Coldman, P.R. Cullis, W. Palaitis, F. Pilkiewicz, N.J. Hudon, J.R. Heggie, J.H. Goldie, J. Pharm. Sci. 82 (1993) 627.
[38] H.M. Dodds, E.T. Walpole, L.P. Rivory, R.W. Strong, S.M. Pond, Ther. Drug Monit. 18 (1996) 537.
[39] A. Rudolphi, S. Vielhauer, K.-S. Boos, D. Seidel, I.-M. Bathge, H. Berger, J. Pharm. Biomed. Anal. 13 (1995) 615.
[40] C. Sottani, E. Leoni, B. Porro, B. Montagna, A. Amatu, F. Sottotetti, P. Quaretti, G. Poggi, C. Minoia, J. Chromatogr. B 877 (2009) 3543.
[41] P. Menna, O. Gonzalez Paz, M. Chello, E. Covino, E. Salvatorelli, G. Minotti, Expert Opin. Drug Saf. (2011), doi:10.1517/14740338.2011.589834.
[42] R. Wall, G. McMahon, J. Crown, M. Clynes, R. O'Connor, Talanta 72 (2007) 145.
[43] Y.H. Yang, Talanta 71 (2007) 596.
[44] P.W. Buehler, S.J. Robles, G.R. Adami, R. Gajee, A. Negrusz, Chromatographia 49 (1999) 557.
[45] L. Alvarez-Cedron, M.L. Sayalero, J.M. Lanao, J. Chromatogr. B 721 (1999) 271.
[46] C.H. Mou, N. Ganju, K.S. Sridhar, A. Krishan, J. Chromatogr. B 703 (1997) 217.
[47] S.K. Cox, A.V. Wilke, D. Frazier, J. Chromatogr. 564 (1991) 322.
[48] R.P. Li, L.L. Dong, J.X. Huang, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 2409.
[49] R.P. Li, L.L. Dong, J.X. Huang, Anal. Chim. Acta 546 (2005) 167.
[50] M. Krogh-Madsen, S.H. Hansen, P.H. Honoré, J. Chromatogr. B 878 (2010) 1967.
[51] S.R. Urva, B.S. Shin, V.C. Yang, J.P. Balthasar, J. Chromatogr. B 877 (2009) 837.
[52] A. Al-Abd, N. Kim, S.-C. Song, S. Lee, H.-J. Kuh, Arch. Pharmacol. Res. 32 (2009) 605.
[53] C.M. Gilbert, R.P. McGeary, L.J. Filippich, R.L.G. Norris, B.G. Charles, J. Chromatogr. B 826 (2005) 273.
[54] R.D. Arnold, J.E. Slack, R.M. Straubinger, J. Chromatogr. B 808 (2004) 141.
[55] P. de Bruijn, J. Verweij, W.J. Loos, H.J. Kolker, A.S.T. Planting, K. Nooter, G. Stoter, A. Sparreboom, Anal. Biochem. 266 (1999) 216.
[56] D.T. Rossi, B.A. Phillips, J.R. Baldwin, P.K. Narang, Anal. Chim. Acta 271 (1993) 59.
[57] R. DiFrancesco, J.J. Griggs, J. Donnelly, R. DiCenzo, J. Chromatogr. B 852 (2007) 545.
[58] O. Kuhlmann, S. Hofmann, M. Weiss, J. Chromatogr. B 728 (1999) 279.
[59] S. Ahmed, N. Kishikawa, K. Ohyama, M. Wada, K. Nakashima, N. Kuroda, Talanta 78 (2009) 94.
[60] J. Dejong, P.A. Maessen, A. Akkerdaas, S.F. Cheung, H.M. Pinedo, W.J.F. Vandervijgh, J. Chromatogr. 529 (1990) 359.
[61] J. van Asperen, O. van Tellingen, J.H. Beijnen, J. Chromatogr. B 712 (1998) 129.
[62] Q.Y. Zhou, B. Chowbay, J. Pharm. Biomed. Anal. 30 (2002) 1063.
[63] J.H. Beijnen, P.L. Meenhorst, R. Van Gijn, M. Fromme, H. Rosing, W.J.M. Underberg, J. Pharm. Biomed. Anal. 9 (1991) 995.
[64] I.K. Barker, S.M. Crawford, A.F. Fell, J. Chromatogr. B 681 (1996) 323.
[65] N.A. Dobbs, C.J. Twelves, J. Chromatogr. 572 (1991) 211.
[66] C.M. Camaggi, P. Carisi, E. Strocchi, F. Pannuti, Cancer Chemother. Pharmacol. 30 (1992) 303.
[67] T. Dine, C. Brunet, M. Luyckx, M. Cazin, P. Gosselin, J.L. Cazin, Biomed. Chromatogr. 4 (1990) 20.
[68] S. Fogli, R. Danesi, F. Innocenti, A. Di Paolo, G. Bocci, C. Barbara, M. Del Tacca, Ther. Drug Monit. 21 (1999) 367.
[69] S. Bermingham, R. O'Connor, F. Regan, G.P. McMahon, J. Sep. Sci. 33 (2010) 1571.
[70] F. Lachâtre, P. Marquet, S. Ragot, J.M. Gaulier, P. Cardot, J.L. Dupuy, J. Chromatogr. B 738 (2000) 281.
[71] R. Ricciarello, S. Pichini, R. Pacifici, I. Altieri, M. Pellegrini, A. Fattorossi, P. Zuccaro, J. Chromatogr. B 707 (1998) 219.
[72] G. Nicholls, B.J. Clark, J.E. Brown, J. Pharm. Biomed. Anal. 10 (1992) 949.
[73] P.A.J. Speth, Q. Vanhoesel, C. Haanen, Clin. Pharmacokinet. 15 (1988) 15.
[74] F.T. Peters, O.H. Drummer, F. Musshoff, Forensic Sci. Int. 165 (2007) 216.
[75] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Guidance for Industry – Bioanalytical Method Validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf, 2001 (accessed 22.06.11).
[76] S.M.R. Wille, F.T. Peters, V. Di Fazio, N. Samyn, Accred. Qual. Assur. 16 (2011) 279.
[77] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, J. Chromatogr. B 785 (2003) 263.
[78] K.E. Maudens, S.M.R. Wille, W.E. Lambert, J. Chromatogr. B 848 (2007) 384.
[79] K.E. Maudens, C.P. Stove, W.E. Lambert, J. Sep. Sci. 31 (2008) 1042.
[80] F. Innocenti, L. Iyer, J. Ramirez, M.D. Green, M.J. Ratain, Drug Metab. Dispos. 29 (2001) 686.
[81] M.J. Zaya, R.N. Hines, J.C. Stevens, Drug Metab. Dispos. 34 (2006) 2097.
[82] L. Sleno, V. Campagna-Slater, D.A. Volmer, Int. J. Mass Spectrom. 255 (2006) 130.
[83] R. Krüger, M. Vogeser, S. Burghardt, R. Vogelsberger, K.J. Lackner, Clin. Chem. Lab. Med. 48 (2010) 1723.
[84] A. Gavenda, J. Sevcik, J. Psotova, P. Bednar, P. Bartak, P. Adamovsky, V. Simanek, Electrophoresis 22 (2001) 2782.
[85] Q. Hu, L. Zhang, T.S. Zhou, Y.Z. Fang, Anal. Chim. Acta 416 (2000) 15.
[86] N. Griese, G. Blaschke, J. Boos, G. Hempel, J. Chromatogr. A 979 (2002) 379.
[87] G. Hempel, S. Haberland, P. SchulzeWesthoff, N. Mohling, G. Blaschke, J. Boos, J. Chromatogr. B 698 (1997) 287.
[88] G. Hempel, P. Schulze-Westhoff, S. Flege, J. Boos, J. Chromatogr. B 758 (2001) 221.
[89] T. Perez-Ruiz, C. Martinez-Lozano, A. Sanz, E. Bravo, Electrophoresis 22 (2001) 134.
[90] N. Siméon, E. Chatelut, P. Canal, M. Nertz, F. Couderc, J. Chromatogr. A 853 (1999) 449.
[91] G. Whitaker, A. Lillquist, S.A. Pasas, R. O'Connor, F. Regan, C.E. Lunte, M.R. Smyth, J. Sep. Sci. 31 (2008) 1828.
[92] H. Lu, G. Yuan, Q. He, H. Chen, Microchem. J. 92 (2009) 170.
[93] J. Fei, X. Wen, Y. Zhang, L. Yi, X. Chen, H. Cao, Microchim. Acta 164 (2009) 85.
[94] Y. Liu, B. Danielsson, Anal. Chim. Acta 587 (2007) 47.
[95] C.H. Grimm, K.S. Boos, in: J. Angerer, K.H. Schaller (Eds.), Analysis of Hazardous Substances in Biological Materials, vol. 7, Wiley VCH, Weinheim, 2001, p. 119.
[96] R. Turci, C. Sottani, A. Ronchi, C. Minoia, Toxicol. Lett. 134 (2002) 57.
[97] A. Pethran, R. Schierl, K. Hauff, C.H. Grimm, K.S. Boos, D. Nowak, Int. Arch. Occup. Environ. Health 76 (2003) 5.
[98] M. Pieri, L. Castiglia, P. Basilicata, N. Sannolo, A. Acampora, N. Miraglia, Ann. Occup. Hyg. 54 (2010) 368.
[99] C. Sottani, P. Rinaldi, E. Leoni, G. Poggi, C. Teragni, A. Delmonte, C. Minoia, Rapid Commun. Mass Spectrom. 22 (2008) 2645.
[100] C. Sottani, G. Tranfo, M. Bettinelli, P. Faranda, M. Spagnoli, C. Minoia, Rapid Commun. Mass Spectrom. 18 (2004) 2426.