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Simultaneous determination of four anthracyclines and three metabolites in human serum by liquid chromatography–electrospray mass spectrometry

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Abstract

A sensitive and very specific method, using liquid chromatography–electrospray mass spectrometry (LC–ES-MS), was developed for the determination of epirubicin, doxorubicin, daunorubicin, idarubicin and the respective active metabolites of the last three, namely doxorubicinol, daunorubicinol and idarubicinol in human serum, using aclarubicin as internal standard. Once thawed, 0.5-ml serum samples underwent an automated solid-phase extraction, using C_{18} Bond Elut cartridges (Varian) and a Zymark Rapid-Trace robot. After elution of the compounds with chloroform–2-propanol (4:1, v/v) and evaporation, the residue was reconstituted with a mixture of 5 m*M* ammonium formate buffer (pH 4.5)–acetonitrile (60:40, v/v). The chromatographic separation was performed using a Symmetry C_{18}, 3.5 μm (150×1 mm I.D.) reversed-phase column, and a mixture of 5 m*M* ammonium formate buffer (pH 3)–acetonitrile (70:30, v/v) as mobile phase, delivered at 50 μl/min. The compounds were detected in the selected ion monitoring mode using, as quantitation ions, *m/z* 291 for idarubicin and idarubicinol, *m/z* 321 for daunorubicin and daunorubicinol, *m/z* 361 for epirubicin and doxorubicin, *m/z* 363 for doxorubicinol and *m/z* 812 for aclarubicin (I.S.). Extraction recovery was between 71 and 105% depending on compounds and concentration. The limit of detection was 0.5 ng/ml for daunorubicin and idarubicinol, 1 ng/ml for doxorubicin, epirubicin and idarubicin, 2 ng/ml for daunorubicinol and 2.5 ng/ml for doxorubicinol. The limit of quantitation (LOQ) was 2.5 ng/ml for doxorubicin, epirubicin and daunorubicinol, and 5 ng/ml for daunorubicin, idarubicin, doxorubicinol and idarubicinol. Linearity was verified from these LOQs up to 2000 ng/ml for the parent drugs ($r \geq 0.992$) and 200 ng/ml for the active metabolites ($r \geq 0.985$). Above LOQ, the within-day and between-day precision relative standard deviation values were all less than 15%. This assay was applied successfully to the analysis of human serum samples collected in patients administered doxorubicin or daunorubicin intravenously. This method is rapid, reliable, allows an easy sample preparation owing to the automated extraction and a high selectivity owing to MS detection.

Keywords: Daunorubicin; Doxorubicin; Epirubicin; Idarubicin; Anthracyclines

1. Introduction

Anthracyclines are a group of anticancer drugs with a potent activity against a wide range of

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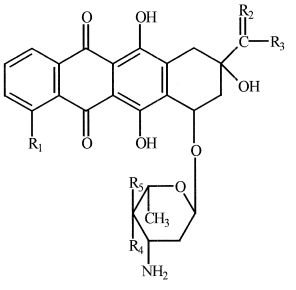
tumours, including lymphomas, Hodgkin's disease, acute leukaemias, bladder carcinoma, soft tissue or osteogenic sarcomas, as well as breast, lung, gastric, testicular or prostate cancers and childhood tumours. Though they present no major contraindication other than pregnancy, lactation or pre-existing cardiac, hepatic or renal disease, they exhibit several adverse effects such as radiosensitisation or common symptomatic effects like nausea, vomiting, diarrhoea, painful oral ulceration (extending to oesophagitis), urticaria at the site of injection and alopecia. The most severe side effects are myelosuppression, particularly affecting neutrophils and platelets and occurring with a nadir at day 8 or 10, and above all cardiotoxicity, leading to arrhythmia and congestive heart failure [1].

The structure of the various anthracyclines is very similar (Fig. 1): daunorubicin, doxorubicin, epirubicin and idarubicin, possessing only one sugar moiety, belong to class I; aclarubicin, with a chain of three sugars, belongs to class II.



	R1	R2	R3	R4	R5
doxorubicin	OCH_3	O	CH_2OH	H	OH
doxorubicinol	OCH_3	OH	CH_2OH	H	OH
daunorubicin	OCH_3	O	CH_3	H	OH
daunorubicinol	OCH_3	OH	CH_3	H	OH
epirubicin	OCH_3	O	CH_2OH	OH	H
idarubicin	H	O	CH_3	H	OH
idarubicinol	H	OH	CH_3	H	OH

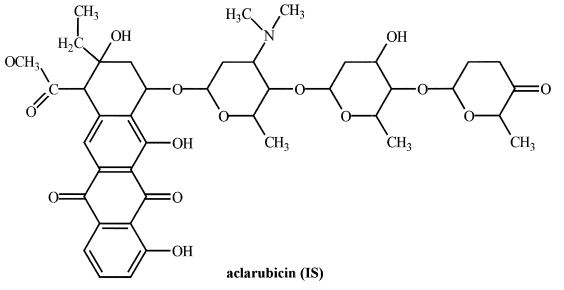


Fig. 1. Chemical structures of the anthracyclines and active metabolites analysed.

Liver is the principal site of anthracycline metabolism and different metabolic steps were demonstrated. Reduction of the ketone function at the C13 position, catalysed by a cytoplasmic aldo-ketoreductase that leads to 13-dihydro metabolites such as doxorubicinol, daunorubicinol and idarubicinol, is the most important one in so far as the metabolites obtained are pharmacologically active. Cleavage is the following step, producing aglycone metabolites whose activity has not been clearly established [2,3].

However, the anthracyclines exhibit specific metabolic and pharmacokinetic features [4]. Indeed, the amount of active metabolite produced in the body depends on the nature of the parent drug, because of different affinity for aldo-ketoreductase: thus, levels of daunorubicinol are higher than those of doxorubicinol for a same administered dose of the parent compounds, because daunorubicin is a better substrate than doxorubicin for the enzyme.

A sensitive analytical technique for the determination of serum levels of these compounds is needed for the routine monitoring of these drugs, because of an important inter-patient heterogeneity of these levels for a same dose [5], and of a wide variability in the relationship between dose and major adverse effects [6]. Indeed, drug administration scheduling was shown to be an important factor for the prevention of anthracycline cardiotoxicity [7], when producing low peak plasma drug concentration [8].

Several methods for the determination of single anthracyclines and their respective metabolites in plasma or tissues have been reported, using liquid–liquid extraction [9–18] or sometimes solid-phase extraction (SPE), and for the large majority, fluorescence detection. Some even included several therapeutic anthracyclines and metabolites [19–21].

The aim of the present study was to obtain a rapid, sensitive and specific technique for the simultaneous determination of four anthracyclines and three active metabolites, using automated extraction and liquid chromatographic–mass spectrometric determination.

2. Experimental

2.1. Chemicals and reagents

Daunorubicin and daunorubicinol as pure standards were kindly supplied by Rhone-Poulenc Rorer

Bellon (Vitry-sur-Seine, France). Pure standards of epirubicin, doxorubicin and doxorubicinol, idarubicin and idarubicinol were obtained from Pharmacia & Upjohn (St. Quentin en Yvelines, France). Aclarubicin (I.S.) was kindly supplied by Medac (Hamburg, Germany) as a commercial lyophilised form, Aclaplastin. All the powders were stored at +4°C in the dark.

HPLC-grade acetonitrile (99.8% pure) and methanol (99.9% pure) were obtained from Carlo-Erba (Milan, Italy). HPLC-grade chloroform (99.3% pure), R.P. Normapur 2-propanol (99.9% pure) and Na_2HPO_4 were purchased from Prolabo (Paris, France). Formic acid and ammonium formate (99% pure) were from Sigma (St. Louis, MO, USA).

2.2. Standards and solutions

A 20 mg/l stock solution of aclarubicin (I.S.) was prepared in a mixture of methanol–water (50:50, v/v). Stock solutions of epirubicin, doxorubicin, daunorubicin, idarubicin, doxorubicinol, daunorubicinol and idarubicinol were prepared at 1 g/l in methanol. A 100 mg/l secondary solution containing the four parent drugs was obtained by mixing the respective stock solutions and adding 5 m*M* formate buffer (pH 4.5); it was immediately aliquoted in small black 1.5-ml Eppendorf vials (C.M.L., Nemours, France) and stored frozen at −18°C. The three metabolite stock solutions were also mixed up, then diluted twice, first with methanol, secondly with the formate buffer, in Falcon polypropylene tubes (Beckton-Dickinson Labware, Meylan, France) in order to obtain a final concentration of 10 mg/l. This secondary solution was aliquoted and stored in the same way as the parent compound solution.

Each day of analysis, working solutions were freshly prepared from thawed aliquots of the secondary solutions, by dilution with the formate buffer to reach concentrations of 40, 20, 10, 2, 1, 0.2, 0.1, 0.05 mg/l for the parent compounds and 4, 2, 1, 0.4, 0.2, 0.1, 0.05 mg/l for the metabolites. In the same way, a 4 mg/l standard solution was prepared from the I.S. stock solution.

2.3. Sample preparation

To 0.5 ml of serum were added 25 μl of I.S. standard solution and 25 μl of each of the appropriate working solutions of parent compounds and metabolites, in order to obtain the following calibrating levels: 0, 2.5, 5, 10, 50, 100, 500, 1000, 2000 ng/ml for parent drugs and 0, 2.5, 5, 10, 20, 50, 100, 200 ng/ml for the metabolites (as several pharmacokinetic studies have shown that the serum concentrations of the metabolites were in a ratio of 1/10 with their respective parent compounds [9,11,14–20]). Then, the tubes were briefly vortex-mixed (10 s) and the samples placed on the Rapid-Trace SPE robot (Zymark Center, Hopkinton, MA, USA), by which they were treated according to the following successive steps: addition of 3 ml of 0.05 *M* Na_2HPO_4 (pH 7.5) to the sample; conditioning of Bond Elut C_{18} (3 ml, 200 mg) cartridges (Varian, St. Quentin-en-Yvelines, France) with 3 ml methanol, 6 ml of 0.05 *M* Na_2HPO_4 (pH 7.5) and 6 ml deionised water, successively; loading of diluted sample on the cartridge; cartridge rinsing with 6 ml deionised water; 12 min drying; elution with 1 ml chloroform–2-propanol (4:1, v/v) in 1.5-ml Eppendorf tubes and evaporation during 10 min under a gentle stream of nitrogen. The dried extracts were redissolved in 25 μl mobile phase, of which 3 μl was injected into the chromatographic system.

2.4. HPLC conditions

The HPLC system consisted of two Series-200 LC micropumps and a Series-200 autosampler (Sciex, Concord, Canada). The chromatographic separation was performed on a Symmetry C_{18}, 3.5 μm (150×1 mm I.D.) reversed-phase column (Waters) together with a mixture of 5 m*M* ammonium formate (pH 3.0)–acetonitrile (70:30, v/v) as mobile phase, delivered at a flow-rate of 50 μl/min. All chromatographic solvents were filtered (0.46 μm) prior to mixing and degassed with helium thereafter.

2.5. Mass spectrometric detection

The instrument used was an API 100 mass spectrometer, equipped with an electrospray-type ionisation device (Sciex). Ultra-high purity nitrogen was used as nebulisation and curtain gas. Calibration of the mass analyser was performed by infusion (5 μl/min) of a commercial mixture of PPGs (polypropylene glycols, Applied Biosystems, St. Quentin-

en-Yvelines, France) using a Harvard Model II syringe pump (Harvard Scientific, South Natick, MA, USA) and monitoring eight m/z ratios in the 55 to 2300 amu mass range.

Ions generated by electrospray (ES) ionisation are generally protonated molecules $[M+H]^+$ or adducts of the molecule with ammonium ions $[M+NH_4]^+$, easily obtained for moderately basic compounds like anthracyclines (average $pK_a=7.5$). The characteristic ions of doxorubicin, daunorubicin, epirubicin, idarubicin, aclarubicin, doxorubicinol, daunorubicinol and idarubicinol were identified by infusing a pure solution of each compound in the mobile phase separately, using full scan acquisition (m/z 50–1000 amu, step size 0.1 amu) (Fig. 2). As ES ionisation is a soft ionisation process which generates few fragment ions, in-source induced fragmentation was used to obtain confirmation ions and optimised by modulating the collision energy through the orifice voltage. A quantification ion (corresponding to the most intense of the high mass ions in the spectrum) and a confirmation ion (free from interfering peak) were chosen for each compound.

2.6. Validation of the method

Recovery was determined in triplicates at concentrations of 5 and 500 ng/ml by extraction of blank serum samples fortified with the analytes (parent drugs and metabolites) and I.S., as well as of blank serum samples spiked only with the same amount of I.S. Fortified extracts were reconstituted with 25 μl pure mobile phase, whereas blank extracts were reconstituted with a solution of parent drugs and metabolites in the mobile phase at concentrations representing 100% recovery. Recovery was calculated by comparison of the analyte/I.S. peak area ratios.

Repeatability, reproducibility and linearity were estimated according to the principles described by Shah et al. [22]: intra-assay precision was assessed at concentration levels of 10 and 500 ng/ml for the parent drugs, 10 and 100 ng/ml for the metabolites, by extraction and analysis, on the same day, of six drug-free serum samples fortified with all the analytes for each level. Intermediate precision was estimated by analysing each day for 6 days a set of calibrating samples spiked at 0, 2.5, 5, 10, 50, 100, 500, 1000, 2000 ng/ml for the parent compounds and 0, 2.5, 5, 10, 20, 50, 100, 200 ng/ml for the metabolites, prepared in advance and stored at −18°C until analysis. The limit of detection (LOD), defined as the lowest concentration yielding a signal-to-noise ratio higher than 3, was determined by analysing samples spiked at 0.1, 0.2, 0.5, 1, 2 and 5 ng/ml of each analyte.

3. Results

For each drug or metabolite, the chromatographic retention times and the ions selected, with their respective optimised orifice voltage (i.e., collision energy), are reported in Table 1.

Fig. 3 shows the chromatogram of a serum sample spiked with 50 ng/ml of each parent drug (doxorubicin, epirubicin, daunorubicin, idarubicin), 20 ng/ml of each metabolite (doxorubicinol, daunorubicinol, idarubicinol), and 200 ng/ml of internal standard, aclarubicin. In extracts of blank serum from healthy volunteers, no chromatographic peak was noted at the retention times of the compounds of interest. Extracts of serum spiked with only one of each anthracycline were also analysed, showing that no peak interfered with the retention times of the other anthracyclins, except for pirarubicin which was part of the assay at the beginning and was excluded because of a rapid and non-negligible degradation into doxorubicin at ambient temperature [16]. Finally, the analysis of serum samples from patients administered doxorubicin or daunorubicin showed no peak from any unidentified metabolite at the retention times of the other compounds of interest (so far, we could not obtain any serum sample from a patient treated with epirubicin or idarubicin). Fig. 4 shows the chromatograms of serum samples collected at 1 h and 72 h after administration of 100 mg daunorubicin (as a 45-min intravenous infusion) to a 58-year-old female patient, treated for a type 3 acute myeloid leukemia.

The extraction recovery for all the analytes ranged between 85 and 105% at a low concentration (5 ng/ml) and between 71 and 97% at a higher concentration (100 ng/ml for metabolites and 500 ng/ml for parents) (Table 2).

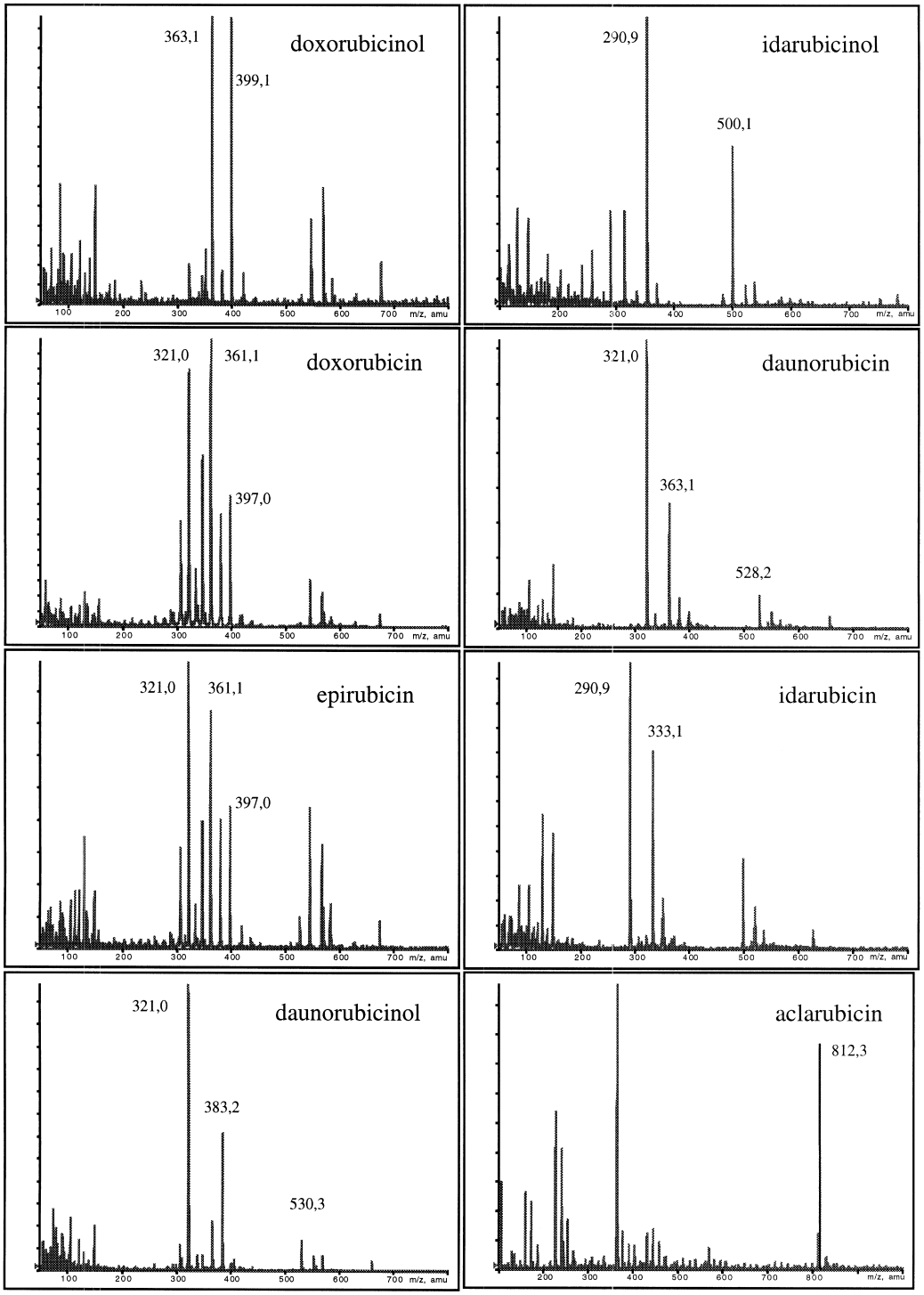


Fig. 2. Mass spectra of the anthracyclines and active metabolites analysed.

Table 1
Retention time, quantitation and confirmation m/z ratios and their respective orifice voltage[a]

Compound	Retention time (min)	Selected ions (amu)	Orifice voltage (V)
Doxorubicinol	10.4	**363**	110
		399	90
Doxorubicin	14.4	**361**	90
		397	90
		321	120
Epirubicin	15.4	**361**	90
		397	90
		321	120
Daunorubicinol	15.8	**321**	120
		383	100
		530	90
Idarubicinol	16.9	**291**	105
		500	65
Daunorubicin	17.2	**321**	120
		528	105
Idarubicin	18.0	**291**	105
		333	110
Aclarubicin (I.S.)	21.2	**812**	85

[a] Quantitation ions are in bold characters.

The measured LOD was: 0.5 ng/ml for daunorubicin and idarubicinol; 1 ng/ml for doxorubicin, epirubicin, idarubicin; 2 ng/ml for daunorubicinol; and 2.5 ng/ml for doxorubicinol. The limit of quantification (LOQ) was 2.5 ng/ml for doxorubicin, epirubicin and daunorubicinol, and 5 ng/ml for daunorubicin, idarubicin, doxorubicinol and idarubicinol (Table 2).

The results of intra-assay precision, intermediate precision and accuracy and correlation coefficients of the regression analysis are reported in Table 2. All of them fulfilled the respective requirements of the Washington consensus conference on Analytical Methods Validation [22]. The linearity was verified, for each compound, from its LOQ up to 200 ng/ml (for the metabolites) or 2000 ng/ml (for parent compounds) and the curvature found not significant.

4. Discussion

The present technique, combining automated SPE and LC–ES-MS, allows the sensitive and specific determination of almost all the anticancer drugs of the anthracycline family, as well as of the active metabolite of three of them. As epirubicinol could not be obtained as a pure standard, either as a gift or as a commercial preparation, it could not be included in this method. In the literature, many analytical papers dealt with a single anthracycline, with none [7,14], one [15,17,18] or several [10,12–14,19,20] of its metabolites but very few included several parent drugs [11,21] and active metabolites and none used such a specific detection technique as mass spectrometry. Indeed, the large majority used fluorescence detection [9–21].

According to information about the instability of anthracyclines provided either by the manufacturer brochures or in the literature [15,17,23], many cautions were taken. In so far as the optimum pH of stability is between 4.5 and 5.5 for daunorubicin, 3 and 7 for doxorubicin, 4 and 5 for epirubicin, 4 and 5 for aclarubicin, working solutions as well as the reconstitution solvent were prepared with pH 4.5, 5 m*M* ammonium formate. Because anthracyclines are light sensitive and adsorb on glass containers [5,23], black Eppendorf tubes (1.5 ml) were used for stock solutions, polypropylene tubes covered with alumin-

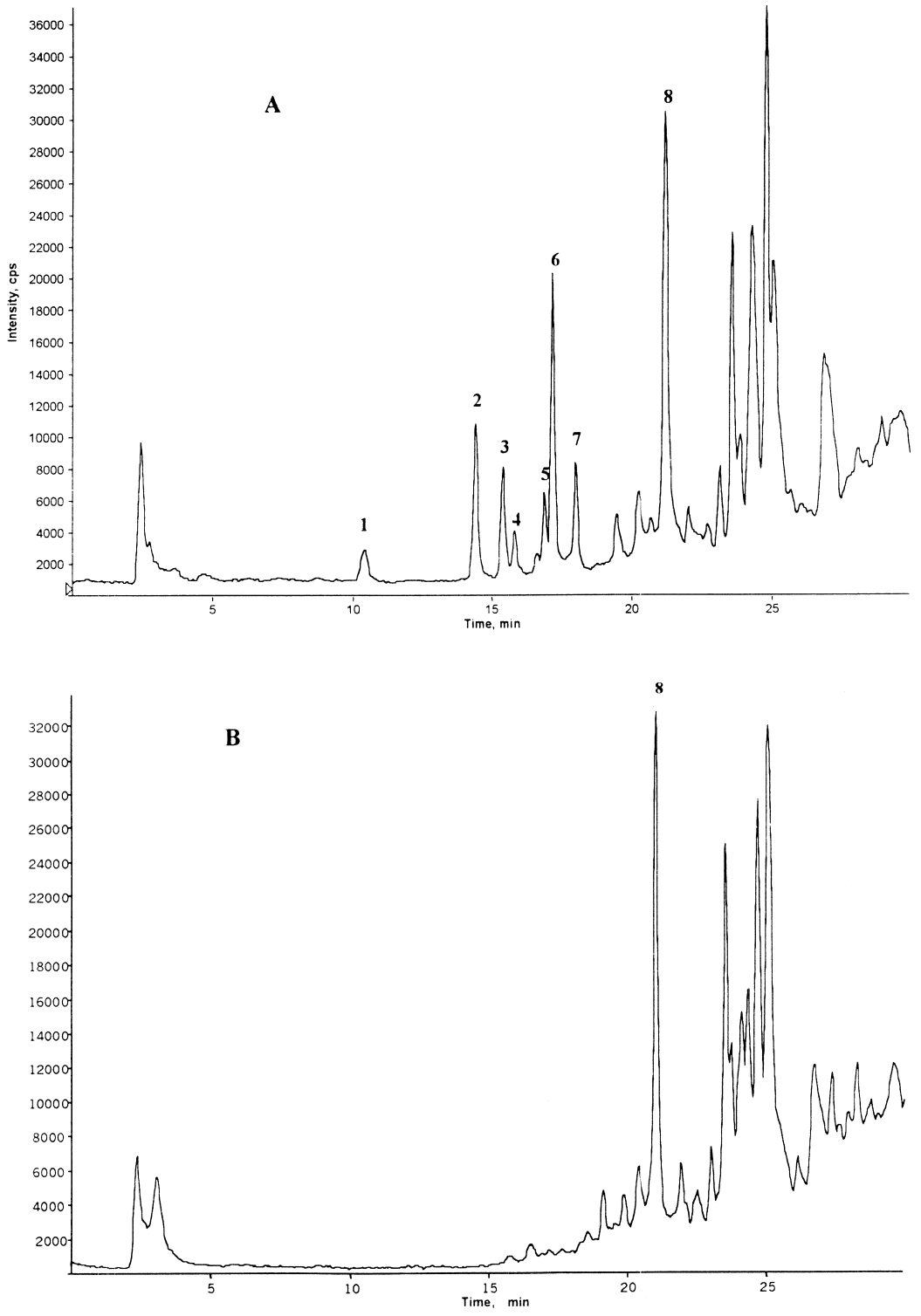


Fig. 3. Total ion chromatograms of: (A) an extract of a serum sample spiked at 50 ng/ml for the parent drugs and 20 ng/ml for the metabolites [1=doxorubicinol, 2=doxorubicin, 3=epirubicin, 4=daunorubicinol, 5=idarubicinol, 6=daunorubicin, 7=idarubicin, 8=aclarubicin (I.S.)]; (B) an extract of a blank serum sample.

Table 2
Validation results of the LC–MS determination of four anthracyclines and three metabolites in serum

Concentration added (ng/ml)	Extraction recovery (%)	Intra-assay precision (n=5) RSD (%)	Inter-assay study (n=6)		
			Mean concentration found (ng/ml)	Precision RSD (%)	Relative mean error (%)
Doxorubicin					
2.5			2.7	19	+8.2
5	104.7		4.7	14.5	−6.1
10		8.8	8.7	5.9	−13.0
50			50.2	13.3	+0.4
100			101.5	10.5	+1.5
500	97.3	10.7	521	5.5	+4.2
1000			1058.2	5.4	+5.8
2000			1920.4	2.2	−4.0
Correlation coefficient				r=0.992±0.006	
Daunorubicin					
2.5			3.7	20.2	+47.8
5	99.7		5.7	7.9	+14.2
10		5.5	8.7	11.5	−13.0
50			44.7	12.8	−10.6
100			90.3	10.4	−9.7
500	89.8	11.4	478.8	11.2	−4.2
1000			963.4	6.4	−3.7
2000			2085.2	5.8	+4.3
Correlation coefficient				r=0.996±0.003	
Idarubicin					
2.5			3	20.7	+21.4
5	84.7		5.3	14.8	+6.1
10		11.3	9.3	10.0	−7.2
50			48.3	7.4	−3.5
100			93.5	10.3	−6.5
500	83.8	12.2	495.1	8.6	−1.0
1000			980.6	7.4	−2.0
2000			2033.4	3.5	+1.7
Correlation coefficient				r=0.994±0.006	
Epirubicin					
2.5			2.9	12.5	+15.1
5	105.5		5.4	14.3	+7.5
10		6.7	9.3	9.2	−7.4
50			48.6	10.1	−2.7
100			88.2	14.4	−11.8
500	85.8	10.9	484.2	6.8	−3.2
1000			1004.9	6	+0.5
2000			2015.5	4.4	+0.8
Correlation coefficient				r=0.996±0.003	

Table 2 (*continued*)

Concentration added (ng/ml)	Extraction recovery (%)	Intra-assay precision (n=5) RSD (%)	Inter-assay study (n=6)		
			Mean concentration found (ng/ml)	Precision RSD (%)	Relative mean error (%)
Doxorubicinol					
2.5			2	29.5	−21.5
5	96.7		5.1	12.5	+1.4
10		9.5	10.2	6.2	+2.2
20			22.1	11.6	+10.5
50			55.6	12.8	+11.2
100	71.0	4.7	97.3	5.7	−2.6
200			195.4	3.6	−2.3
Correlation coefficient				r=0.995±0.004	
Daunorubicinol					
2.5			2.5	12.5	+0.7
5	95.6		5.5	13.9	+9.4
10		5.7	9.1	9.2	−9.2
20			20.5	7.9	+2.8
50			48.8	11.4	−2.4
100	75.0	14.1	94.5	8	−5.5
200			206.9	4.2	+3.5
Correlation coefficient				r=0.985±0.012	
Idarubicinol					
2.5			1.8	19.6	−26.5
5	94.5		5.4	9.2	+8.3
10		8.6	9.8	10.4	−2.3
20			22.1	7.1	+10.3
50			54.7	10.4	+9.5
100	91.1	13.5	104.9	2.7	+4.9
200			188.9	3.6	−5.5
Correlation coefficient				r=0.985±0.010	

ium paper for working solutions, black Eppendorf tubes for the collection of extracts, whereas (brown) glass vials were only used in the auto-sampler. Finally, because of thermal instability, all the stock and secondary solutions were aliquoted and stored at −18°C, then thawed only once.

Though secondary and working solutions in pure methanol would have had some advantages for the compound stability, they would have been hardly compatible with SPE. For this reason, they were prepared in a mixture of methanol and formate buffer, so that the highest fortified serum of the calibrating set contained only 0.8% of methanol.

SPE, based on the HPLC behaviour of compounds, avoids the drawbacks of manual techniques based on partition between aqueous and organic liquid phases, necessitating careful and sometimes difficult removal of the organic layers [21]. Besides, the automation of the technique would normally increase its reproducibility, which was probably impeded in the present study by the instability of the drugs. Furthermore, this method renders the possibility to run the extraction of the whole calibrating set and samples at the same time (with a sufficient number of extraction modules) without any human intervention, and allows its use even by a limited or not specialised technical staff. Moreover, extraction recovery was globally higher than those reached with the previously published techniques using liquid–liquid extraction. Nevertheless, some of these, gener-

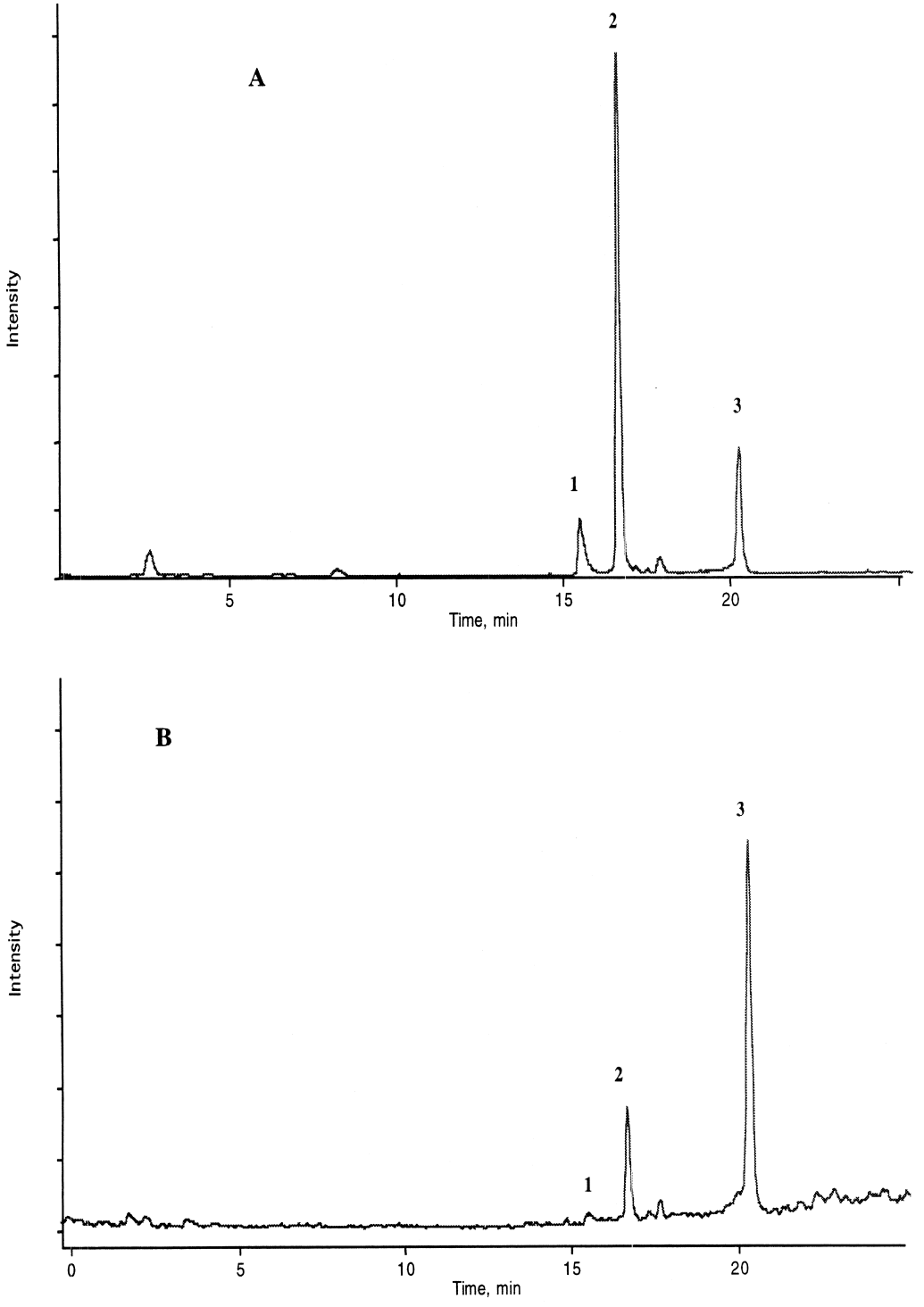


Fig. 4. Selected ion chromatograms of serum samples collected at (A) 1 h and (B) 72 h after administration of 100 mg daunorubicin, as a 45-min intravenous infusion, to a 58-year-old female patient, treated for type 3 acute myeloid leukemia. The concentrations measured were: daunorubicin 1078 ng/ml at 1 h and 76 ng/ml at 72 h; daunorubicinol 148 ng/ml at 1 h and 9 ng/ml at 72 h [1=daunorubicinol; 2=daunorubicin; 3=aclarubicin (I.S.)].

ally dealing with only one anthracycline and its metabolites, reached limits of detection as low [10,12,15,19] or even lower [9,11,13,14,16–18,20] than ours, which is probably due to a limited ionisation rate of these molecules in the electrospray source. However, the LODs and LOQs reported herein are low enough for pharmacokinetic studies in humans.

Linearity was verified over a large concentration range: 0 to 200 ng/ml for the metabolites and 0 to 2000 ng/ml for the parent drugs, while most of the previous methods had a linearity range limited to 1000 ng/ml [12,14,20], 500 ng/ml [11,13] or less [9,10,15–18]. The interest of such a large concentration range is, for pharmacokinetic studies as well as for routine therapeutic drug monitoring, to determine in a single run samples with high (e.g., end of infusion) or low concentrations (e.g., trough level) of a given drug.

This study shows that this method is accurate, repeatable and reproducible over the range of concentrations usually found in clinical blood samples. Finally, it renders it possible to analyse in a single run samples from different patients treated with anyone of the four anthracyclines concerned, using a single set of calibrating standards and quality controls. It can also be useful to study the pharmacokinetic properties of each drug and to relate them to its effects both as clinical response and toxicity.

5. Conclusion

The present method is the first one using liquid chromatography coupled to mass spectrometry for the determination of anthracyclines in serum samples. It is rapid and simple due to automated SPE extraction, highly specific and selective owing to mass spectrometry.

This technique can be applied to the therapeutic drug monitoring of four parent drugs and the respective active metabolite of three of them, allowing the samples from patients treated with any of these drugs to be analysed in a same series.

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