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Coupled-column liquid chromatographic analysis of epirubicin and metabolites in biological material and its application to optimization of liver cancer therapy*

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Abstract: A specific, sensitive and fully automated coupled-column LC method for the determination of the anthracycline cytostatic epirubicin and four metabolites in the biological materials human plasma, liver homogenate and liver tumour homogenate has been developed. System-integrated sample processing was achieved using a new restricted access silica precolumn packing. This porous Alkyl-Diol Silica (ADS) was specially designed for the direct and repetitive injection of proteinaceous samples. It consists of a hydrophilic and electroneutral external particle surface (glyceryl-residues) and a hydrophobic reversed-phase internal surface (butyryl-, octanoyl- or octadecyl-residues). These bimodal chromatographic properties allow retention of low molecular analytes by classical RP-chromatography exclusively at the lipophilic pore surface. Macromolecular constituents of the sample matrix (e.g. proteins) are size-excluded by 5 nm pores and quantitatively eliminated in the interstitial void volume. On-line analysis was performed by coupling a C_4-Alkyl-Diol precolumn (20 × 4 mm i.d., particle size 25 μm) and a LiChrospher RP Select B analytical column (250 × 4 mm i.d., particle size 5 μm) via an electrically driven six-port valve. Separation of the parent compound and its metabolites was achieved with a mobile phase consisting of water (0.1% triethylamine, v/v, pH 2.0 adjusted with trichloroacetic acid)–acetonitrile (70:30, v/v) at a flow rate of 1 ml min^{-1}. The analytes were detected using their natural fluorescence (excitation 445 nm, emission 560 nm). The method described is used for the determination of pharmacokinetics of epirubicin and its metabolites in order to evaluate and optimize treatment regimen of liver cancer chemoembolization therapy.

Keywords: *Coupled-column LC; epirubicin; restricted access materials; Alkyl-Diol Silica; pharmacokinetics.*

Introduction

Epirubicin belongs to the family of anthracycline cytostatics which are used against a broad spectrum of cancer diseases. It was first synthesized in 1975 by Arcamone *et al.* [1] by epimerization of the hydroxyl group in the 4′-position of the amino sugar moiety of doxorubicin (adriamycin), the best known anthracycline. The structures of epirubicin, its metabolites and doxorubicin are presented in Fig. 1.

Epirubicin and doxorubicin are the most utilized cytotoxic agents for systemic chemotherapy of primary hepatocellular carcinoma (HCC). HCC is one of the worst types of cancer as it is usually inoperable at diagnosis and is a rapidly fatal disease with a median survival time of approximately 2 months for untreated patients. Most other antineoplastic drugs or their combinations have proved to be ineffective. Moreover, even the anthracyclines generally provide objective response rate of only 10–15%, and adverse effects like alopecia, myelosuppression and myocardial damage often require termination of therapy [2]. Therefore, alternatives to systemic treatment in order to increase therapeutic efficacy, reduce side-effects and improve life quality are being tried. One promising effort is locoregional chemotherapy of the liver by transcatheter arterial chemoembolization (TACE). Administration of a mixture of the cytostatic with an embolizing agent leads to the reduction of the hepatic arterial blood flow. Thus the local drug concentration and the contact time of the cytostatic with the target organ are

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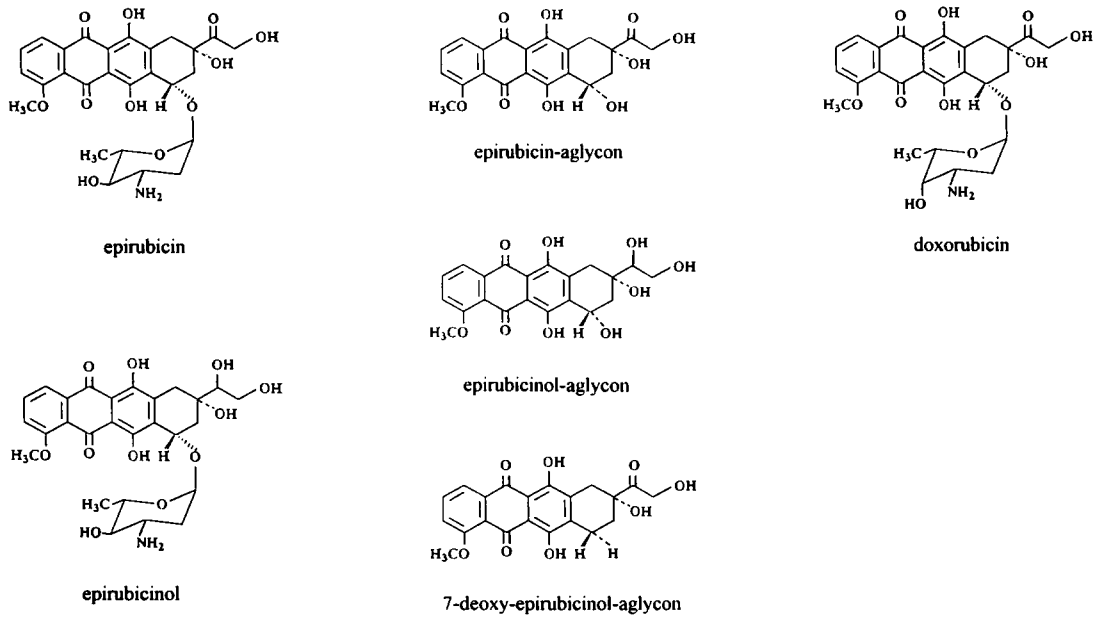

Figure 1
Structures of doxorubicin, epirubicin and its metabolites.

augmented. As hepatic tumours are supplied by 80% with blood from the hepatic artery, occlusion of this feeding vessel results in tumour ischaemia and improved drug exposure within the target region while reducing systemic drug availability and, therefore, preventing toxic side-effects. Two different embolizing agents (Lipiodol® and Spherex®) were examined. Monitoring of epirubicin plasma levels and evaluation of the pharmacokinetic data obtained allowed judgement of the efficiency of locoregional treatment in comparison to systemic drug application.

Various LC methods have been applied to determine epirubicin and its metabolites in biological fluids [3–14]. All of them involve time consuming and error-prone sample pretreatment steps such as liquid–liquid and manual or semi-automated solid-phase extraction to remove the protein matrix which otherwise adsorbs to the silica packings of the analytical columns used. Consequently, an increase in back pressure, a loss of column performance and finally column deterioration occur.

LC-integrated sample processing of complex biological matrices (plasma, serum, tissue homogenates, urine) can be achieved by coupling a sample processing precolumn to an analytical column as described e.g. in [15–21]. The precolumn eliminates the destructive macromolecular matrix compounds while extracting the analytes of interest. Special packing materials referred to as restricted access supports have been designed during the last decade [22–25] for the direct injection of untreated biological samples. Recently, our group introduced a new family of restricted access supports for bioanalytical LC, the Alkyl-Diol Silica (ADS) materials [26, 27]. These internal-surface reversed-phase supports have the ability to extract and enrich hydrophobic analytes by classical RP-chromatography (C_4-, C_8- or C_{18}-partitioning chromatography) exclusively at the internal surface while size-excluding macromolecules by 5-nm pores in the interstitial void volume. This paper describes the application of C_4-ADS in a fully automated coupled-column LC-system for the analysis of epirubicin in different biological materials.

Materials and Methods

Reagents and standard solutions

All anthracyclines were kindly donated by Farmitalia Carlo Erba (Freiburg, Germany). Methanol, acetonitrile, water (all HPLC-grade) and triethylamine, trichloroacetic acid, orthophosphoric acid (85%, v/v) and potassium dihydrogen phosphate (all analytical grade) were obtained from E. Merck (Darmstadt, Germany).

Stock solutions of epirubicin and metabolites in methanol were prepared, aliquoted and stored at −20°C, protected from light. Working standards of all anthracyclines were derived from the stock solutions by diluting aliquots with aqueous buffered solution (potassium dihydrogen phosphate 10 mM, pH 2.0 adjusted with ortho-phosphoric acid), covering the following concentration ranges: epirubicin 3.1–414.4 ng ml^{-1}; epirubicinol 4.1–205.8 ng ml^{-1}; epirubicin-aglycon 2.8–140.7 ng ml^{-1}; epirubicinol-aglycon 3.2–161.5 ng ml^{-1}; 7-deoxy-epirubicinol-aglycon 4.0–207.9 ng ml^{-1}; and stored at 4°C, protected from light. At pH 2.0, no adsorption of the anthracyclines on glassware and polypropylene tubes, as described for other pH values [29, 30], was observed. All working standards were stable under these storage conditions for at least 2 months.

Sampling

Human blood (2 ml) was collected into EDTA-tubes (Primavette, B. Braun Melsungen, Germany) from the vena cephalica of patients and centrifuged immediately for 5 min at 4000*g*. The plasma fraction obtained was stored at −20°C until analysis. At this temperature samples were stable for a period of at least 1 month.

For analysis, plasma was allowed to thaw at room temperature, centrifuged again for 3 min (4000*g*) and an aliquot transferred into an autosampler glass vial. Vials were thermostated at 4°C by the autosampler sample rack.

Human tumour and liver tissue were weighed, homogenized for about 2 min by an UltraTurrax T25 homogenizer (Janke und Kunkel, Staufen, Germany) at 13 500 rpm in a 1:5 (weight:volume) ratio with water–methanol (95:5, v/v). After centrifugation for 2 min at 3200*g*, the supernatant was transferred to another tube, centrifuged again for 2 min at 4000*g*. The resulting supernatant was diluted in a 1:10 (weight:volume) ratio with the water–methanol mixture above and analysed as described for plasma samples.

Apparatus

The chromatographic system (instrumental set-up see Fig. 2) consisted of an E. Merck (Darmstadt, Germany) model L-6200 gradient pump (P1), a L-6200A gradient pump (P2), an AS-2000 autosampler (AS), a F-1050 fluorescence detector (FD), a D-2500 integrator, a Krannich (Göttingen, Germany) model ELV 7000 automatic switching valve (ASV), an analytical column LiChrospher 60 RP-Select B, 5 μm, 250 × 4 mm i.d. (E. Merck, Darmstadt, Germany) (AC) and a dry-packed pre-

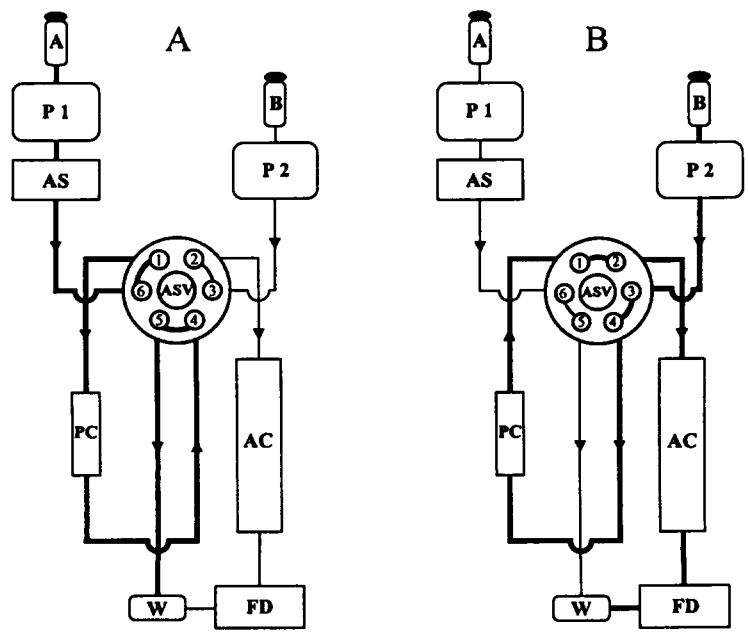

Figure 2
Instrumental set-up and flow diagram for coupled-column analysis. (A) Valve position for precolumn loading step (LOAD); (B) valve position for transfer step (INJECT). W = waste, P = pump, AS = autosampler, ASV = automatic switching valve, PC = precolumn, AC = analytical column, FD = fluorescence detector.

column C_4-Alkyl-Diol Silica, 25 μm, 20 × 4 mm i.d. (PC).

Alkyl-Diol Silica (ADS)

Alkyl-Diol Silica is a new family of chemically and enzymatically modified precolumn packing materials for bioanalytical LC belonging to the group of restricted access supports [26–28]. In accordance with the internal-surface reversed-phase materials introduced by Pinkerton *et al.* in 1985 [31], ADS is characterized by bimodal chromatographic properties, i.e. different bonded phases at the external (particle) and internal (pore) surface. The external bonded phase (glycerylpropyl-groups) is hydrophilic, electroneutral and non-adsorptive towards proteins, whereas the n-alkyl chains at the internal surface allow classical RP-chromatography of small solutes by hydrophobic interactions. As the pore size of ADS is 5 nm, the molecular weight cut-off of ADS is approximately 15000 Dalton. Thus macromolecules such as proteins (e.g. albumin: $M_r \approx 65\,000$ Dalton) are size-excluded and quantitatively eliminated in the interstitial void volume.

An ADS precolumn (25 × 4 mm i.d.) packed with the octanoyl-derivatized material was previously shown to tolerate over 200 analysis cycles (500 μl plasma samples) [27].

Chromatography

The sample (injection volume: 50 μl) is loaded to the C_4-ADS precolumn by pump 1 which delivers eluent A (water–methanol, 95:5, v/v) at a flow rate of 1 ml min^{-1}. A mobile phase of acetonitrile–water (0.1% triethylamine, v/v, pH 2.0 adjusted with trichloroacetic acid), 30:70, v/v, was delivered by pump 2 at a flow rate of 1 ml min^{-1} (eluent B). Analytes were detected by their natural fluorescence with the excitation and emission wavelengths set at 445 and 560 nm, respectively.

Calibration

The working standards of epirubicin and metabolites were analysed in the coupled-column mode to obtain the calibration curve by calculating the peak areas. When linearity was confirmed an external three-point calibration was used for quantification of drug concentrations in the plasma samples.

Chemoembolization

TACE was performed and epirubicin levels were monitored for a total of 19 patients with primary HCC (33 chemoembolizations). Embolizing materials were either Lipiodol® Ultra-Fluid (Byk Gulden, Konstanz, Germany), an iodized poppyseed oil (iod content 480 mg ml^{-1}) usually used as a lymphographic contrast medium, or Spherex® (Kabi Pharmacia, Erlangen, Germany), a sterile suspension of degradable starch microspheres (DSM) with a mean particle diameter of 45 μm. Epirubicin (Farmorubicin®, Farmitalia Carlo Erba, Freiburg, Germany) was administered as a standardized dose of 1 mg kg^{-1} body weight either as an aqueous suspension with Spherex (ratios ranging from 1:5 to 1:10, w/w) or as a W/O-emulsion with Lipiodol. For the latter, epirubicin, dissolved in 1 ml aqua ad injectabilia, was emulsified in a 10:1 ratio (mg substance/ml oil). Mixtures were selectively injected in the arteria hepatica dextra and sinistra via a catheter inserted into the femoral artery by Seldinger's method. Blood samples were drawn at 0, 15, 30, 45, 60 min and 3, 5, 15, 21, 27, 39, 45 and 63 h after injection. Pharmacokinetic data were calculated from epirubicin and metabolites plasma levels by means of the computer program RSTRIP II, version 2.02 (MicroMath Scientific Software, Salt Lake City, USA).

Results and Discussion

Analysis cycle

The coupled-column analysis cycle of the determination of epirubicin and its metabolites can be subdivided into three different phases (Table 1).

Sample loading. The sample (standard sol-

Table 1
Timetable for switching-valve positions

Time (min)	Position	Precolumn	Analytical column
0.0–10.0	LOAD	Sample loading	(Re)conditioning
10.0–15.0	INJECT	Transfer	Transfer, separation
15.0–25.0	LOAD	Reconditioning	Separation

ution, plasma or supernatant of tissue homogenates) is loaded with eluent A via the autosampler through valve positions 6–1 onto the C_4-ADS precolumn. While quantitatively eliminating the sample matrix (proteins and other interfering compounds which are not retained by the butyryl-ligands under the selected conditions) for a duration of 10 min, the anthracyclines are simultaneously enriched on top of the precolumn by hydrophobic partitioning. During this precolumn sample processing step, the analytical column is equilibrated with eluent B delivered by pump 2.

Analyte(s) transfer. Switching the automatic valve into position INJECT, eluent B is delivered through valve connections 3–4 to the precolumn (backflush mode). The retained anthracyclines are eluted from the precolumn and transferred in a narrow elution band through valve positions 1–2 to the analytical column. After 5 min the valve is switched back into position LOAD.

Separation. While the analytes are separated on the analytical column under isocratic conditions by eluent B, the precolumn is reconditioned with eluent A for the next sample injection. As the second sample can be loaded onto the precolumn and extracted simultaneously to the first sample being separated on the analytical column, analysis cycles can be shortened. Thus sample throughput is increased by 30%.

In Fig. 3 chromatograms of an aqueous standard solution of epirubicin and metabolites, a human plasma blank and a plasma sample of a patient 15 min after chemoembolization are shown.

Validation

The coupled-column method was validated with respect to the following parameters.

Linearity. Good linearity between peak areas and concentrations in plasma samples spiked with known amounts of epirubicin and metabolites (ranges see Table 2) was obtained ($r = 0.999$).

Recovery. The recoveries of epirubicin from spiked plasma samples were calculated by comparing peak areas in the chromatograms of the samples with those of aqueous solutions (buffer pH 2.0) with identical concentrations. Recoveries ranged from 98 to 106% (Table 3). These values demonstrate the excellent efficiency of the ADS precolumn. Such high

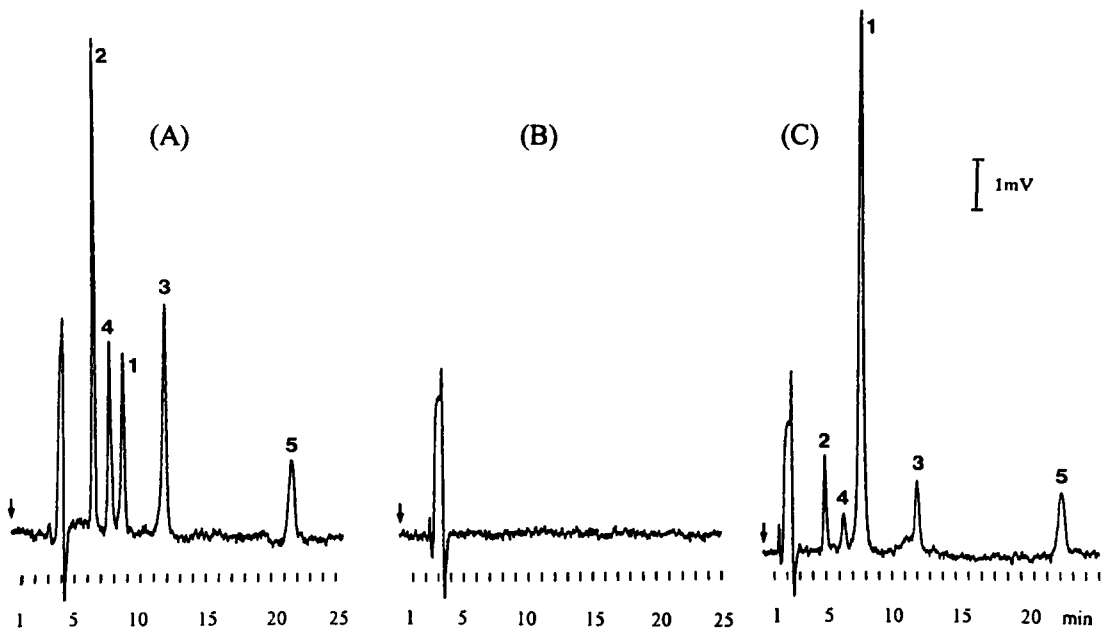

Figure 3
Coupled-column analysis of epirubicin and metabolites (1 = epirubicin, 2 = epirubicinol, 3 = epirubicin-aglycon, 4 = epirubicinol-aglycon, 5 = 7-deoxy-epirubicinol-aglycon). (A) 50 µl of aqueous standard solution (1, 2, 3, 4, 5 = 12.4, 16.4, 11.2, 12.8, 16.0 ng ml^{-1}); (B) 50 µl of human plasma; (C) 50 µl of patient's plasma 15 min after chemoembolization (1, 2, 3, 4, 5 = 40.6, 3.8, 5.3, 2.8, 7.1 ng ml^{-1}).

Table 2
Linearity (injection volume: 50 μl)

	Range (ng ml^{-1})	r
Epirubicin	0.5–620	0.9995
Epirubicinol	0.5–205	0.9993
Epirubicin-aglycon	0.5–410	0.9997
Epirubicinol-aglycon	2.4–480	0.9989
7-Deoxy-epirubicinol-aglycon	2.0–800	0.9995

Table 3
Recovery of epirubicin from plasma samples (injection volume: 50 μl)

Conc. epirubicin (ng ml^{-1})	Recovery (%)	RSD (n = 5) (%)
9.69	102.6	4.6
19.38	103.8	1.4
38.75	103.8	1.4
77.50	98.3	1.7
155.00	105.9	0.7

yields have not been achieved with the sample preparation procedures reported earlier [3, 4, 6, 9, 12, 13].

As the recovery from plasma is quantitative, addition of an internal standard to control the analyte recovery is not necessary.

Precision. The within-day precision of the method, as indicated by the relative standard deviations (RSDs), was studied by repeating the analysis of three plasma samples spiked with varying amounts of epirubicin and metabolites ten times within one day. The day-to-day RSD was calculated from the daily mean of duplicate analyses of plasma samples containing the analytes at the same concentrations as for the within-day study, analysed over ten consecutive days. All RSD values were less than 4% for all compounds (Table 4) except for the within-day RSD for 7-deoxy-epirubicinol-aglycon. Within-day RSD did not remarkably differ from day-to-day RSD.

Limit of detection/limit of quantification. The limits of detection (LOD) and quantification (LOQ) of the method for the parent drug and metabolites were determined according to [32] and are shown in Table 5. Additionally, LOQ values were experimentally proven by analysis of spiked plasma samples containing the LOQ amounts of the anthracyclines (Table 5).

Comparison of peak area in coupled-column and single-column mode. In order to control the accuracy of the coupled-column system, aqueous solutions of 7 different concentrations (0.5–434 ng ml^{-1}) of epirubicin were analysed in duplicate via the coupled-column mode and via direct injection onto the analytical column. The linear regression equation of peak areas for single-column vs coupled-column mode was $y = 1.034 \times -920.85$ ($r = 0.9999$), whereby areas ranged from 11 000 to 6.8×10^6.

Pharmacokinetics

Monitoring epirubicin plasma levels revealed that a certain amount of the cytostatic reached the circulation because the blood flow of the hepatic artery did not stop immediately

Table 4
Within-day and day-to-day precision (injection volume: 50 μl)

	RSD within-day (n = 10) (%)	RSD day-to-day (n = 10) (%)
Epirubicin	1.43	1.11
Epirubicinol	2.42	2.52
Epirubicin-aglycon	1.27	1.66
Epirubicinol-aglycon	3.49	3.71
7-Deoxy-epirubicinol-aglycon	6.80	3.76

Table 5
Limits of detection/limits of quantification (injection volume: 50 μl)

	LOD (pg on column)	LOQ (pg on column)	RSD (n = 10) (%)
Epirubicin	25	50	3.2
Epirubicinol	23	51	7.5
Epirubicin-aglycon	31	53	9.2
Epirubicinol-aglycon	65	120	13.1
7-Deoxy-epirubicinol-aglycon	93	200	9.6

after administration of the chemoembolizing mixture. In most cases, a triexponential decrease in the plasma levels of the unaltered drug with a long terminal plasma half-life was observed as described for i.v.-bolus injection of epirubicin [33]. This disposition is interpreted in terms of the classical multicompartment model, the high-capacity deep compartment of which is responsible for the slow elimination of the drug. In accordance with the literature [34], large intra- and interindividual differences could be seen in the mean c_{max}- and AUC-values of all 19 patients receiving Lipiodol.

$$c_{max} = 456.1 \pm 367.2 \text{ ng ml}^{-1} \quad (n = 22)$$

$$\text{AUC}[t_0 \rightarrow t_\infty] = 482.4 \pm 258.6 \text{ ng ml}^{-1} \text{ h}^{-1} \quad (n = 16).$$

The 4'-glucuronide conjugates of epirubicin and epirubicinol can also be analysed under the conditions presented in this paper. As none of the patients showed significant concentrations of these metabolites at any time after treatment they were not taken into account for method validation and study evaluation.

In 24 of 26 evaluable metabolite profiles epirubicin-aglycon and not epirubicinol as reported [35] was found to be the main metabolite. Calculated curves fit closely to experimental drug concentrations within the time interval investigated (0 to about 70 h). Representative plasma profiles of parent drug and metabolites are shown in Fig. 4.

In order to examine the properties of both Lipiodol and Spherex, nine patients out of the collective received two to four TACE treatments, alternatingly in iodized oil or in DSM (22 chemoembolizations in total). Mean c_{max}- and AUC-values are presented below.

$$\text{Lipiodol: } c_{max} = 420.5 \pm 312.4 \text{ ng ml}^{-1}$$

$$\text{AUC}[t_0 \rightarrow t_\infty] = 421.5 \pm 282.4 \text{ ng ml}^{-1} \text{ h}^{-1} \quad (n = 11)$$

$$\text{Spherex: } c_{max} = 1018.55 \pm 679.4 \text{ ng ml}^{-1}$$

$$\text{AUC}[t_0 \rightarrow t_\infty] = 476.3 \pm 179.1 \text{ ng ml}^{-1} \text{ h}^{-1} \quad (n = 11).$$

Comparing the AUC-values after chemoembolization with those after i.v.-infusion therapy (1305 ng ml^{-1} h^{-1}) [36] shows that

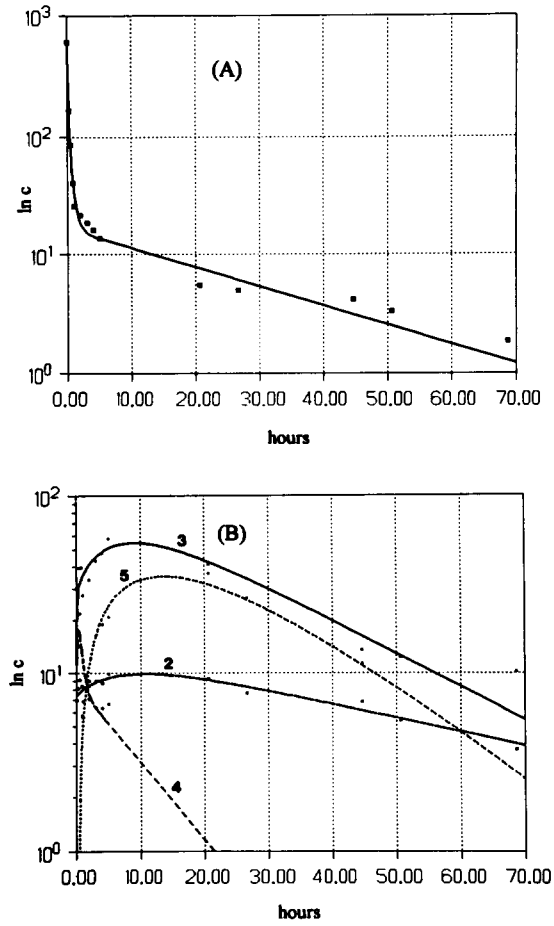

Figure 4
Plasma profiles of epirubicin and metabolites after chemoembolization. Transcatheter injection of a mixture of 52 mg epirubicin in 5.2 ml Lipiodol. (A) Kinetics of epirubicin; (B) kinetics of metabolites (2 = epirubicinol, 3 = epirubicin-aglycon, 4 = epirubicinol-aglycon, 5 = 7-deoxy-epirubicinol-aglycon).

TACE reduces the systemic availability of epirubicin.

The oily contrast medium Lipiodol has been reported to be selectively incorporated in liver tumour cells [37–39]. Therefore, it might function as a targeting agent for cytocoxic drugs to their site of action, thus increasing drug concentration in the tumour cell and improving efficacy of antineoplastic treatment.

In our study, seven patients had a lower Lipiodol-c_{max} than Spherex-c_{max}. In one of the two remaining cases, Lipiodol-c_{max} exceeded Spherex-c_{max}, but in comparison to the other patients Spherex-c_{max} was also elevated. The high c_{max}-values in both treatments were due to arteriovenous shunts as documented by

angiography. Through these shunts the epirubicin-formulation in part reached the venous system and therefore the circulation. The inverse ratio of c_{max}-values is explained by the faster occlusion of Spherex because of its particulate structure.

The key point of chemoembolization therapy is to make the anticancer drug selectively retainable at a high concentration in tumour tissue for a long time. From normal liver parenchyma Lipiodol is removed within several days [40] by the reticulo-endothelial system (e.g. Kupffer cells) which lacks in tumour cells. Examination of liver tissue and liver tumour tissue of the same patient being resected 8 weeks after Lipiodol-chemoembolization revealed that epirubicin was still present in the tumour tissue [2.5 ng ml^{-1} protein; c.f. Fig. 5] but not in the normal liver tissue. This fact suggests that the cytostatic is stored for a longer time in the tumour due to the presence and carrier effect of Lipiodol. The corresponding chromatograms are presented in Fig. 5.

Conclusions

Analytical method

This paper describes the application of the novel precolumn packings Alkyl-Diol Silica for LC-integrated sample processing in a coupled-column HPLC system. The method allows the direct and repetitive injection of untreated biological samples as well as the separation and trace analysis (lower ppb level) of drugs and their metabolites on a routine basis. No manual sample pretreatment steps except for centrifugation are required and the contact time with the biological fluid and thus danger of contamination (e.g. HCC patients often are hepatitis-B positive) is minimized. Compared to the conventional methods described, precision as well as accuracy are improved and an internal standard is no longer necessary. Finally, the chromatographic properties of the C_4-ADS precolumn packing allow the performance of at least 500 analysis cycles when injecting and processing 50-µl plasma samples.

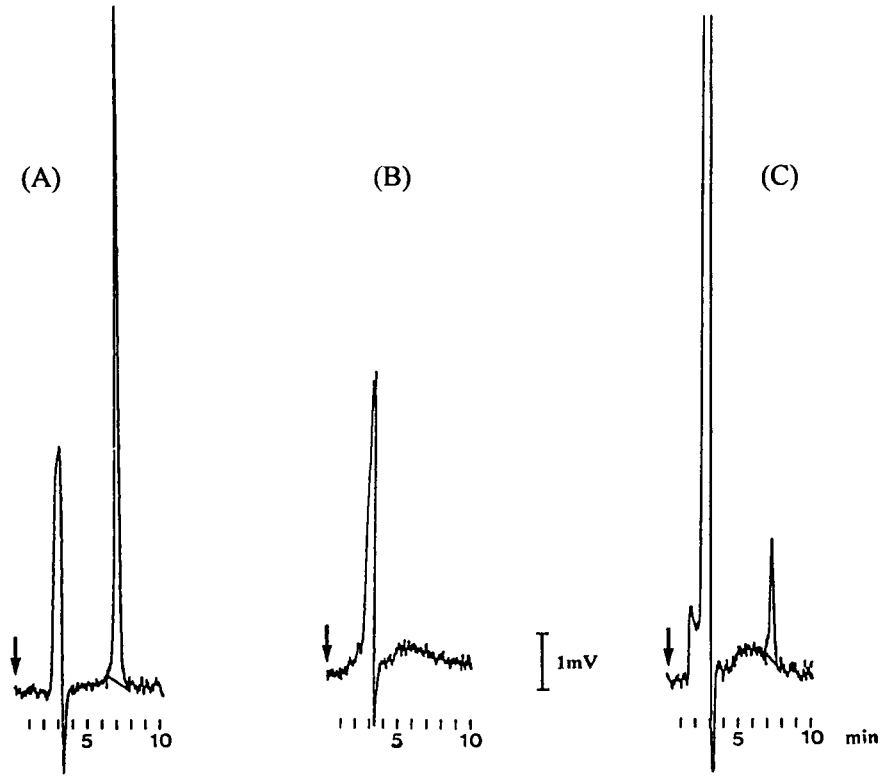

Figure 5
Coupled-column analysis of liver tissue 8 weeks after Lipiodol-TACE. (A) 50 µl of aqueous epirubicin standard solution (31.0 ng ml^{-1}); (B) 50 µl liver homogenate supernatant (protein 2.07 mg ml^{-1}); (C) 50 µl tumor homogenate supernatant (protein 1.34 mg ml^{-1}, epirubicin: 2.5 ng mg^{-1} protein).

Therapy optimization

As shown by the pharmacokinetic data, a lower systemic epirubicin plasma level is present (lower c_{max}-values) when applying Lipiodol instead of Spherex as the occlusive agent. This fact and the presence of epirubicin in tumour tissue even 8 weeks after Lipiodol-chemoembolization suggest that Lipiodol serves as a carrier for the uptake of epirubicin in the tumour cell and causes a prolonged residence time of the cytostatic in the tumour compared to normal liver tissue. This drug targeting effect favours Lipiodol as occlusive agent for chemoembolization therapy of HCC.

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