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A validated assay for measuring doxorubicin in biological fluids and tissues in an isolated lung perfusion model: matrix effect and heparin interference strongly influence doxorubicin measurements

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Abstract

Doxorubicin is an antineoplasic agent active against sarcoma pulmonary metastasis, but its clinical use is hampered by its myelotoxicity and its cumulative cardiotoxicity, when administered systemically. This limitation may be circumvented using the isolated lung perfusion (ILP) approach, wherein a therapeutic agent is infused locoregionally after vascular isolation of the lung. The influence of the mode of infusion (anterograde (AG): through the pulmonary artery (PA); retrograde (RG): through the pulmonary vein (PV)) on doxorubicin pharmacokinetics and lung distribution was unknown. Therefore, a simple, rapid and sensitive high-performance liquid chromatography method has been developed to quantify doxorubicin in four different biological matrices (infusion effluent, serum, tissues with low or high levels of doxorubicin). The related compound daunorubicin was used as internal standard (I.S.). Following a single-step protein precipitation of 500 μl samples with 250 μl acetone and 50 μl zinc sulfate 70% aqueous solution, the obtained supernatant was evaporated to dryness at 60 °C for exactly 45 min under a stream of nitrogen and the solid residue was solubilized in 200 μl of purified water. A 100 μl-volume was subjected to HPLC analysis onto a Nucleosil 100–5 μm C18 AB column equipped with a guard column (Nucleosil 100–5 μm C_6H_5 (phenyl) end-capped) using a gradient elution of acetonitrile and 1-heptanesulfonic acid 0.2% pH 4: 15/85 at 0 min → 50/50 at 20 min → 100/0 at 22 min → 15/85 at 24 min → 15/85 at 26 min, delivered at 1 ml/min. The analytes were detected by fluorescence detection with excitation and emission wavelength set at 480 and 550 nm, respectively. The calibration curves were linear over the range of 2–1000 ng/ml for effluent and plasma matrices, and 0.1 μg/g–750 μg/g for tissues matrices. The method is precise with inter-day and intra-day relative standard deviation within 0.5 and 6.7% and accurate with inter-day and intra-day deviations between −5.4 and +7.7%. The in vitro stability in all matrices and in processed samples has been studied at −80 °C for 1 month, and at 4 °C for 48 h, respectively. During initial studies, heparin used as anticoagulant

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was found to profoundly influence the measurements of doxorubicin in effluents collected from animals under ILP. Moreover, the strong matrix effect observed with tissues samples indicate that it is mandatory to prepare doxorubicin calibration standard samples in biological matrices which would reflect at best the composition of samples to be analyzed. This method was successfully applied in animal studies for the analysis of effluent, serum and tissue samples collected from pigs and rats undergoing ILP.
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1. Introduction

Doxorubicin (Fig. 1) is an antitumor anthracycline antibiotic first isolated in *Streptomyces peucetius* var. *caesius* more than 30 years ago [1].

Since then, despite of its extensive clinical use in multiple malignancies, doxorubicin's mechanisms of action remain uncertain and complex. Doxorubicin intercalates between adjacent DNA base pairs, which interferes with DNA strand separation and inhibits DNA topoisomerase II, as well as DNA and RNA polymerases and helicase activities, consequently inhibiting DNA replication and transcription, which ultimately induces DNA fragmentation. Conversely, doxorubicin-mediated free radical formation and lipid peroxidation, which produce direct membrane damage, are more directly responsible for the cardiotoxic effects of doxorubicin than its antitumor effects [2–4]. Doxorubicin is an essential component, as a single agent or in combination regimen, of the treatment of numerous malignancies such as non-Hodgkin's lymphoma, breast cancer, osteosarcomas and soft tissue sarcomas [4].

Soft tissue sarcomas are rare tumors accounting for approximately 1% of all adult cancers [5]. They mostly metastasize in lungs even when the primary tumor is controlled locally. The median survival from the time of metastatic disease's diagnosis is about 8–12 months [6]. Only a few drugs have shown as single-agent an activity above 15–20%, among them ifosphamide, DTIC and the anthracyclic drugs doxorubicin and epirubicin [6]. Nowadays, the combination regimens using the most active single agents at present give response rates in the range of 40% [6]. Interestingly, O'Bryan et al. have demonstrated in a dose–response study that the response rates of sarcomas raised with the total dose of doxorubicin administered as a single agent [7]. However, the use of high doses of doxorubicin in patients is hampered by a dose-dependant myelosuppression and by the risk of an irreversible cardiotoxicity occurring above a cumulative life-time dose of 450–550 mg/m^2 [3,4].

Consequently, isolated lung perfusion (ILP) of doxorubicin may be a promising approach for the treatment of metastases localized in lung tissues which are surgically unresecable and unresponsive to conventional chemotherapy. This approach involves a vascular isolation of the lung, enabling to deliver an agent locoregionally, circumventing the systemic exposure of the organism to high drug

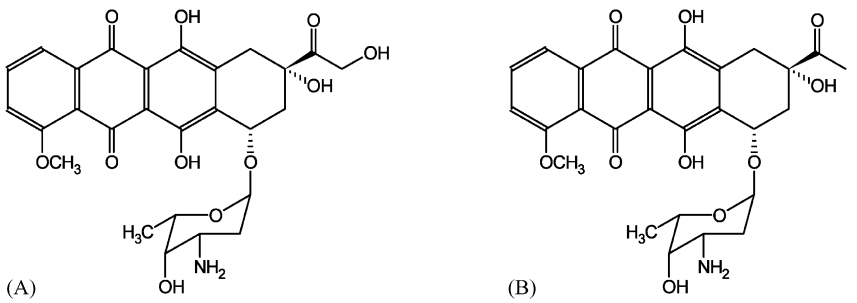

Fig. 1. Structure of (A) doxorubicin and (B) daunorubicin (I.S.).

levels [8]. Indeed, ILP of doxorubicin has been demonstrated to reduce the cardiotoxicity and the hematologic toxicity observed when doxorubicin is administered systematically [9]. However, despite encouraging results in preclinical studies [10,11], there was only modest tumor response to ILP with doxorubicin or other agents in human studies [12–15]. The reasons for the poor responses observed in the clinical setting are still unclear. Considering that the bronchial territories (irrigated by the bronchial artery) are not perfused with the anterograde (AG) mode (i.e. perfusion through the pulmonary artery (PA)), it has been hypothesized that a non homogenous distribution of the drug inside the lung could be at the origin of this failure. A retrograde (RG) perfusion mode (i.e. perfusion through the pulmonary vein (PV), which drains the pulmonary, and the bronchial artery) could presumably improve drug distribution. To that end, the Thoracic Surgery Department at CHUV has initiated a research program for optimizing the ILP technique [16], using two animal models, the pig and the rat, to study in detail the influence of the mode of administration on doxorubicin pharmacokinetics, compartmental drug distribution in lung tissue, systemic leakage, perfusion related lung injury, extra-pulmonary toxicity and anticancer activity. Therefore, the development of a simple and sensitive assay for measuring doxorubicin in various biological matrices (plasma, effluent and tissues) was required.

Various methods for the quantification of doxorubicin in biological fluids have been reviewed recently [17,18]. The sample pretreatment can be performed by solid-phase extraction [19–21] or liquid–liquid extraction procedures [22,23]. Since a large number of samples were to be analyzed in our study, a fast, simple and economic sample pre-treatment such as a single-step protein precipitation was chosen [24–28]. Protein precipitation with $ZnSO_4$ and acetone has been previously proposed for plasma samples [24], but had to be adapted for all the biological matrices considered in our investigation, such as serum, ILP effluents and tissue samples.

Reversed-phase liquid chromatography coupled with fluorescence detection is the method of choice for the assay of doxorubicin [20–28], even though alternate detection such as electochemical [19], UV spectrophotometric [29,30] detection, or mass spectrometry [21] have been used. However, fluorescence detection is probably the most appropriate detection method for doxorubicin, considering its simplicity of use, selectivity and sensibility.

During the feasibility study with rats, some spurious analytical results were observed which finally proved to be due to an interference between doxorubicin and heparin, used as anticoagulant during effluent sample collection in our first series of animal experiments. Considering that some published methods have used heparin as an anticoagulant agent for sample collection [19,23,24], or did not report which type of anticoagulant was used [22], our initial observations of this phenomenon prompted us to examine the importance of this interference, and part of this report is consequently devoted to study the influence of this interaction heparin–doxorubicin, highlighting a potential critical problem in the analytical method development of an assay for doxorubicin.

2. Experimental

2.1. Chemicals

Doxorubicin HCl, (8-hydroxyacetyl (8S,10S)-10-[(3-amino-2.3.6-trideoxy-a-L-lyxo-hexopyranosyl)oxy]-6.8.11-trihydroxy-1-methoxy-7,8,9,10-tetrahydronaphtacene-5,12-dione hydrochloride), was generously supplied as solution ready for use (2000 μg/ml) by Pharmacia & Upjohn (Dübendorf, Switzerland). Daunorubicin was purchased from Aventis Pharma (Zürich, Switzerland). Potassium dihydrogenphosphate p.a., Zinc sulfate heptahydrate and acetic acid (glacial) 100% GR for analysis were obtained from E. Merck (Darmstadt, Germany). 1-heptanesulfonic acid sodium salt monohydrate was purchased from Fluka (Buchs, Switzerland) and acetonitrile from Mallinckrodt Baker (Deventer, Holland). Purified water was obtained from a Milli-Q® UF-Plus apparatus (Millipore, Bedford, USA) and acetone (number article 010400, Schweizerhall Chimie SA, Switzerland).

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett–Packard 1050 (Hewlett–Packard, Germany) connected to an HP 1050 online degazer, an HP 1050 autosampler, an HP 35900 interface and a LC 240 fluorescence detector (Perkin–Elmer, Beaconsfield, UK) with excitation and emission wavelengths set at 482 and 550 nm, respectively. The attenuation factor of the spectrofluorimeter was set at 256. The software HPCHEMSTATION A.06.03 (Hewlett–Packard 1990–1998) loaded on a Compac Desk Pro was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation and samples analysis.

The separations were performed at room temperature on a ChromCart® cartridge column (125 × 4 mm I.D.) Nucleosil 100–5 μm C18 AB (Macherey-Nagel, Oensingen, Switzerland) equipped with a guard column (8 × 3 mm I.D.) Nucleosil 100–5 μm C_6H_5 (Phenyl) end-capped (Macherey-Nagel). The injection volume was 100 μl and the mobile phase was delivered at 1 ml/min, with the following step-wise gradient elution program: acetonitrile/1-heptanesulfonic acid 0.2% pH 4; 15/85 at 0 min → 50/50 at 20 min → 100/0 at 22 min → 15/85 at 24 min → 15/85 at 26 min (the 24–26 min elution corresponds to the re-equilibration step). The 1-heptanesulfonic acid 0.2% solution pH 4 was prepared by dissolving 2.2 g of 1-heptanesulfonic acid sodium salt monohydrate in 1000 ml of purified water. The pH was adjusted to 4.00 with acetic acid 0.1 M.

2.3. Stock solution, calibration and control standards

2.3.1. Stock solutions

Four standard stock solutions (I, II, III and IV) containing, respectively, 100, 50, 10 and 0.1 μg/ml of doxorubicin were freshly prepared by diluting the 2000 μg/ml-doxorubicin mother solution with purified water. These standard stock solutions were used to prepare the calibrations and control standards in the respective biological matrix. The internal standard (I.S.) daunorubicin (100 μg/ml in ultrapure water) was stocked at −80 °C as 1 ml aliquots and diluted with purified water at concentration of 2.5 μg/ml prior to sample processing.

2.3.2. Calibration and control samples in "effluent" solution

Effluent calibration standards were prepared at concentrations of 2, 100, 400, 700, 1000 ng/ml along with control standards at concentrations of 50, 350, 750 ng/ml. An appropriate volume of the respective stock solution was added to 50 μl of citrated plasma and diluted with purified water in batches of 10 ml (total plasma content 1:200). This preparation procedure enables to obtain calibration samples in a matrix similar to that encountered in our animal study during the locoregional perfusion (i.e. "effluent solution", see below). Calibration standards and control samples were stored as 500 μl aliquots in polypropylene Eppendorf tubes at −80 °C until use and thawed the day of analysis.

2.3.3. Plasma calibration and control samples

Plasma calibration samples were prepared at concentrations of 2, 100, 400, 700, 1000 ng/ml, along with control standards at concentrations of 50, 350, 750 ng/ml, by diluting the appropriate volume of stock solutions with human plasma, collected from Vaquez diseases patients, at the occasion of their regular phlebotomy. Calibration and control samples were prepared in batches of 10 ml, and stored as 500 μl aliquots in polypropylene Eppendorf tubes at −80 °C until use and thawed the day of analysis.

2.3.4. Homogeneized tissue suspension A and B

Lung tissues specimens were obtained from untreated white pigs subjected to experimental surgery, taken after the sacrifice of animals, and were kept frozen at −20 °C. Aliquots of lung tissue samples (1 and 0.2 g, Samples A and B, respectively) were individually introduced into 6 ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) and homogenized for 90 s using a tissue tearor, (speed set at 3, Model 985370, Biospec Products, Breda, The Netherlands) in 3.8 and 2 ml, respectively, of a KH_2PO_4 0.2 mM pH 3.8 solution. The homogenized tissue suspension, desig-

nated tissue suspension A (3.8 ml), contained 0.25 g tissue per ml of suspension, and was directly used for the preparation of calibration and control standards in lung tissue, by adding appropriate volumes of doxorubicin stock solution. The second tissue suspension (2 ml, tissue suspension B) was further diluted to 38 ml with the KH_2PO_4 0.2 mM pH 3.8 solution and aliquoted as batches of 3.8 ml. After dilution, the tissue suspension B contains 0.005 g tissue per ml of suspension.

2.3.5. Calibration and control standard in tissue suspension A matrix (with low level of doxorubicin)

Calibration standards samples with low tissular levels of doxorubicin, were prepared by adding appropriate volume of doxorubicin stock solution into the homogenized tissue suspension A to obtain samples containing doxorubicin at concentrations of 0.1, 2, 5, 10, 15 μg doxorubicin per gram of tissue in the homogenates, along with control samples at concentrations of 2.5, 6.5, 12.5 μg/g tissue. Calibration standards and control samples were prepared as 4 ml batch homogenates, and stored as 500 μl aliquots in polypropylene Eppendorf tubes at −80 °C until use and thawed the day of analysis.

2.3.6. Calibration and control standard in tissue suspension B matrix (with high level of doxorubicin)

Calibration standard were similarly prepared using the tissue suspension B at concentrations of 5, 100, 250, 500, 750 μg per gram of tissue, along with control samples at concentrations 125, 325, 625 μg/g of tissue in the homogenates.

2.4. Experimental animals

White pigs (25–30 kg) were bred by Eric Pavillard (Orny (Geneva), Switzerland) and male Fisher rats (150–300 g) were obtained from Charles River (Sultzfeld, Germany). Animal experiments were approved the 1.2.2000 by the Competent Veterinary Authority and the experiments were conducted in compliance with the Swiss regulations (art. 13a de la loi fédérale sur la protection des animaux, LPA, et art. 60 à 62 de l'ordonnance fédérale sur la protection des animaux, OPAn).

2.4.1. Standard operating procedures for anterograde and retrograde isolated lung perfusion (ILP)

2.4.1.1. ILP in white pigs. After induction of anesthesia, the lungs were ventilated with a volume controlled ventilator. The chest was opened and the left Pulmonary artery (PA) and the left superior and inferior Pulmonary vein (PV) were clamped following intravenous administration of heparin (100 UI/kg). For AG ILP, a right angled cannula was introduced into the left PA. For RG ILP, the inferior and superior PV were separately cannulated with two cannulas that were joined through a Y-connector. A 500 ml-solution of doxorubicin at 320 μg/ml (total dose 160 mg) in buffered HAES 6% (HydroxyEthyl Amidon Solution, hydroxyethyl starch), or two solutions of 250 ml were administered by gravity infusion during 20 min through the PA (AG ILP) or through the two PV (RG ILP), respectively. Dual ILP consisted in 10 min perfusion with the AG mode, followed by 10 min perfusion with the RG mode. The outcoming perfusate, called effluent, was drained through a cannula placed at the PV or PA for AG ILP, and RG ILP, respectively. At the end of infusion, the anesthesized animal was sacrificed and the perfused and contra-lateral unperfused lungs, heart, kidney, liver, fat, muscle tissue samples were harvested for the measurement of doxorubicin tissular levels.

2.4.1.2. ILP in male Fisher rats. Fisher rats were intubated after induction of anesthesia and were connected to a standard rodent ventilator (Harvard apparatus, Inc., Model 683). First an arterial line was placed in the right carotid artery. A left sided thoracotomy was then performed and a small retractor was introduced. The PA and PV were encircled by use of sutures and clamped at their base. The PA was cannulated for AG ILP and the PV was cannulated for RG ILP. Single-pass AG or RG ILP of 5.0 ml of doxorubicin at the concentration of 20, 80 and 320 μg/ml (total dose: 100, 400 and 1600 μg, respectively) in

buffered HAES 6% was infused at a flow-rate of 0.25 ml/min during 20 min, followed by a 20 min-washing period with buffered HAES 6%. The effluent was drained by an arteriotomy for RG and a venotomy for AG ILP, respectively, and was collected through a cannula every 2 min. After completion of ILP, the cannulas were removed and the pulmonary artery or vein was repaired. After restoration of the pulmonary circulation, peripherical blood was taken from the arterial line placed in the right carotid at predetermined times for 1 h. The anesthesized animal was then sacrificed, and the perfused and contra-lateral unperfused lungs, the heart, the chest wall, the mediastinum, and the liver were collected for doxorubicin assay in tissue.

2.5. Sample collection

2.5.1. Blood and effluent samples

Blood and effluent samples were collected from pigs and rats during ILP experiments. The samples were collected in polypropylene Eppendorff tubes into which no anticoagulant was added and were stored on ice before centrifugation within 2 h at 1500 g for 10 min at $+4\,^{\circ}C$. The serum and the effluent were immediately frozen at $-80\,^{\circ}C$ in 2 ml microtubes from Sarstedt until analysis.

2.5.2. Tissue samples

At the end of the ILP experiments, animals were sacrificed and the following tissues were taken: lung, heart, mediastinum, liver, chest wall muscles, and fat. The samples were stored on ice before being frozen within 30 min at $-80\,^{\circ}C$.

2.6. Sample processing

2.6.1. Serum and effluent samples processing

Frozen samples were thawed and homogenized by vortex. First experiments indicated that effluent samples had expectedly doxorubicin levels much higher than the concentration range of the calibration curve into which the response signal of the fluorescence detector was linear. Thus, samples were diluted with purified water according to the calculated concentration of the ILP perfusion. An appropriate volume of citrated plasma corresponding to 2.5 μl of the final 500 μl sample (1:200) was added to all effluent samples before dilution, in order to standardize at best the heterogeneous biological matrix. The volume of serum obtained with the rat experiments was sometimes smaller than the 500 μl-volume required for processing, and these were, therefore, made up to this volume with citrated plasma. To 500 μl of the sample, 100 μl of I.S. daunorubicin solution (2.5 μg/ml in water) was added prior to the protein precipitation step. The tubes were briefly vortex-mixed (5 s) and 250 μl of acetone, and 50 μl of a zinc sulfate 70% aqueous solution were added prior being vortex-mixed for another 5 s. Samples were centrifuged at $22\,000 \times g$ for 10 min at $+4\,^{\circ}C$. The supernatant was transferred into a 5 ml polypropylene tube and evaporated to dryness at $60\,^{\circ}C$ for exactly 45 min under a stream of nitrogen. The solid residue was solubilized in 200 μl water, introduced into HPLC 0.5 ml crim vials (amber, i.e. protected form light) (Laubscher Labs, Miecourt, Switzerland), and a volume of 100 μl was used for HPLC analysis.

2.6.2. Tissue sample processing

Frozen samples were allowed to thaw at room temperature. A 0.5 g-aliquot was taken from tissues with expected low doxorubicin levels, designated as tissue A samples, which included the contralateral-unperfused-lung, the heart, the liver, the mediastinum, the chest wall muscles and the fat tissues. A lesser amount (0.2 g) was taken from the perfused lung samples, with an expected higher doxorubicin content (tissue B samples). Tissue A and B samples were both homogenized in 2 ml KH_2PO_4 0.2 mM pH 3.8 solution for 90 s using a tissue tearor. After homogenization, 500 μl of tissue A samples were used directly, and 50 μl of tissue B homogenates samples were diluted to 500 μl with KH_2PO_4 0.2 mM pH 3.8 solution and aliquoted in Eppendorff tubes. All samples were processed in parallel along with their respective calibration and control standards. Samples were placed in a water-bath at $37\,^{\circ}C$ during 30 min, after which 100 μl of aqueous daunorubicin aqueous solution (I.S., 2.5 μg/ml) was added. Tubes were briefly (5 s) vortex-mixed prior to a first incubation at $37\,^{\circ}C$ for 15 min. A volume of

250 μl acetone and 50 μl of aqueous zinc sulfate 70% solution were then added to homogenated samples which were vortex-mixed for 5 s prior to another incubation period at 37 °C for 15 min. Samples were centrifuged at 22 000 × *g* for 10 min at +4 °C and the supernatant was transferred into a 5 ml-polypropylene tube and dried at 60 °C for exactly 45 min under a stream of nitrogen. The residue was solubilized in 200 μl water and introduced into HPLC 0.5 ml light-proof amber vials. A volume of 100 μl was used for HPLC analysis.

2.7. Calibration curves

Quantitative analysis of doxorubicin was performed using the I.S. method. Calibration curves were obtained by linear weighted (1/x) least-squares linear regression analysis of the peak ratio of doxorubicin to I.S., versus the ratio of the injected amount of doxorubicin to I.S. in each standard solution.

2.8. Validation of the method

The calibration curve was established using two sets of calibration standard samples: one set at the beginning and the second at the end of the HPLC run. Throughout the routine analysis of samples collected in these animal studies, control samples at the three relevant concentration levels (see above) were assayed every five samples in maximum.

The control samples were used for determination of precision and accuracy of the method, precision being calculated as the coefficient of variation (C.V.%) within a single run and between different assays, and accuracy as the percentage of deviation between nominal and experimental concentration calculated with the established calibration curves.

The lower limit of quantitation (LOQ) was experimentally determined by analyzing spiked samples for each matrix ($n = 5$), as the concentrations which provided measurements with a precision and accuracy within the recommended ±20% from their nominal values, in accordance with the guidelines of the Washington Conference [31].

2.9. Stability

The stability of doxorubicin in frozen serum, effluent, and tissue samples stored at −80 °C was assessed using the respective control standards ($n = 3$) after 1 month. The stability of doxorubicin in processed samples (i.e. reconstituted in water in HPLC microvials) was also examined after 48 h at +4 °C. Long-term stability has not been determined.

The stability of doxorubicin in serum, effluent, tissue A and B homogenates samples after multiple freeze–thaw cycles was studied using the respective control standards ($n = 3$), subjected to a total of three freeze–thaw cycles. Samples were successively stored 2 h at −20 °C and were allowed to thaw at room temperature for 1 h.

2.10. In vitro studies on the doxorubicin–heparin interference

2.10.1. In aqueous solutions

Series of aliquots (450 μl) of aqueous doxorubicin solutions at various concentrations (0.5, 5, 100 μg/ml) were placed into polypropylene Eppendorff tubes, onto which 50 μl of the following medium were added: (a) purified water (duplicate controls); (b) sodium heparinate 31.25 UI/ml, (c) sodium heparinate 62.5 UI/ml, (d) sodium heparinate 125 UI/ml, (e) sodium heparinate 250 UI/ml, (f) sodium heparinate 500 UI/ml, resulting in a final sodium heparinate concentrations of 3.125, 6.25, 12.5, 25 and 50 UI/ml, respectively. These concentrations are around those present in lithium heparinate Monovettes® Sarstedt that contains 15 UI/ml. The tubes were vortex-mixed and were left for 30 min in the dark at room temperature, with a vortex-mixing after 15 min. Heparinized samples and one of the aqueous samples, used as control, were transferred into the upper reservoir of a Microcon® YM-10 Centrifugal Filter Devices (Milipore) with a cellulose filter with a cut-off of 10 000 Da. These tubes were centrifuged during 30 min at 10 000 × *g*. After appropriate dilution, the ultrafiltrates were placed in HPLC 0.5 ml light-proof vials. A volume of 100 μl was used for HPLC analysis.

2.10.2. In citrated human plasma

Similarly, six series of plasma calibration and control standards were spiked in duplicate either with 50 μl of purified water, or sodium heparinate 125 and 2500 UI/ml, resulting in an heparin concentration of 12.5 and 250 UI/ml, respectively. After vortex mixing, the samples were left for 30 min in the dark at room temperature, before being subjected to the single-step protein precipitation, as described in Section 2.6.1.

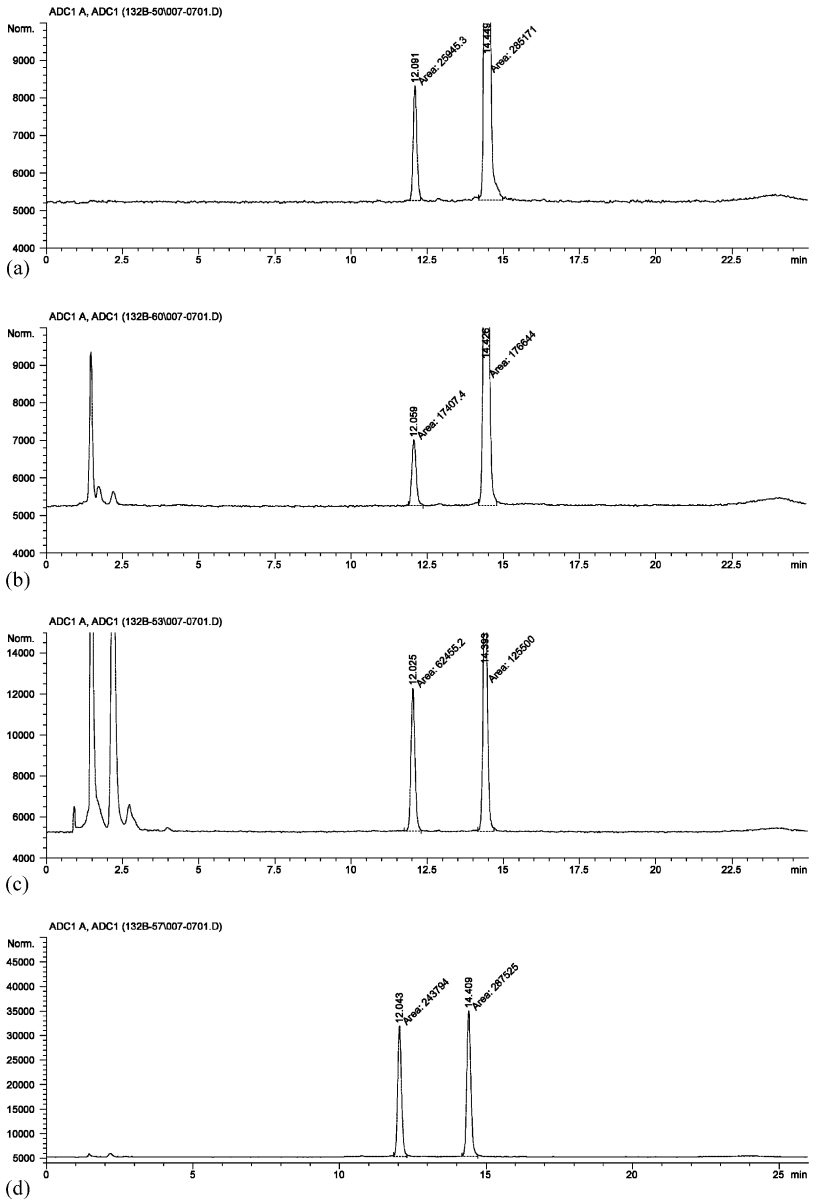

Fig. 2. (a) Chromatographic profile of an effluent control at 50 ng/ml spiked with IS (daunorubicine at 500 ng/ml). (b) Chromatographic profile of a plasma control at 50 ng/ml spiked with IS (daunorubicine at 500 ng/ml). (c) Chromatographic profile of a tissue A control at 2.5 μg/g spiked with IS (daunorubicine at 2 μg/g). (d) Chromatographic profile of a tissue B control at 125 μg/g spiked with IS (daunorubicine at 100 μg/g).

2.10.3. In freshly collected blood

Human blood from one healthy volunteer was collected into tubes containing five different medias: (a) lithium heparinate 15 UI/ml, (b) sodium heparinate 12.5 UI/ml, (c) sodium heparinate 250 UI/ml, (d) EDTA 1.6 mg/ml, and (e) no anticogulant added. Aliquots (3.8 ml) from each batch were spiked with doxorubicin solutions (200 μl) to yield a doxorubicin concentration in blood of 100 ng/ml, 5 and 100 μg/ml. After gentle agitation for 5 min, the samples were left for 30 min on ice, and were centrifuged at $1500 \times g$. An aliquot (500 μl) of plasma or serum was subjected in duplicate to single-step protein precipitation, as described in Section 2.6.1.

2.11. Animal studies

Briefly, six groups of six rats ($n = 36$) underwent AG or RG ILP with increasing doses of doxorubicin (100, 400, 1600 μg) administered for 20 min in order to assess the influence of the mode of infusion on doxorubicin's pharmacokinetic and distribution. In the same time, five groups of three pigs ($n = 15$) underwent (a) AG (b) RG (c) dual ILP (d) i.v. or (e) Swan-Ganz (direct injection in the PA of the left lung) infusion of 160 mg of doxorubicin during 20 min to study the pattern of doxorubicin's distribution in lung according to the mode of administration.

3. Results

3.1. Chromatograms

The proposed HPLC method enables the quantitation of doxorubicin in effluent, serum and in tissue suspensions A and B, as shown in the chromatograms of Fig. 2(a–d). Doxorubicin is eluted at 12.1 ± 0.5 min and is well separated from the I.S. daunorubicin eluted at 14.4 ± 0.5 min. The overall chromatographic run time is 26 min. No interfering peaks were observed in the chromatograms of blank effluent, plasma and tissue suspensions A and B (Fig. 3a–d). The fluorimetric detection at an emission wavelength of 480 nm and a detection wavelength of 550 nm provided a satisfactory selectivity and adequate sensitivity down to 2 ng/ml for the effluent and plasma samples and 0.1 μg per gram of tissue samples.

3.2. Calibration curves

The calibration plots for doxorubicin in the various matrices were satisfactorily described by linear weighted (1/x) least-squares linear regression analysis over their respective concentration ranges with mean coefficient of correlation (r) ($n = 5$) of 0.9997, 0.9998, 0.997, 0.9995 for the effluent, plasma and tissue suspensions A and B calibrators, respectively. The slope of the calibration curve obtained throughout the method validation is stable (Table 1) with values averaging 0.95 ± 0.035, 0.95 ± 0.058, 0.37 ± 0.025, 0.81 ± 0.039 for the effluent, plasma and tissue suspensions A and B calibrators, respectively. These slopes remained stable during the routine sample analysis.

3.3. Validation of the HPLC method: precision, accuracy, LOQ and LOD

The precision and accuracy of the control samples are given in Table 2. The concentrations of doxorubicin in effluent and plasma control samples were chosen at 50, 350 and 750 ng/ml to encompass the range of concentrations presumably present in plasma or diluted effluent. The tissue suspension A concentrations were selected at 3.5, 6.5 and 12.5 μg/g, and tissue suspension B at 125, 325 and 625 μg/g.

Throughout these concentration ranges, the mean intra-assay ($n = 5$) precision was always lower than 3.3, 3.5, 4.4, 6.1% for the effluent, plasma and tissue suspensions A and B samples, respectively. Overall, the mean inter-day precision was similar for all matrices, with mean C.V.% within 2.9 and 6.7%.

The accuracy was good for all matrices. The intra-assay deviation from nominal concentrations were always $< 7.7\%$ and the range of inter-day deviation was between -4.8 and $+6.3\%$.

The LOQ in effluent, plasma and Tissue suspensions A and B was experimentally found to be 2, 2 ng/ml, 0.1 and 5 μg/g, respectively. The intra-assay and inter-assay precisions and accuracies (devia-

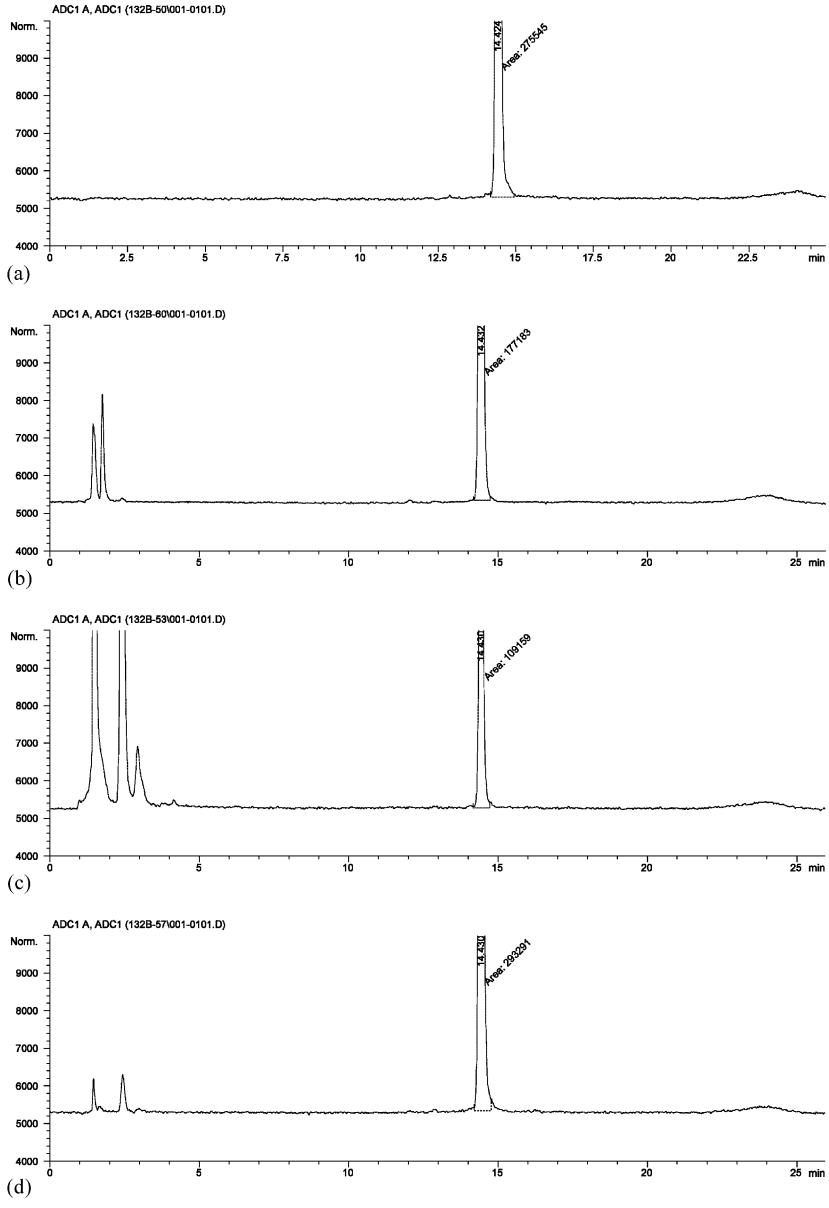

Fig. 3. (a) Chromatographic profile of a blank effluent spiked with IS (daunorubicine at 500 ng/ml). (b) Chromatographic profile of a blank plasma spiked with IS (daunorubicine at 500 ng/ml). (c) Chromatographic profile of a blank tissue A spiked with IS (daunorubicine at 2 μg/g). (d) Chromatographic profile of a blank tissue B spiked with IS (daunorubicine at 100 μg/g).

tion between nominal and measured values) at these low concentrations are reported in Table 3. As clearly shown, such values are well below the recommended allowances [31] that the accuracy and precision of samples at the LOQ must not exceed $\pm 20\%$.

The LOD for doxorubicin was 1 ng/ml for both effluent and plasma samples, and 0.05 and

Table 1
Mean parameters of the calibration curves for doxorubicin

	y = mx + b				Correlation coefficient (r)	
	m		b			
Effluent ($n = 5$)	0.97	±0.021	−0.012	±0.011	0.9997	±0.00017
Plasma ($n = 5$)	0.95	±0.058	0.00092	±0.00090	0.9998	±0.00016
Tissue A ($n = 5$)	0.37	±0.025	−0.014	±0.014	0.997	±0.0025
Tissue B ($n = 5$)	0.81	±0.039	−0.012	±0.0029	0.9995	±0.00034

0.625 μg/g for tissue suspension A and B samples, respectively.

3.4. Sample stability

Doxorubicin's stability in processed samples (i.e. extracted samples into vials, ready for HPLC analysis) up to 48 h at +4 °C was evaluated to ascertain that any spontaneous degradation would not occur during the storage of HPLC vials before the analysis. Since the matrix composition is expectedly different, doxorubicin stability was assessed separately in triplicate with the control samples in the four matrices. The results reported in Table 4 indicate that all processed samples were stable when placed at +4 °C in the fridge up to 48 h.

Moreover, doxorubicin was found to be stable in the different matrices placed at −80 °C for at least 1 month (Table 4). In addition, the concentrations of the control samples used in our semi-routine analysis remained stable up to 12 months, with slopes of the calibration curves remaining constant, confirming stability study performed up to 3 months, reported in details elsewhere [19].

The variations of doxorubicin levels when submitting control samples to successive freeze–thaw cycles are reported in Table 4. This indicates that no significant loss of doxorubicin is expected when the plasma and the tissue suspension B samples are thawed up to three times. The effluent control sample at the lower concentration (50 ng/ml) appear to be slightly affected by the third thawing cycle. Some decay of doxorubicin could be observed in the tissue suspension A controls.

3.5. In vitro studies of the doxorubicin–heparin interference

3.5.1. In aqueous solutions

The free doxorubicin (i.e. unbound doxorubicin) was assessed by ultrafiltration of aqueous solutions of doxorubicin, into which increasing concentrations of heparin were added. The results are expressed as percent of the doxorubicin levels in aqueous solutions (control, with no heparin added) subjected to the same ultrafiltration (for correcting the unspecific doxorubicin adsorption onto the filter membrane), and are shown in Fig. 4. A pronounced decrease in free doxorubicin is already noticeable at the lowest concentration of heparin (3.125 UI/ml). At concentrations higher than 12.5 UI/ml, heparin markedly affected doxorubicin's disposition in aqueous solution, and only extremely low concentrations (< 4.7% of the control with no heparin) of free doxorubicin could be measured in the ultrafiltrates.

3.5.2. In plasma calibration and controls

The calibration curve parameters and the doxorubicin levels found in plasma controls with (12.5 or 250 UI) or without heparin are given in Table 5, using the $ZnSO_4$–acetone extraction procedure. Neither the slope of the calibration curve (0.9672 vs. 0.9924) nor the measurement of control samples, with mean accuracy deviations included between −1.8 and +5.5%, seemed to be affected by the addition of 12.5 or 250 UI/ml heparin.

3.5.3. In freshly collected blood

Various volumes of doxorubicin stock solutions were added to freshly collected whole blood, collected in tubes containing either heparin (at

Table 2
Precision and accuracy of the HPLC assay for doxorubicin in effluent, plasma, tissue A and B

Nominal concentration	Concentration found	Precision (C.V.%)	Accuracy (deviation %)[a]
Effluent (*ng/ml*)			
Intra-assay ($n=5$)			
50	48±1.6	3.3	−4.0
350	331±5.3	1.6	−5.4
750	782±15.5	2.0	4.3
Inter-assay ($n=5$)			
50	53±3.0	5.6	6.3
350	333±10.5	3.1	−4.8
750	736±27.3	3.7	−1.8
Plasma (*ng/ml*)			
Intra-assay ($n=5$)			
50	49±1.7	3.5	−1.9
350	347±1.8	0.5	−0.8
750	791±7.2	0.9	5.4
Inter-assay ($n=5$)			
50	48±2.6	5.3	−3.5
350	344±22.3	6.5	−1.8
750	761±23.8	3.1	1.4
Tissue A (*μg/g*)			
Intra-assay ($n=5$)			
2.5	2.55±0.056	2.2	2.1
6.5	6.6±0.24	3.6	2.1
12.5	12.8±0.56	4.4	2.3
Inter-assay ($n=5$)			
2.5	2.4±0.14	5.9	−4.0
6.5	6.7±0.30	4.4	3.4
12.5	13.0±0.93	7.2	3.9
Tissue B (*μg/g*)			
Intra-assay ($n=5$)			
125	125±5.1	4.1	−0.2
325	338±10.6	3.1	4.0
625	673±40.9	6.1	7.7
Inter-assay ($n=5$)			
125	119±3.5	2.9	−4.5
325	317±17.2	5.4	−2.4
625	636±42.4	6.7	1.8

[a] (Found − nominal)/nominal × 100.

12.5, 15 and 250 UI/ml), EDTA or in the absence of anticoagulant. Whole blood was centrifuged and the collected plasma/serum were subjected to protein precipitation procedure with $ZnSO_4$–acetone (see Section 2.6.1). With such a procedure, there was not a significant difference in doxorubicin levels in the various plasma samples, collected either in the presence or absence of heparin, for the samples at the lowest concentration levels (100 and 5000 ng/ml in whole blood) (Table 6). However, a decrease of doxorubicin was noticeable at the highest level of 100 μg/ml in whole blood, a concentration unlikely to occur in the clinical setting (see below).

Table 3
Precision and accuracy of the HPLC assay for doxorubicin at its LOQ

	Nominal concentration	Concentration found	Precision C.V.%	Accuracy deviation %
Intra-assay ($n=5$)				
Effluent (ng/ml)	2	2.0 ± 0.28	14.2	−1.8
Plasma (ng/ml)	2	2.0 ± 0.26	12.9	−0.4
Tissue A (μg/g)	0.1	0.11 ± 0.013	11.6	11.2
Tissue B (μg/g)	5	5.4 ± 0.22	4.0	8.2
Inter-assay ($n=5$)				
Effluent (ng/ml)	2	2.1 ± 0.19	9.1	6.1
Plasma (ng/ml)	2	2.11 ± 0.099	4.7	5.5
Tissue A (μg/g)	0.1	0.105 ± 0.0060	5.7	5.0
Tissue B (μg/g)	5	5.2 ± 0.28	5.4	4.9

3.6. Applications of the method to animal studies

This method was applied to the analysis of serum, effluent and tissue samples from pigs and rats subjected to AG or RG ILP, according to the above-mentioned procedure. The results of these animal studies will be reported in details elsewhere (Krueger et al., Dusmet et al., in preparation). Briefly, the Fig. 5 shows the mean effluent's concentration–time curves in rats treated with 100, 400 or 1600 μg of doxorubicin, according to the AG or RG mode ILP. The steady-state effluent concentration was reached after about 5 min of a 20 min-long infusion, irrespectively, of the infusion mode (AG vs. RG) and administered dose (100, 400, and 1600 μg). Similarly, the doxorubicin levels in the effluents, measured during the washing period (20–40 min) also became stable after about 5 min in all groups.

Fig. 6 shows the mean doxorubicin levels measured in tissues from perfused lungs (i.e. subjected to ILP) in our experiments with rats. Doxorubicin concentrations in lung tissues were 17 μg per gram of tissue (10.4–26.7, range) versus 15 μg/g (10.8–21.5); 46 μg/g (26.2–81.7) versus 80 μg/g (57.4–110.5); 189 μg/g (112.3–316.6) versus 227 μg/g (128.3–403.0), for the 100 μg-AG versus -RG; 400 μg-AG versus -RG; and 1600 μg-AG versus -RG infused groups, respectively. The effluent's AUCs and the lung extraction ratio (μg in perfused lung concentration/dose) were calculated and analyzed statistically using the t-test. No statistically significant difference could be observed between the groups perfused with the 100 and 1600 μg dose. However, in the group perfused with 400 μg doxorubicin, a statistically significant difference ($P<0.05$) was noticeable between the AG- and RG-ILP group, both for the AUCs $_{0-20\ \mathrm{min}}$ (12 (9.9–13.6) vs. 8.4 (7.0–10.1) mg h/l, respectively), and for the extraction ratio (0.08 (0.042–0.146) vs. 0.18 (0.146–0.229), for AG- and RG-ILP, respectively.

Fig. 7 shows the mean doxorubicin levels measured in the perfused lung tissues, in our animal experiments with pigs. Doxorubicin levels in lung tissues were comprised between 10 and 930 μg/g (mean ± S.D.; 402 ± 245), 8–1566 μg/g (480 ± 423), 7–1520 μg/g (443 ± 379), 13–1180 μg/g (570 ± 630), 14–88 μg/g (47 ± 20) using the AG-, RG- and dual ILP mode, Swann-Ganz and i.v. infusions, respectively. The discussion of the results of these animal studies are beyond the scope of the present report and will be reported elsewhere.

4. Discussion and conclusion

This HPLC method has been demonstrated to provide a fairly simple, rapid, and sensitive procedure for determining doxorubicin levels in various biological matrices.

Sodium heptanesulfonate was chosen as an ion-pairing agent to retain doxorubicin and the I.S. daunorubicin—both cationic at pH 4.0—on the reversed-phase column long enough to achieve an

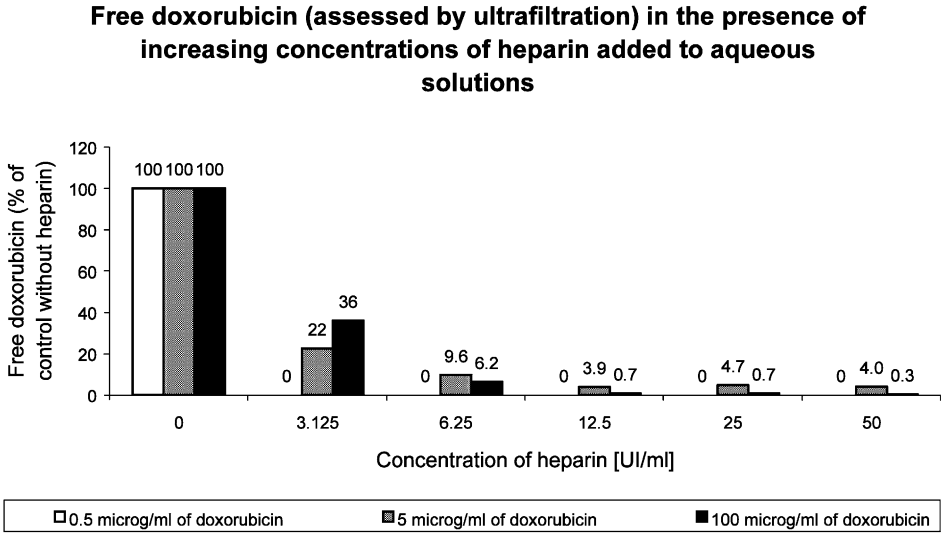

Fig. 4. Free doxorubicin (assessed by UF) in the presence of increasing concentrations of heparin added to aqueous solutions with 0.5 μg/ml (□), 5 μg/ml (▨) and 100 μg/ml (■) doxorubicin.

efficient separation. As expected with charged molecules, careful control of pH (4.00±0.02) of the heptanesulfonate aqueous solution was crucial for obtaining reproducible retention times.

The calibration curves validated for four different biological matrices remained stable during the routine analysis of a large number of samples. Both, accuracy and precision were always $<15\%$,

Table 4
Stability of doxorubicin in various matrices (percentage of the initial concentration)

Nominal concentration	Storage of the standards samples for 1 month at −80 °C	Storage of the processed standards samples for 48 h at 4 °C[a]	Freeze–thaw cycles		
			1 cycle	2 cycles	3 cycles
Effluent (*ng/ml*)					
50	100±4.0	99±2.7	95±4.3	93±5.4	89±4.0
350	99±1.6	96±1.5	99±2.3	97±2.0	100±1.8
750	95±2.9	98±2.9	103±3.0	103±1.1	104±0.4
Plasma (*ng/ml*)					
50	97±1.7	97±2.8	98±2.2	98±2.8	99±1.5
350	94±5.2	101±0.8	101±2.5	102±1.3	104±2.8
750	98±2.4	101±2.4	98±5.9	100±4.2	100±7.3
Tissue A (*μg/g*)					
2.5	113±8.8	100±7.9	105±6.3	107±6.8	107±8.2
6.5	95±2.2	99±2.3	105±4.6	101±9.6	106±2.6
12.5	92±3.9	99±4.0	101±6.5	115±9.5	117±7.3
Tissue B (*μg/g*)					
125	99±5.0	99±4.2	103±6.3	101±1.7	96±7.7
325	105±2.5	99±2.8	107±10.6	98±7.3	101±8.5
625	95±7.0	100±7.3	100±7.6	97±0.8	101±5.6

[a] Extracted samples into HPLC vials (i.e. ready for HPLC analysis).

Table 5
Calibration curve parameters and doxorubicin levels in plasma controls with or without addition of heparin

	Calibration curve parameters ($y = mx + b$)			Control samples								
	m	B	r	F (50 ng/ml)			G (350 ng/ml)			H (750 ng/ml)		
				Measured concentration	C.V. %	Accuracy deviation %	Measured concentration	C.V. %	Accuracy deviation %	Measured concentration	C.V. %	Accuracy deviation %
Without heparin	0.9924	0.00085	0.9998	49.7 ± 0.78	1.6	−1.8	361 ± 1.6	0.5	2.9	738 ± 13.7	1.9	−2.9
Heparin (Na) 12.5 UI	0.9897	0.00075	0.9999	51.2 ± 0.28	0.6	2.8	354 ± 1.7	0.5	1.6	737 ± 11.7	1.6	−0.6
Heparin (Na) 250 UI	0.9672	0.00004	0.9999	52.9 ± 1.13	2.1	4.2	367 ± 3.8	1.0	5.5	764 ± 16.3	2.1	0.3

in accordance with published recommendations [31]. There was a very large difference (up to 160 000-fold) in doxorubicin concentrations in the various samples analyzed during these animal studies, wherein very high (perfused lung tissue, effluent) or low (other unperfused tissues, rats serum) doxorubicin levels were found. A weighting factor of 1/x was applied to the linear regression of the calibration curve, with LOQs (2 ng/ml in plasma) similar to previously published methods.

Doxorubicin is known to be notoriously light-sensitive. In our study, all precautions were taken to avoid samples to be exposed to light, which results in an acceptable stability of doxorubicin, as shown in Table 4. Doxorubicin appears to be stable in all the raw matrices during at least 1 month storage at −80 °C. Moreover, it was observed during our routine analysis that calibration standards (prepared in batches stored at −80 °C) could be used for as long as 12 months without any noticeable changes in the calibration curve parameters. Nevertheless, it was of importance to verify doxorubicin's stability in the processed samples (i.e. in vials ready for HPLC analysis) at +4 °C during 48 h, since large series of samples were prepared simultaneously, and sometimes temporarily stored in the fridge prior to their HPLC analysis. The freeze–thaw cycles performed with QC samples did not reveal any change in doxorubicin levels except for the samples in effluent medium, at the lowest concentration, and at the highest concentration of the QC samples prepared in tissue A matrix. More generally, doxorubicin is expected to be less stable in matrices either with a low buffering capacity such as the effluent, or at lower concentrations, as previously reported [32]. An apparent increase of the measured concentrations was noticeable over time in the tissue A control sample at the highest concentration. This increase arises presumably from the alteration of the superstructure of the matrix, which may result in a diminution of doxorubicin's affinity to the tissular macromolecules.

More generally, our method development has revealed that the HPLC doxorubicin response is strongly influenced at every analytical step, either by the samples themselves (biological matrix

Table 6
Doxorubicin levels in plasma collected from whole blood into which various heparin concentrations were added

Nominal concentration (doxorubicin added in fresh whole blood) (ng/ml)	Concentration in plasma				In serum
	Heparin (Na) 250 UI/ml	Heparin (Na) 12.5 UI/ml	Lithium heparinate Monovette®	EDTA Monovette®	
100	60 ± 2.1	52 ± 4.6	48 ± 1.6	63 ± 4.2	45 ± 2.2
5000	3397 ± 38	3381 ± 161	3388 ± 223	3774 ± 192	2998 ± 124
100 000	24 508 ± 1275	28 620 ± 882	35 830.5 ± 599	107 855 ± 3900	99 358 ± 919

composition, the so-called "matrix effect"), the anticoagulant, and the reagent used for the protein precipitation ($ZnSO_4$–acetone). $ZnSO_4$ and acetone used for the protein precipitation were found to markedly increase (up to 4-fold) the fluorescence signal intensity of doxorubicin, regardless of

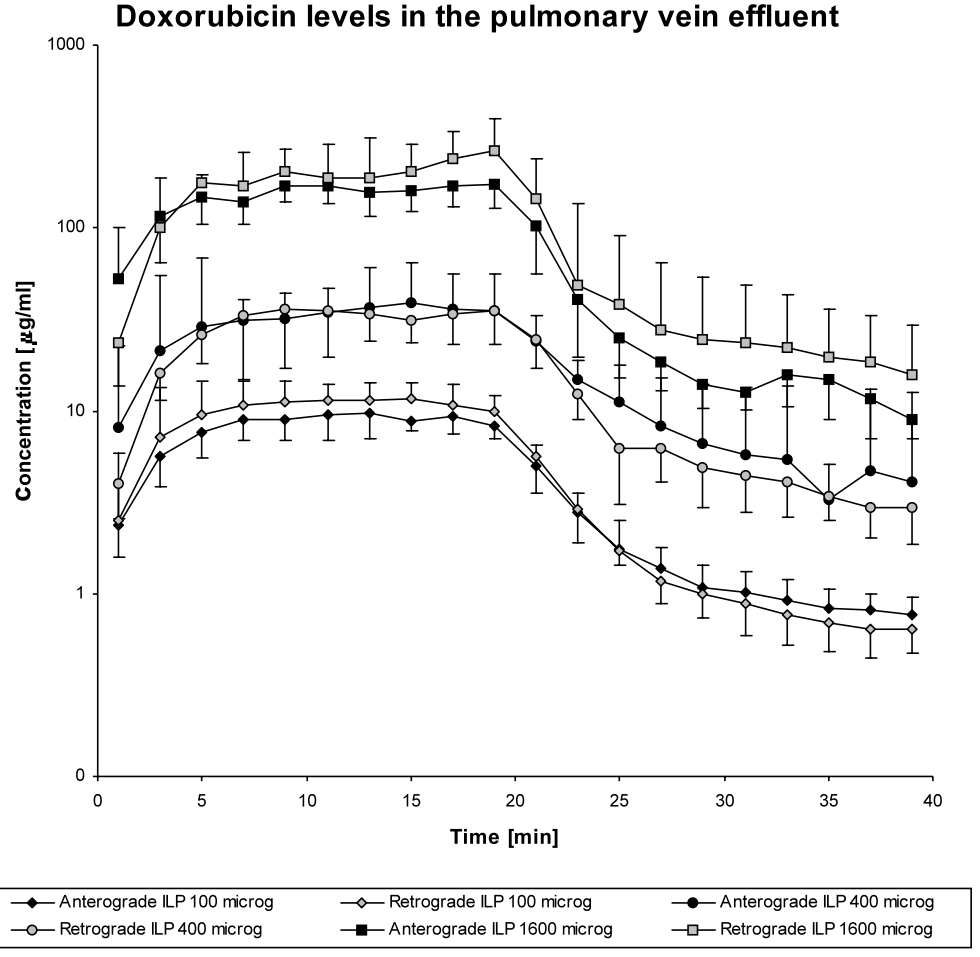

Fig. 5. Doxorubicin levels in the PV effluent during AG ILP with 100 μg (◆), RG ILP with 100 μg (◇), AG ILP with 400 μg (●), RG ILP with 400 μg (○), AG ILP with 1600 μg (■) and RG ILP with 1600 mg (□) doxorubicin.

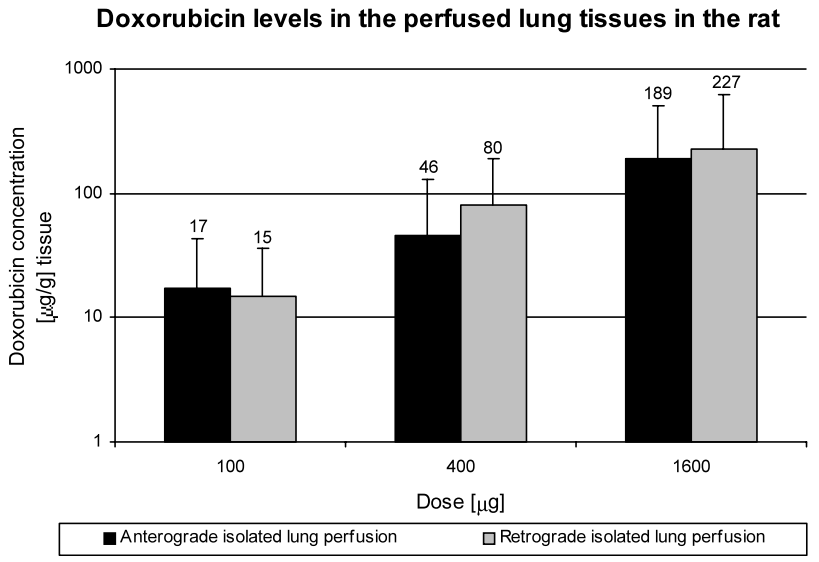

Fig. 6. Doxorubicin levels in the perfused lung tissues in the rat after AG ILP (black) and RG ILP (grey).

their direct effect for biological matrix elimination, since this anecdotal phenomenon was also observed with aqueous doxorubicin solution into which $ZnSO_4$ and acetone—individually or together—were added.

The effluent matrix composition was found to vary during the course of ILP—as testified by the presence (or absence) of blood in the collected samples—resulting in an highly variable signal for the daunorubicin signal, used as I.S. For eliminat-

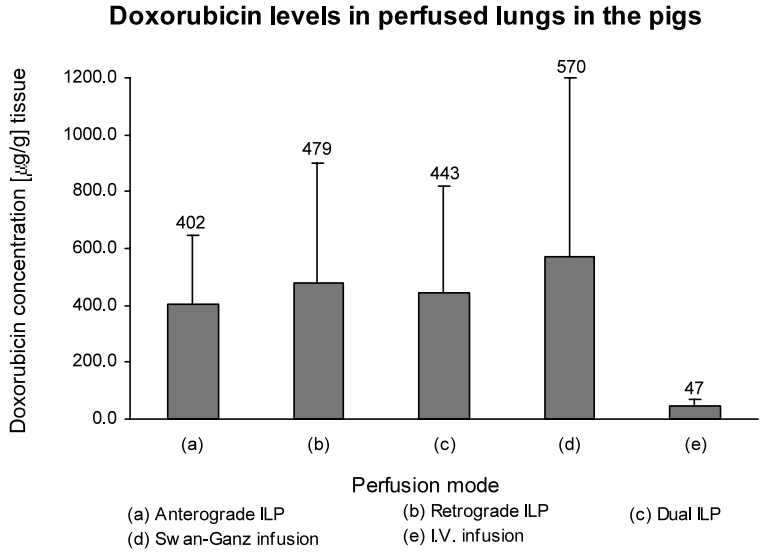

Fig. 7. Doxorubicin levels in perfused lungs in the pigs after AG ILP (a), RG ILP (b), Dual ILP (c), Swan-Ganz infusion (d) and I.V. infusion (e).

ing the matrix variability in effluent samples, the addition of as much as 2.5 μl plasma to each 500 μl-sample could satisfactory standardize the fluorescence signal intensity, and hence analytical reproducibility.

For measuring the high levels of doxorubicin in the perfused lung tissue, the tissue suspensions were homogenized and further diluted to bring the fluorescence signal of doxorubicin within the linearity range of the detector. Such a dilution procedure decreases the lung tissue content in the suspensions, which was shown to strongly influence the calibration curve of the extracted samples, with an increase of doxorubicin response signal (slope). This observation indicated that an accurate measurement of the high levels of doxorubicin in these tissues subjected to ILP, required the preparation of a separate series of calibration and control samples prepared in a diluted matrix, with lower tissue content (i.e. tissue B suspension).

Since doxorubicin was also measured in various other tissues (heart, mediastinum, chest, fat and liver), this would in principle have required the preparation of calibrations for each type of tissue. Due to the limited availability of these tissues in the present study, calibration curves established with homogenates from lung tissues were used for the assay of doxorubicin in other tissues.

Of importance, spuriously low doxorubicin levels were measured in a first series of experiments with rats whose effluent samples were collected in tubes containing heparin as anticoagulant. Several potentially important physico-chemical interactions between heparin and various drugs (aminoglycosides, vancomycin, cytarabine, erythromycin, tetracycline, and any large molecules containing an amino group [33]) have been already reported in the literature. However, the potential interference between doxorubicin and heparin, and its clinical importance, have been addressed in a limited way in a few conflicting reports. An interference between doxorubicin and heparin was first described by Cofrancesco et al. [34], suggesting that a reduction of the anticancer activity of doxorubicin could be feared. Despite contradictory results in vitro [35–37], the interaction between doxorubicin and heparin has been reported not to significantly influence its pharmacological activity [35,38]. Indeed, the macromolecular complex formed between one molecule of heparin and 16 molecules of doxorubicin [39] is apparently labile and reversible at equilibrium and should not modify the doxorubicin's tissue distribution nor its antitumor activity [35]. However, the doxorubicin–heparin complex was reported to reduce doxorubicin's acute and chronic cardiotoxicity [40]. Thus, a physico-chemical interaction during the processing of biological samples could not be fully excluded, as a precipitation within i.v. tubing containing heparin and doxorubicin has already been documented [41]. Our in vitro studies indicate that the doxorubicin–heparin interference does not appear to significantly affect the measurement of clinically relevant concentrations of doxorubicin in blood collected on heparin. However, the heparin–doxorubicin interference was noticeable in aqueous solutions and at very high doxorubicin levels, such as those encountered in the ILP effluents samples, wherein this observation was initially made. Of importance, this phenomenon could have profoundly influenced our analytical results, and the significance of the animal studies, wherein the observation of spuriously low levels of doxorubicin in the effluents in ILP could have led to an overestimation of the lung extraction ratios.

In conclusion, this method was successfully applied to the analysis of serum, effluent and tissue samples from pigs and rats undergoing ILP. Overall, given the specificity and sensitivity achieved by HPLC coupled to spectrofluorimetric detection, the assay of doxorubicin is hardly to be considered as an analytical challenge. Our studies, however, confirm the critical influence of the matrix effect on doxorubicin's peak signal intensity, and highlight the importance of preparing calibration samples in a matrix composition similar to that wherein doxorubicin is to be measured. To the best of our knowledge, very few methods have been reported so far for the assay of doxorubicin in tissues [23,25,28,42] using calibration standards prepared either in plasma [23,25] or in tissues homogenates [28]. Given the pronounced matrix effect observed in our study, which was also observed by van Asperen et al. [23], there are at present some uncertainties whether the concentrations calculated using calibration samples pre-

pared in plasma confidently reflect the actual tissular doxorubicin concentrations.

Finally, the observed doxorubicin-heparin does not seem to affect the measurement of clinically relevant concentrations, but may influence the analysis of very high doxorubicin levels, as those encountered in the ILP effluents. Indeed, this doxorubicin–heparin interference may have also clinical consequences, possibly influencing doxorubicin's biodisposition in patients receiving anticancer chemotherapy through heparinized i.v. tubing [41], but this remains to be formally demonstrated.

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