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Simultaneous measurement of doxorubicin and reduced metabolite doxorubicinol by UHPLC–MS/MS in human plasma of HCC patients treated with TACE

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ABSTRACT

A sensitive, selective, accurate and precise method for simultaneous quantification of doxorubicin (DOX) and doxorubicinol (DOXol) in human plasma of patients diagnosed as having intermediate stage unresectable hepatocellular carcinoma (HCC) was developed. The method was based on electrospray tandem mass spectrometry in selected reaction monitoring mode. DOX, DOXol and trofosfamide, an internal standard, were extracted from plasma by using a simple solid phase extraction (SPE) procedure after the addition of 0.1 M hydrochloric acid.

A 200-μL aliquot of the extracted sample reconstituted in mobile phase was analyzed on a Zorbax SB-C18 UHPLC column (50 mm × 2.1 mm, 1.8 μm particle size) in 8 min. The mobile phase consisted of acetonitrile and 0.1% formic acid pH 4.5 (95:05 v/v). Good accuracy and precision of this method were demonstrated by determination of spiked plasma QC samples in four consecutive days. The SPE extraction recoveries ranged from 72.3 to 77.3% and 75.5 to 98.4% for doxorubicin and doxorubicinol, respectively. The intra-day and inter-day precisions were less than 11.4%. The limit of quantitation was 1.0 ng/mL for both compounds. The calibration curves of DOX and DOXol were analyzed by weighted linear regression with $1/x$ as a weighting factor. They were linear over the concentration range of 1.0–100.0 ng/mL with R^2 greater than 0.99.

This developed method was successfully applied to study plasma pharmacokinetics in patients affected by HCC and treated with transarterial chemoemolization practices (TACEs) using HepaSphere™ preloaded with DOX in a standardized procedure.

1. Introduction

Doxorubicin (DXR) is an anthracycline antibiotic that possesses broad spectrum antineoplastic activity and is by far the most frequently administered drug to treat a wide variety of adult solid tumors [1,2]. The main metabolic pathway of doxorubicin yields secondary alcohol compounds, such as 13-S-dihydro metabolite, generally denoted with the suffix-ol, doxorubicinol (DOXol) [3].

Various analytical methods have been reported for quantification of doxorubicin and doxorubicinol with detection limits that are adequate for analyzing these drugs in plasma or serum samples of patients receiving conventional chemotherapeutic treatments. The reported methods have mostly used high performance liquid chromatography (HPLC) coupled with fluorescence (FL) [4–12], electrochemical [13] and chemiluminescence detection [14]. Since the reduction of doxorubicin to doxorubicinol involves the addition of only two mass units, the specificity of tandem mass spectrometry (MS/MS) is a prerequisite for chromatography methods aimed at quantifying DOX and DOXol in complex biological samples [15–17]. These LC–MS methods provide improved sensitivity with LLOQ of less than or equal to 0.2–1.0 ng/mL for DOX and DOXol respectively, but exhibit low selectivity as the baseline separation between the two analytes is not always well optimized [3,15]. Nowadays, new targeted LC-FL [18] and LC–MS methods [19,20] have been developed to study pharmacokinetics (PK) of doxorubicin and/or its metabolite, doxorubicinol, administered by infusion of chemotherapeutic agents followed by embolization. In fact, in an attempt to overcome toxic side effects of systemic chemotherapy and to enhance antitumor selectivity, new drug delivery technologies such as peptide-conjugates, special pharmaceutical formulations (pegylated liposomalor micellas) and embolization systems like transarterial chemoembolization (TACE) are currently in use. For the treatment of unresectable hepatocellular carcinoma

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(HCC), TACE with drug eluting beads (DEB-TACE) has become a standard loco-regional therapy [21]. The eluting beads mostly used include DC Bead® and HepaSphere™. Pharmacokinetic studies have been carried out for quantifying doxorubicin in animal models of liver cancer [20,22–24] and in patients treated with TACE [19,25,26]. A new UHPLC–MS/MS method has been recently developed to measure doxorubicin uptake in rabbit VX2 tumor HCC after chemoembolization. This study showed no correlation between tissue iodine (lipiodol) and doxorubicin levels in treated tumor tissue [27]. Other *invitro–in vivo* kinetic studies on the release-profiles of drugs have demonstrated an initial burst release followed by a slow release of drugs for up to at least 48 h [21]. Similarly, a PK study in HCC patients treated with TACE showed that after an earlier release effect, epirubicin continued to be detectable in blood samples [28]. While big efforts have been made to optimize new carrier-based strategies, clinical studies reported with drug eluting beads are at this point lacking in terms of PK assessment. It is clinically relevant to measure the sum of the parent compound and its major metabolite in order to provide data on the active fraction of this drug in TACE procedures.

The objective of this study was to develop and validate a sensitive, specific and rapid UHPLC-MS/MS method for simultaneous determination of DOX and DOXol in human plasma of HCC patients treated with HepaSphere™. Since doxorubicin pharmacokinetic is characterized by a tri-compartment model with a rapid initial distribution phase lasting up to 1 h, an intermediate phase and, a much slower terminal elimination phase, apparently established after 12–24 h [29–31], this method has been applied to study the plasma-concentration–time curves of DOX and DOXol over seven days. The present study reveals that when doxorubicin was administered by TACE, the plasma concentrations of doxorubicinol were actually five times less than those of the parent compound in the first 2 h. On the day 2, the metabolite concentration increased and was 2.5 times fold of doxorubicin.

2. Materials and methods

2.1. Standards and chemicals

Reference standard doxorubicin hydrochloride (batch K1G106), doxorubicinol citrate (batch 2-MMH-152-2) and the internal standard (IS), trofosfamide (TR, 99.7% purity), were supplied by Nova Chimica (Milan, Italy) as 50, 2 and 250 mg powder, respectively. Acetonitrile, methanol, 2-propanol, methylene chloride and formic acid (HCOOH), all of HPLC gradient grade, were purchased from VWR International Ltd (Merck House, Poole, UK). Deionized water was generated from Milli-Q Plus water purifying system purchased from Millipore Corporation (Milford, MA, USA). Eppendorf (15.0 mL) and micro-test tubes (1.5 mL) as well as pipette models (from P20 to P5000) were purchased from Eppendorf (Nethelar-Hinz-GmbH, Hamburg, Germany). Disposable pipette tips were obtained from Rainin Instruments, Woburn, MA, USA. A Visiprep™ DL model Solid Phase Extraction (SPE) Vacuum Manifold for developing biological samples purification was supplied by Supelco (Sigma–Aldrich, Inc., USA). The DL device (Disposable Liner) was used to eliminate the possibility of contamination from one sample to the next in the same manifold port. The SPE cartridges were OASIS® hydrophilic–lipophilic balance (HLB) cartridges (10 mg, 1 mL) purchased from Waters Associates Milford, MA, USA. The dry extracts were reconstructed in mobile phase and then filtered through syringe filter devices (0.45 μm pore size, Whatman Inc., Clifton, NJ, USA). Citrated blank (drug free) human plasma to prepare daily standard calibration curves and quality control samples (QCs) were obtained from volunteers. DC-Beads (Biocompatible UK Limited, Surrey, UK) and HepaSphere™ were purchased from Biosphere Medical, France.

Table 1
Multiple reaction monitoring transitions and optimized potentials: fragmenter voltage (FG) and collision energy (CE) for each analyte and internal standard, tofosfamide (TR).

Compound name	Mass	Precursor	Product ion	FG	CE
DOX	543	544	361[b]	108	20
DOX	543	544	397[a]	108	4
DOX	543	544	321	108	20
DOX	543	544	379	108	12
DOXol	545	546	399[a]	88	4
DOXol	545	546	363[b]	88	16
DOXol	545	546	351	88	16
TR	322	323	154	123	20

[a] Product ion used as quantifier ion.
[b] Product ion used as qualifier ion.

2.2. Chromatography

The UHPLC system consisted of an Agilent Technologies 1200 series system equipped with a degasser, binary pump and an high-performance autosampler (HiP ALS SL+) with a thermostatic column compartment. Separation of the analytes from plasma was achieved on a Zorbax SB C18 column (50 mm × 2.1 mm i.d., 1.8 μm; Agilent Technologies, Inc., Santa Clara CA, USA). The mobile phase consisted of 0.1% formic acid in water (v/v) as solvent A and acetonitrile as solvent B. The initial conditions were 95% A and 5% B. A linear gradient elution was applied: 5% B up to 1 min, 40% B from 1 to 8 min. Finally, an isocratic condition (100% B) was held for up to 13 min. The column was re-equilibrated prior to injection of the next sample to initial conditions for 3 min. UHPLC flow rate was 0.4 mL/min at 40 °C with a total run time of 13 min. This applied gradient mobile phase has been a necessary prerequisite to obtain a good baseline separation between the main compound and the reduced metabolite since the mass unit difference is only 2 with an isotope distribution that overlaps for 6.7% [3].

2.3. Mass spectrometry

The UHPLC system was coupled with a 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Inc.) equipped with an electrospray ionization (ESI) source operating with the Jet Stream technology and utilizing a super-heated sheath gas to enhance sensitivity. An Agilent Mass Hunter work station was used for the control of equipment, data acquisition, and analysis. This software allowed the optimization of MS/MS parameters using the working standard solutions of doxorubicin, doxorubicinol, and internal standard injected at the concentration of 1 μg/mL.

Finally, the instrument was operated using nitrogen as nebulizer gas set at 45 psi with a flow rate of 5 L/min. The sheath gas had a flow rate of 11 mL/min, with a temperature of 350 °C. The nozzle voltage was set at 1 kV and the capillary voltage at 3.5 kV.

Quantification was performed in selected reaction monitoring (SRM) mode with the following transitions: m/z 544 → 361, 397 for doxorubicin; m/z 546 → 363, 397 for doxorubicinol, and m/z 323 → 154 for trofosfamide, the internal standard. All the optimized potentials in relation to the product ions are reported in Table 1.

2.4. Stock and working standard solutions

The stock solutions of doxorubicin and doxorubicinol were separately prepared in methanol at the concentration of 1.0 and 0.2 mg/mL from each powder. The internal standard stock solution was obtained in methanol at the concentration of 5.0 mg/mL. To prepare the standard points of the calibration curves and quality controls containing the mixture of DOX and DOXol different aliquots of these stock solutions were used. A final stock solution at the concentration of 50 μg/mL was obtained. Afterward, the

working stock solutions were prepared in methanol at the concentrations of 0.1, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, and 10.0 μg/mL by serial dilution of the mixture of DOX and DOXol. Similarly, the IS working solution was prepared in methanol at the concentration of 200 ng/mL by diluting the stock solution. All these solutions were stored at +4 °C.

2.5. Standards and quality control sample

Eight-point plasma calibration curves were freshly prepared every day during the validation study. Each calibration standard was prepared by adding 100 μL of each working stock solution to 10 mL of blank plasma samples to obtain the final calibration curve for doxorubicin and doxorubicinol with the following concentrations: 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 and 100 ng/mL.

Each calibration curve included a blank sample (plasma processed without IS) and zero blank sample (plasma processed with the IS). Three quality control samples (QCs) were used for each calibration curve at the concentration of 3.0, 15.0 and 50 ng/mL for DOX and DOXol. The lowest level of quantification (LLOQ) was determined by analyzing spiked samples ($n = 4$) at the concentration of 1.0 ng/mL. The samples of calibration standards and QCs were divided into 1.0 mL aliquots and stored at −20 °C before the analysis.

2.6. Solid phase extraction (SPE) procedure

Calibration points, QCs, blanks and actual samples were prepared for SPE by adding 100 μL of the internal standard solution and 2.0 mL of 0.1MHCl. The samples were vortexed after each addition. To avoid losses due to protein binding, the dilution of samples in presence of hydrochloric acid was applied before starting with the SPE procedure. For each sample, Oasis HLB 1 mL extraction cartridges were conditioned with methanol, water, and methylene chloride. Sample solution was loaded on the cartridge and washed with water. The final sample was eluted using 2 mL rinses of the solvent organic mixture composed of methanol, isopropanol and methylene chloride (25:25:50, v/v/v). The eluted solution was dried under a gentle stream of nitrogen at room temperature and the residue was reconstituted with 200 μL of mobile phase A before the injection in the UHPLC–MS/MS system.

3. Validation study

3.1. Extraction recovery and matrix effect

The developed LC–MS/MS method was validated according to the Food and Drug Administration (FDA) guidance on bioanalytical method validation [32]. The percentage extraction recovery (RE) was calculated at the lower level of quantification for DOX and DOXol and at three plasma concentrations, 3.0, 15.0, 50.0 ng/mL, prepared in quintuplicate. The mean peak areas of analytes spiked before SPE extraction (set C) were compared with those of analytes spiked in the matrix of extracted blank plasma (set B).

For both analytes matrix effect (ME) was also studied at LLOQ and QC levels. ME was assessed by comparing the mean peak areas of analytes post-spiked in blank extracted plasma with those of DOX and DOXol standard solutions prepared in mobile phase (set A). The loss of signal expressed as a percentage represents the ion suppression as given by the formula $[100 - (B/A) \times 100]$. Similar procedure was used to assess these performances of IS at the concentration of 20 ng/mL.

The potential for relative matrix effect on the assay selectivity was also evaluated using six independent sources of extracted blank human plasma. A single 1 mL aliquot was post-spiked with DOX and DOXol at the concentration of the lowest calibrator (1.0 ng/mL) and analyzed for both analytes.

3.2. Linearity

The linearity of calibration curves was validated for different working days ($N = 4$). For each standard point, the peak area ratios of DOX and DOXol to IS were plotted against the concentrations of each analyte. The linearity of the standard curves was checked by regression analysis and the goodness of the regression by calculating the Pearson's determination coefficient R^2 and by comparison of the actual and back-calculated concentrations of the calibration standards.

The calibration curves were considered acceptable if the calculated accuracies of >75% of standards were within 15% and a minimum of six standards had to meet these criteria, including the LLOQ and the calibration standard at the highest concentration.

3.3. Precision, accuracy, and LLOQ

The precision and accuracy of the method were assessed by intra- and inter-day validation over four non-consecutive days. The intra-day accuracy and precision were evaluated by processing QCs in five replicates ($n = 5$) at three concentration levels. The concentration of the QCs was calculated according to the daily calibration curves. The inter-day accuracy and precision were determined by analyzing QCs in twenty replicates of each concentration. Accuracy was determined as the ratio between the back-calculated concentration and the actual value and expressed as a percentage. The coefficient of variation (CV%) was used as a measure of precision. The intra- and inter-assay accuracies should be within the recommended ranges and reported in the international guidelines [32]. Only one QC level could be excluded at each concentration. The lower limit of detection (LOD) level was obtained as three times the standard deviation of the peak areas detected at the retention times of the analyte of interest.

As indicated in the report from the third AAPS/FDA Bioanalytical Workshop, the evaluation of bioanalytical methods by reanalysis of "incurred" or study samples is recommended and can be considered an additional measure of assay reproducibility [33]. In particular, the incurred samples from drug studies can vary in their composition when compared with the standards and quality control samples used to validate the method. They may have metabolites that neither the standards nor the quality control samples contain; for example, the drug metabolite may interfere with the assay or may revert to its parent drug in vitro, causing non reproducible results. The accuracy of the present method was therefore assessed by reanalyzing incurred plasma samples of one patient from the pharmacokinetic study in a further analytical session. The analyses can be considered equivalent if at least 2/3 of the reanalyzed samples had concentrations within 20% of the original analysis values [34].

3.4. Stability

The stability of DOX and DOXol in plasma was assessed by analyzing QC samples at two concentration levels (3.0 and 50.0 ng/mL) during storage and handling. Bench-top stability was determined after 4 h at room temperature. Stability in the autosampler was also assessed at room temperature by reanalyzing the processed QC samples 72 h after the first injection.

Long-term stability was studied in plasma and in working solutions stored at −20° C. Freeze–thaw stability was studied by analysing the low and high QCs that were frozen overnight, at normal storage temperature (−20 °C) and thawed unassisted at room temperature. When completely thawed, the samples were

frozen again at the same temperature for 24 h and thawed. This freeze–thaw cycle was repeated 2 more times. After the third cycle (4 days), the samples were analyzed. To check freeze–thaw stability, an aliquot of each QC sample concentration was freshly prepared, processed and analyzed. The analytes were considered stable at each concentration when the differences between the freshly prepared samples and the stability testing samples did not deviate more than ±15% from the nominal concentrations.

3.5. Application to clinical samples

The method is being used to explore the pharmacokinetics of DOX and DOXol in HCC cancer patients administering DOX by DEB-TACE. Blood samples were collected at 0, 5, 10, 20, 40 min and 1, 2, 3, 4, 6 and 12 ± 2 h after dosing. Additional samples were collected on days 1–7. Samples were collected in tubes containing sodium citrate as anticoagulant because doxorubicin may interact directly with heparin and the complex DOX-heparin may influence its analysis [3]. Plasma samples were immediately centrifuged at +4 °C for 10 min at 1500 × *g*. Then the plasma was stored at −20 °C pending analysis.

4. Results and discussion

4.1. Mass spectrometry

The protonated molecules [M + H]+ of doxorubicin and its metabolite doxorubicinol at *m/z* 544 and *m/z* 546 were generated in the positive ESI mode. These ions were used as precursor ions to obtain the product ion spectra for DOX and DOXol, as reported in Table 1. The fragmentation pattern relative to the MS/MS spectrum of DOX has been already described in a previous paper [35]. Fig. 1B shows the MS/MS spectrum of the pharmacologically active 13-S-dihydro metabolite, DOXol characterized by a four ringed 7,8,9,10-tetrahydrotetracene-5,12-quinone structure bound to an aminoglycoside sugar. The loss of the glycoside portion gave a product ion at *m/z* 399 and with a further loss of two water molecules led to a product ion of *m/z* 363. The transitions from *m/z* 546 to 363 and from *m/z* 546 to 399 were used in our SRM analysis for detection of DOXol (Table 1). Representative SRM chromatograms for doxorubicinol (I), doxorubicin (II), and trofosfamide (IS, III) in human plasma are shown in Fig. 2. SRM chromatograms of a blank plasma sample without the IS are reported in panel A. SRM chromatograms of a blank plasma sample spiked with the analytes at the LLOQ level (1 ng/mL), and trofosfamide (20.0 ng/mL) are shown in this figure (panel B). The SRM profiles of a plasma sample obtained at 24 h after the starting of TACE procedure evidenced the presence of doxorubicin and doxorubicinol at the concentration of 16.3 and 44.7 ng/mL, respectively (panel C). In an analytical run of 8 min the detection of the two compounds occurs at the retention times (R_t) of 4.6 (doxorubicinol) and 5.2 min (doxorubicin). This well optimized peak-to-peak baseline separation of DOX and DOXol from the plasma matrix is suitable for an unambiguous quantification of the metabolite. The SPE sample clean-up carried out in the present method avoids the mostly used deproteinization procedure based on the use of zinc sulfate that causes severe contamination of the mass spectrometric detector [36]. The SPE extracted plasma samples were very clean sample solutions minimizing possible interferences. Fig. 2 (panel A) shows that one interference peak occurs at 5.8 min with the transition at *m/z* 544 > 397. To confirm that neither this interference nor other ones from sample-related products at the retention times of DOX and DOXol may affect the quantification of these analytes, the chromatograms of blank plasma samples and signal-to-noise ratio of spiked blank plasma samples at the LLOQ level (1.0 ng/mL) are reported in this figure (panels A/B). The selectivity of the method was confirmed by analyzing six independent sources of blank human plasma.

4.2. Validation of the method

The mean peak area values for extracted samples and matrix of extracted plasma at LLOQ and QCs are reported in Table 2. Recovery was evaluated over three concentrations and in quintuplicate and was 72.3–77.3 for DOX and 75.5–98.4% for DOXol (Table 2). The recovery of IS was 95.9%.

The process efficiency (PE%) values obtained comparing the peak-areas of the post-extracted samples with those in UHPLC mobile phase ranged between 58.0% and 59.6% for DOX and from 63.1% to 71.5% for DOXol at the three QC levels (Table 2). Therefore, the variability in the process efficiency of the developed method may be considered acceptable. The method was not affected by different human matrices; on spiking six different sources of human plasma with 1.0 ng/mL of DOX and DOXol (the LLOQ) the precision was 12%. There were therefore no significant differences (<15%) in the peak area of the analytes, so we could exclude the presence of any relative matrix effect of ion suppression. The variability observed for DOXol recovery at the three levels of quality control does not affect precision and accuracy parameters because the extent of recovery of the analytes and of the internal standard was consistent and reproducible. The ratio between the peak-area of DOXol and the internal standard was linear along the dynamic range of the calibration curve. Table 3 reports the results for the calibration curves of DOX and DOXol prepared each day during the validation study and the accuracy and precision for each standard point. As shown by data reported in Table 3 the precision ranged from 1.6% to 5.4% and from 2.8 to 12.2 for DOX and DOXol, respectively. The mean accuracy was always close to 100% (range 91.2–113.1%). The peak-area ratios of the analyte/IS compared with the nominal concentrations were plotted, and a weighted regression function ($1/x$) was applied to generate the calibration curves. The calibration curves prepared on four different days showed excellent linearity and good results of the back-calculated concentrations over the validated range of 1.0–100.0 ng/mL. Pearson's coefficient of determination R^2 was 0.997 ± 0.0027 for DOX and 0.997 ± 0.0023 for DOXol. The mean weighted regression function was $y = 0.042 \pm (0.010)x - 0.014 \pm (0.008)$ for DOX and $y = 0.019 \pm (0.006)x - 0.005 \pm (0.005)$ for DOXol. The minimum R^2 value for each analytical session was 0.993 for both compounds. The carryover effect was excluded by injecting samples of mobile phase between successive test samples and extracted blank plasma. The precision and accuracy of the method were evaluated analyzing five replicates of the QC samples at concentrations of 3.0, 15.0, and 50.0 ng/mL within a single-run analysis for intraday assessment and three replicates over four consecutive runs for interday assessment. The accuracy and precision (CV%) are shown in Tables 4 and 5 for the main compound and its metabolite. The method was precise, with interday CV ≤11.4%for DOX and ≤10.1% for DOXol, and with intraday CV ≤8.2% (DOX) and ≤8.8%(DOXol). Since CVs of the analyte/IS ratios for samples spiked before extraction and the analogous CVs for samples spiked after extraction were shown to be similar, the variability in the recovery on the overall method precision and matrix effect may be considered negligible. The accuracy ranged between 102.3% and 107.1% for the parent drug, and 96.3–104.6% for the metabolite. The LLOQ was fixed at 1.0 ng/mL and was validated through analysis of four replicates. The CVs% were always less than 6.5% for both compounds. As shown in Fig. 2, with the high signal-to-noise ratio (>100 for DOXol and >300 for DOX), it would have been possible to fix a lower LLOQ, given the limit of detection of 0.03 ng/mL for DOXol and 0.01 ng/mL for DOX; however, we used the higher LLOQ of 1.0 ng/mL in view of the levels of the analyte expected in plasma from patients. The good

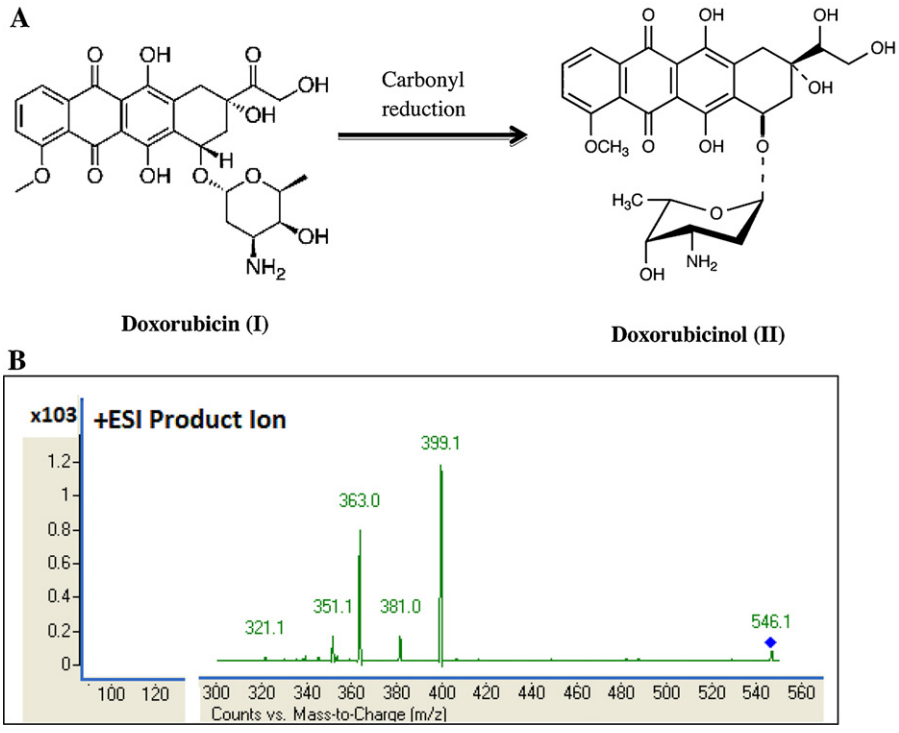

Fig. 1. Structure of doxorubicin and doxorubicinol (panel A), electrospray ionization mass spectrum of product ion of doxorubicinol (panel B).

Table 2
Doxorubicin and doxorubicinol mean peak areas of the standard solutions of analytes in the mobile phase (set A); analytes post-spiked in extracted blank plasma samples (set B); SPE extracted plasma samples (set C).

	Doxorubicin			PE%	RE%	ME%
	Mean area ± SD					
	Set A	Set B	Set C			
LLOQ	2091.4 ± 112.9	1536.2 ± 134.0	1186.6 ± 127.7			
QC1	6617.5 ± 286	5166.8 ± 914.2	3849.3 ± 298.7	58.2	74.5	21.9
QC2	35,237.4 ± 1297.4	29,064.5 ± 700.7	21,007.8 ± 1738.5	59.6	72.3	17.5
QC3	125,058.0 ± 1057.5	98,162.0 ± 724.1	72,534.5 ± 2360.6	58.0	73.9	21.5
	Doxorubicinol			PE%	RE%	ME%
	Mean area ± SD					
	Set A	Set B	Set C			
LLOQ	813.3 ± 50.9	649.9 ± 82.8	490.6 ± 32.8			
QC1	2594.3 ± 115.5	2030 ± 296.1	1637.1 ± 65.2	63.1	80.6	21.7
QC2	13,214.0 ± 235.0	10,867.1 ± 455.5	8448.5 ± 244.9	63.9	77.8	17.8
QC3	37,776 ± 180.2	27,441.2 ± 155.3	27,012.6 ± 1628.7	71.5	98.4	22.3

Table 3
Linearity, accuracy, and precision of calibration curves of DOX and DOXol.

Concentration (ng/mL)	DOX (ng/mL)			DOXol (ng/mL)		
	Mean ± SD	CV%	Accuracy%	Mean ± SD	CV%	Accuracy%
1.0	1.0 ± 0.04	4.0	100.3	1.02 ± 0.08	7.8	100.9
2.5	2.5 ± 0.10	4.0	100.6	2.36 ± 0.29	12.2	94.6
5.0	4.5 ± 0.19	3.5	91.2	4.64 ± 0.20	4.3	92.9
10.0	8.99 ± 0.49	5.4	90.0	9.32 ± 0.51	5.4	93.2
20.0	18.91 ± 0.83	4.3	94.6	18.44 ± 1.24	6.7	92.2
40.0	41.61 ± 0.77	1.8	104.0	39.46 ± 2.55	6.4	98.7
80.0	90.5 ± 6.72	7.4	113.1	90.79 ± 6.07	6.6	104.6
100.0	100.6 ± 1.64	1.6	100.6	101.86 ± 2.88	2.8	101.9
Day 1	$y_1 = 0.034x - 0.0076$			$y_1 = 0.013x - 0.0021$		
Day 2	$y_2 = 0.035x - 0.0081$			$y_2 = 0.014x - 0.0020$		
Day 3	$y_3 = 0.046x - 0.0134$			$y_3 = 0.021x - 0.0045$		
Day 4	$y_4 = 0.054x - 0.0256$			$y_4 = 0.026x - 0.0126$		

Table 4
Intra- and interday validation of the method for quantitative determination of DOX in human plasma.

Concentrations	LLOQ 1.00	QC1 3.00	QC2 15.00	QC3 50.00
Interday				
Measured concentration				
Day 1	0.99	2.82	17.68	46.01
	1.13	2.74	15.46	46.31
	1.04	2.94	14.86	57.93
	0.98	3.28	17.72	55.34
		3.32	16.20	
Day 2	0.98	2.80	17.96	44.78
	1.13	3.08	17.30	47.64
	1.05	3.57	17.98	45.56
	0.98	3.58	17.38	58.35
		3.39	16.95	59.07
Day 3	1.07	3.21	16.34	55.71
	1.02	3.13	16.43	55.15
	1.12	2.97	13.78	56.54
	1.12	3.30	15.73	47.22
		3.19	14.46	48.75
Day 4	1.16	3.16	14.69	58.95
	1.16	2.96	14.09	47.02
	1.16	2.77	12.49	48.20
	1.05	3.07	12.50	48.17
		2.98	13.57	54.78
Mean ± SD (QCs; *n*=20)	1.07 ± 0.068	3.11 ± 0.25	15.68 ± 1.79	51.66 ± 5.28
Precision (%)	6.3	7.9	11.4	10.2
Accuracy (%)	108.1	103.7	104.1	103.3
Intraday				
Mean ± SD (*n* = 5)	1.08 ± 0.051	3.16 ± 0.12	15.35 ± 1.18	52.67 ± 4.34
Precision (%)	4.7	3.8	7.6	8.2
Accuracy (%)	107.10	105.3	102.3	105.6

reproducibility and accuracy of the method was further demonstrated by the reanalysis of incurred plasma samples collected on the first day of therapy from one patient given 50 mg/vial in the initial phase of the study. The concentrations of DOX and DOXol determined on the two occasions were practically identical in all samples. The second analysis obtained data included between 85.7% and 108.7%. DOX and DOXol in human plasma were stable at least for 4 h at room temperature, and at least 72 h in the autosampler after extraction in dark conditions. In the autosampler at room temperature, DOX and DOXol exhibited changes from control, which were acceptable because the analytes decreased in concentration by 4.5% at the two levels of the quality controls (3.0 and 50.0 ng/mL). The analytes were stable in human plasma over three freeze–thaw cycles because the concentration left was more than 95% of the nominal concentration. DOX decreased in concentration by 3.9% and DOXol by 4.5%. After three months at −20 °C, the

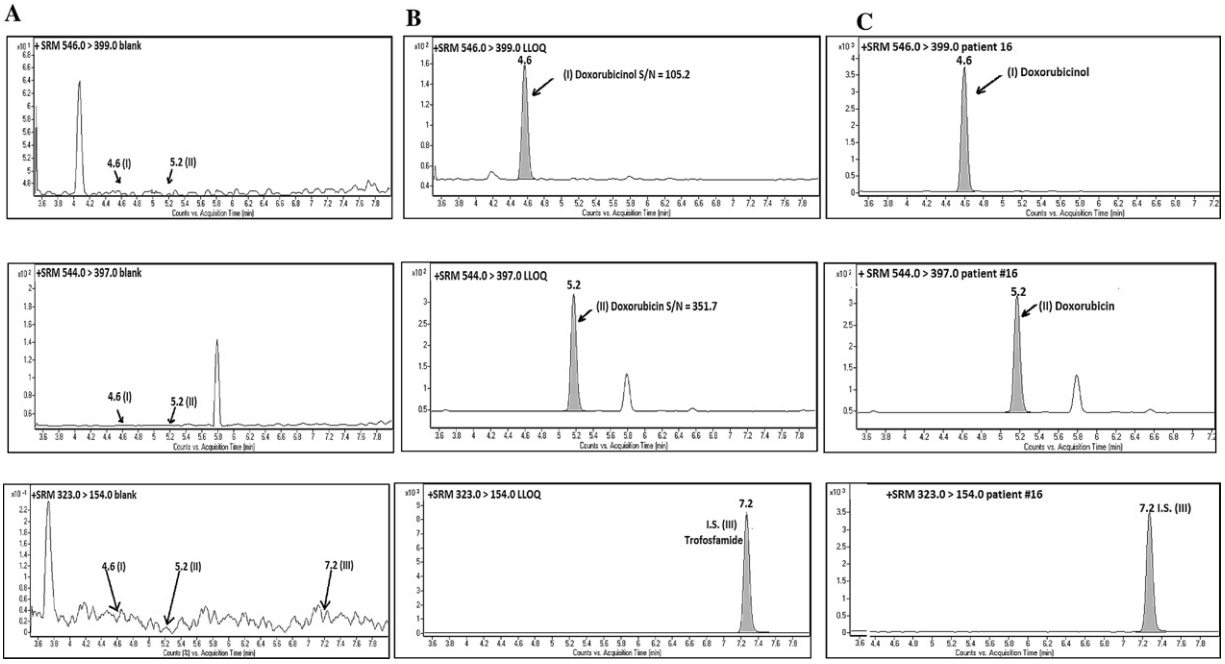

Fig. 2. Representative SRM chromatograms for doxorubicinol (I), doxorubicin (II), and trofosfamide (IS, III) in human plasma; (A) blank plasma sample; (B) blank plasma sample spiked with doxorubicin, its metabolite doxorubicinol (1 ng/mL), and trofosfamide (20.0 ng/mL), (C) plasma sample obtained at 24 h after the starting of TACE procedure in one patient (patient #1) receiving 50 mg/vial.

Table 5
Intra- and interday validation of the method for quantitative determination of DOXol in human plasma.

Concentrations	LLOQ 1.00	QC1 3.00	QC2 15.00	QC3 50.00
Interday				
Measured concentration				
Day 1	1.00	2.98	15.38	54.10
	1.09	3.18	15.21	46.98
	1.05	3.42	14.65	58.06
	0.97	3.47	17.35	58.61
		3.30	16.53	45.17
Day 2	1.05	2.91	16.84	47.87
	1.12	3.13	16.91	45.28
	0.96	3.58	17.19	49.42
	0.96	3.37	16.65	56.69
		3.43	15.79	53.98
Day 3	1.05	3.42	16.45	54.21
	0.97	3.07	15.99	54.69
	1.07	2.96	14.08	45.12
	1.10	3.37	15.58	47.26
		3.22	14.04	52.46
Day 4	1.13	3.06	14.48	43.88
	1.16	2.77	13.53	44.26
	1.16	2.69		
	1.06	3.02	13.16	45.33
		2.90	12.83	52.58
Mean ± SD (QCs; $n = 20$)	1.06 ± 0.07	3.16 ± 0.25	15.26 ± 1.52	50.00 ± 5.06
Precision (%)	6.5	7.9	9.9	10.1
Accuracy (%)	105.6	104.7	101.7	96.1
Intraday				
Mean ± SD (QCs; $n = 5$)	1.05 ± 0.05	3.21 ± 0.19	15.23 ± 1.11	51.05 ± 4.51
Precision (%)	5.2	6.1	7.3	8.8
Accuracy (%)	104.6	96.3	101.5	102.9

concentrations were 80.2%, 92.5%, and 86.6% of the nominal value of the QC samples prepared at 3.0, 15.00, and 50.0 ng/mL, respectively. The standard working solutions of analytes and IS, prepared in methanol and stored at −20 °C, were stable after 6 months (range 93.4–96.4% and 98.8% for the IS).

4.3. Pharmacokinetic study

Fig. 3 shows representative plasma concentration-versus-time curves of DOX and DOXol in one patient receiving 50 mg/vial of DOX in TACE treatment.

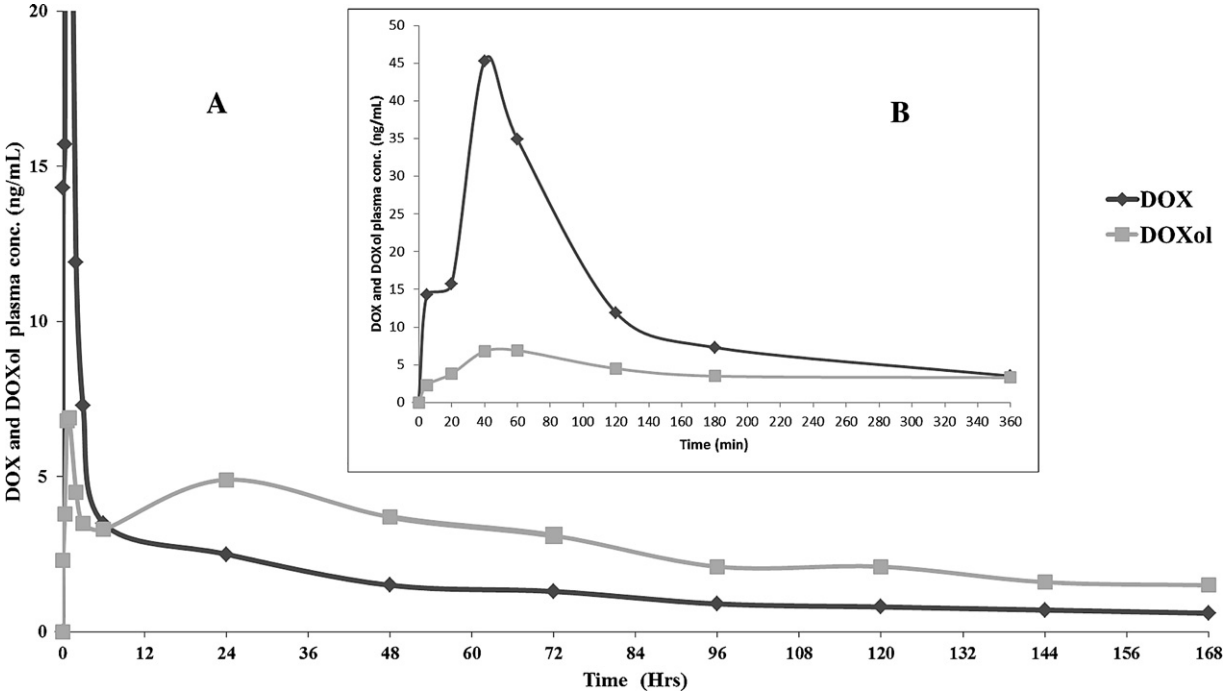

Fig. 3. Plasma concentration-versus-time profiles of DOX and DOXol in one patient (patient #16) receiving 50 mg/vial of the drug loaded in HepaSphere™, reconstituted in 20 mL 0.9% normal saline and administered by TACE procedure. (A) Full DOX and DOXol profiles (time, 0–168 h), (B) zoomed DOX and DOXol profiles (time, 0–6 h).

For the parent drug, the maximum plasma concentration (C_{max}) was 45.2 ng/mL and the time at which the concentration is maximum (T_{max}) was observed at 40 min, followed by a slow decrease in a time-period of 96 h (Fig. 3, panels A and B).

For the metabolite, C_{max} was 6.9 ng/mL and T_{max} was observed at 1 h (T_{max}). The concentrations of DOXol in this patient was longer than 24 h, so DOXol, the metabolite, was detectable for up to 7 days, at levels approximately 1–5 times the LLOQ (Fig. 3, panel A). On day 5, the doxorubicin had levels below the LLOQ. The present findings have proven that using drug-loaded particles or microspheres a sustained and somewhat controlled drug delivery is achieved with very low plasma levels. In agreement with our preliminary data regarding the pharmacokinetic profile of the pre-loaded epirubicin, this study confirmed a similar PK profile also for doxorubicin with a continuous drug elution from microspheres. The plasma concentration of the main metabolite, DOXol, mirrored that of doxorubicin parent compound with an initial increase followed by a slow decline up to 6 h when the concentrations of the two compounds in the systemic circulation were the same (~3.5 ng/mL). Then, the pharmacokinetic profile of doxorubicinol was different from that of doxorubicin with a prolonged increase particularly over the time-period included between 6 and 24 h of the PK study. After 24 h, the circulating plasma level of the metabolite was 2.5 times-fold of the parent compound and was 2.0 ng/mL on day 7 from the beginning of TACE procedure. In the present study the increase of DOXol plasma concentrations was combined with a decrease in plasma doxorubicin levels as previously reported in a rabbit model of liver cancer study [24]. Also, in accordance with preclinical and clinical studies [18,20,21,23–25], the doxorubicin pharmacokinetic profile provided by this loco-regional drug delivery system exhibited lower systemic drug concentrations compared with conventional TACE and a more classic intra-arterial injection of drug alone.

5. Conclusion

The described method based on a simple SPE procedure and HPLC–MS/MS determination quantifies DOX and its reduced metabolite, DOXol, in human plasma of HCC patients. The method, which has been successfully validated, is rapid, selective, sensitive, precise, and accurate. The present study showed that when doxorubicin is loaded in HepaSphere and delivered transarterially, the plasma concentration of doxorubicinol is lower than or equal to its parent compound up to 6 h. Then, the metabolite increases and is detectable over the pharmacokinetic study for at least seven days post dosing.

This method is currently in use to measure plasma concentrations of DOX and DOXol in samples from cancer patients, providing the first pharmacokinetic profile of doxorubicinol during TACE procedures.

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