Separation and Determination of Liposomal and Non-Liposomal (Free) Doxorubicin from Human Plasma by SPE and LC-MS/MS

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Key Words
SPE, SOLA, Accucore C18, solid core, doxorubicin, daunorubicin

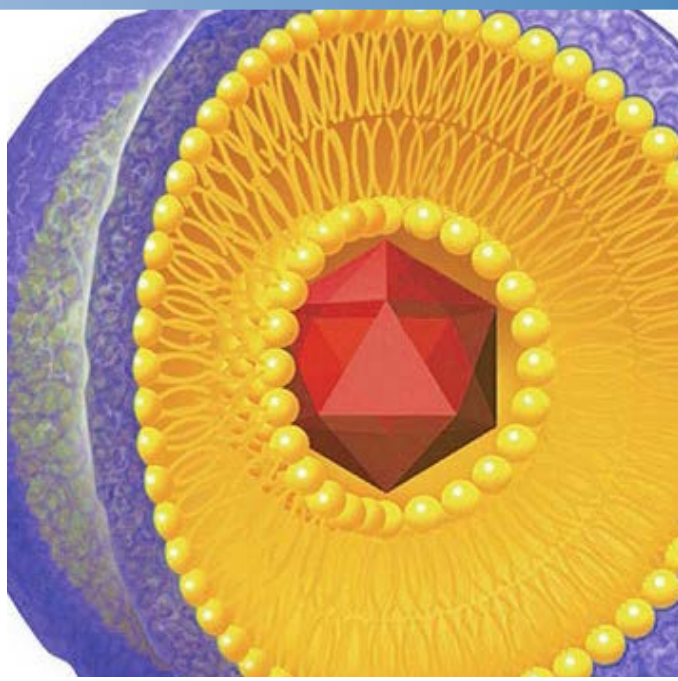
Abstract
A separation and determination of liposomal (encapsulated) and non-liposomal (free) doxorubicin in human plasma is described. The separation was based upon the selective retention of liposomal (encapsulated) doxorubicin and non-liposomal (free) doxorubicin on the hydrophobic reversed-phase Thermo Scientific™ SOLA™ HRP solid phase extraction (SPE) cartridge. The former exhibited no retention, while the latter was retained on the stationary phase and eluted with acidified methanol.

After separation, the liposomal (encapsulated) fraction was treated with a protein precipitation methodology to disrupt the liposome with subsequent SPE clean up. The resultant extracts were separated on a Thermo Scientific™ Accucore™ C18 HPLC column under reversed-phase gradient conditions. Detection was performed on a Thermo Scientific™ TSQ Vantage™ triple quadrupole mass spectrometer using positive polarity, heated electrospray ionization (HESI) conditions operating in selected reaction monitoring (SRM) mode.

Daunorubicin was used as an internal standard. Excellent peak shape and linearity over the dynamic range 1 to 500 ng/mL was achieved for doxorubicin.

Introduction
A pegylated liposomal formulation of doxorubicin has been approved worldwide for the treatment of a variety of human tumors. Preclinical and clinical studies have shown this liposomal formulation decreases the cardiotoxicity and improves the therapeutic index of the drug compared with conventional doxorubicin formulation.

To understand the efficacy and toxicity of liposomal drugs, it is essential to establish a method for separation and determination of liposomal and non-liposomal (free) doxorubicin present in circulating blood. The purpose of this particular study is to demonstrate the effectiveness of combining SOLA, a revolutionary solid phase extraction (SPE) device, and an Accucore C18 HPLC column for the determination of liposomal and non-liposomal (free) doxorubicin in human plasma with tandem mass spectrometry detection. The structures of doxorubicin and the internal standard, daunorubicin, are shown in Figure 1.



The first-in-class SOLA SPE product range introduces next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products. These include:

- Higher levels of reproducibility
- Higher levels of extract cleanliness
- Reduced sample and solvent requirements
- Increased sensitivity

SOLA products have significant advantages for the analyst when processing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical laboratories where reduced failure rate, higher analysis speed, and lower sample/solvent requirements are critical.

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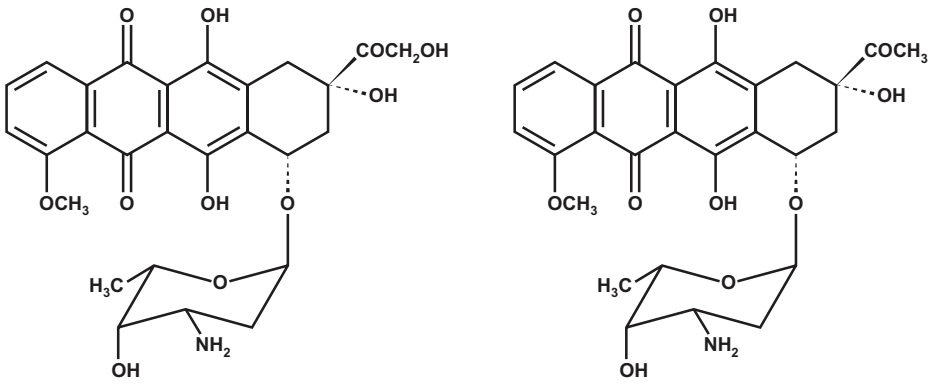

(a) Doxorubicin

(b) Daunorubicin

Figure 1. Structures of doxorubicin (a) and daunorubicin (IS) (b)

The increased performance of SOLA SPE gives higher confidence in analytical results and lowers cost without compromising ease of use or requiring complex method development.

Accucore HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6 µm diameter particles are not totally porous, but instead have a solid core and a porous outer layer. The optimized phase bonding creates a series of high coverage, robust phases. This coverage results in a significant reduction in secondary interactions and thus yields highly efficient peaks with very low tailing.

Experimental Details

Consumables	Part Number
Fisher Scientific™ Optima™ LC/MS grade methanol	A456-1
Purified water	
SOLA HRP SPE cartridge 10 mg/1 mL	60109-001
Fisher Scientific Optima formic acid, 90%, LC/MS grade	A117-50
Doxorubicin and daunorubicin, kindly supplied by a customer	
Thermo Scientific borosilicate glass vials (2 mL, 12 × 32 mm) with 8 mm black screw top fitted with a silicone/PTFE seal	60180-600

Sample Handling Equipment	Part Number
Thermo Scientific™ FinnPipette™ (100–1000 µL)	4642090
FinnPipette (20–200 µL)	4642080
FinnPipette (2–20 µL)	4642060
Thermo Scientific™ Finntip™ Flex™ 1000	94060720
Finntip Flex 200	94060320

Sample Pretreatment
A standard spiking solution of doxorubicin was prepared in methanol / water (50:50 v/v) at a concentration of 0.2 mg/mL. An internal standard solution (daunorubicin hydrochloride) was prepared in methanol / water (50:50 v/v) at a concentration of 0.1 mg/mL.
Blank human plasma (285 µL) was added to 300 µL of phosphate buffered saline, pH 7.4. For standards and quality control (QC) samples, 15 µL of standard spiking solution and 15 µL of internal standard solution were added to 285 µL of human plasma. For blanks, 30 µL of water was added.
To prevent liposomal doxorubicin rupture, vortex mixing was not used.

Extraction Procedure
To separate liposomal and non-liposomal (free) doxorubicin the following procedure was used and is described in Figure 2.
a. Separation of liposomal and non-liposomal (free) doxorubicin
(A1) Condition: Add 0.5 mL methanol (to waste)
(A2) Equilibrate: Add 0.5 mL water (to waste)
For the next steps, collect the effluent of A3 and A4 as this contains the liposomal doxorubicin fraction. This fraction requires further clean up, described below.
(A3) Application: load pre-treated sample (collect)
(A4) Wash 1: 2 × 0.5 mL phosphate buffered saline, pH 7.4 added sequentially (collect)
Allow A5 to go to waste but Collect A6 as this fraction contains non-liposomal (free) doxorubicin
(A5) Wash 2: 2 × 1 mL water / methanol (90:10 v/v) added sequentially (to waste)
(A6) Elution: 0.5 mL methanol + 0.1% formic acid (collect)
Dilute the eluent (A6) with an equal amount of water (0.5 mL) prior to LC-MS/MS analysis.
b. Further clean up of liposomal doxorubicin fraction
To a 25 µL aliquot of vortex-mixed liposomal doxorubicin fraction (combined effluent from A3 and A4), add 15 µL of internal standard solution. Add 75 µL of acetonitrile to disrupt the liposome structure and dilute with 800 µL water prior to loading on the SPE cartridge.
(B1) Condition: Add 0.5 mL methanol (to waste)
(B2) Equilibrate: Add 0.5 mL water (to waste)
(B3) Application: load combined A3 and A4 effluent (to waste)
(B4) Wash: 2 × 1 mL water / methanol (90:10 v/v) added sequentially (to waste)
(B5) Elution: 0.5 mL methanol + 0.1% formic acid (collect)
Dilute the eluent (B5) with an equal amount of water (0.5 mL) prior to LC-MS/MS analysis.

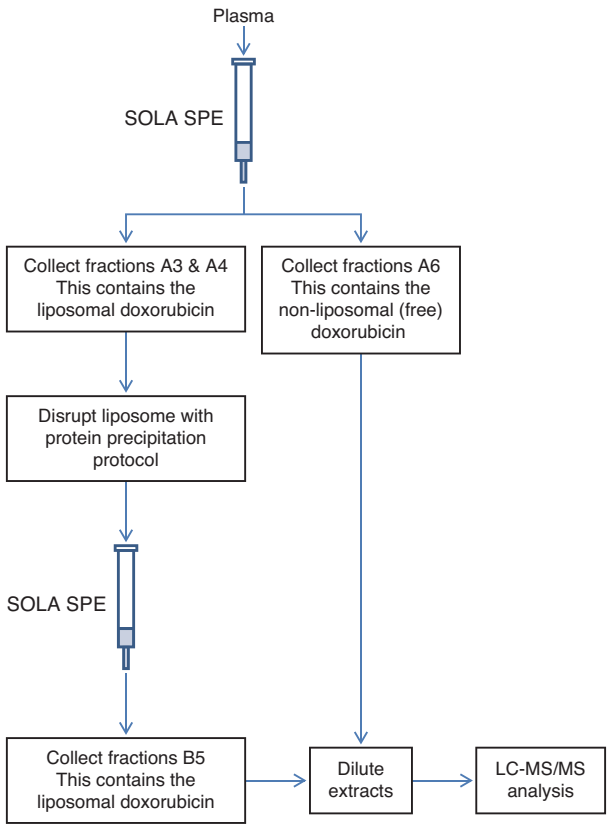

Figure 2: General scheme for the separate extraction of liposomal and non-liposomal (free) doxorubicin from plasma

Verification of Liposomal and Non-Liposomal (Free) Doxorubicin Separation by SPE

To verify the suggested protocol (liposomal and non-liposomal (free) doxorubicin separation by SPE) meets the intended results, doxorubicin hydrochloride liposome injection, 2 mg/mL (pegylated liposomal), was spiked in plasma at L/M/H levels (3,000, 30,000, and 60,000 ng/mL) and liposomal (encapsulated) and non-liposomal (free) doxorubicin content was estimated using the doxorubicin calibration curve. If the estimated results of non-liposomal (free) doxorubicin content are close to the label claim of 1.5–2.5% inherent free doxorubicin in spiked doxorubicin hydrochloride liposome injection, it can be concluded that the suggested separation protocol met the intended results.

A 5% dextrose solution was employed as diluent for serial dilutions of doxorubicin hydrochloride liposome injection.

Separation Conditions		**Part Number**
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000RS system	
Column:	Accucore C18 HPLC column, 2.6 µm, 50 × 2.1 mm	17126-052130
Mobile phase A:	Water + 0.1% formic acid	
Mobile phase B:	Methanol + 0.1% formic acid	
Mode:	Gradient (Refer to Table 1)	
Flow rate:	0.4 mL/min	
Column temperature:	30 °C	
Injection details:	10 µL	

Time (min)	% B
0	10
0.2	10
4.0	90
4.2	10
5.0	10

Table 1: Mobile phase gradient

MS Conditions	
Instrumentation:	TSQ Vantage mass spectrometer
Ion source type:	HESI-2
Polarity:	Positive
Spray voltage:	3,000 V
Vaporizer temperature:	250 °C
Sheath gas pressure:	50 arb
Ion sweep gas pressure:	0 arb
Auxiliary gas pressure:	10 arb
Capillary temperature:	350 °C
Declustering voltage:	0 V
Collision pressure:	1.5 mTorr
Scan width:	0.02 *m/z*
Scan time:	0.1 s
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7

The transition details for doxorubicin and daunorubicin are provided in Table 2.

Compound	Doxorubicin	Daunorubicin (IS)
Parent (*m/z*)	544.2	528.2
Products (*m/z*)	361.1	321.1
Collision energy	24	25
S-lens	77	76

Table 2: Compound transition details

Results

Doxorubicin standards extracted from human plasma gave a linear calibration curve over the dynamic range of 1 to 500 ng/mL with an r^2 coefficient of 0.999 (Figure 3 and Table 3). The chromatography of the limit of quantitation (LOQ) at 1 ng/mL is shown in Figure 4.

QC samples were analyzed in replicates of six at concentrations of 3, 200, and 400 ng/mL (Table 4). Overspikes (of doxorubicin) were analyzed at concentrations of 3, 200, and 400 ng/mL and used to calculate recovery and matrix interference (Table 5).

In addition, the label claim that the doxorubicin hydrochloride liposome injection contains between 1.5–2.5% of free doxorubicin was tested. Blank plasma was spiked with doxorubicin hydrochloride liposome injection at concentrations of 3,000, 30,000, and 60,000 ng/mL and the level of free doxorubicin was calculated. The non-liposomal (free) doxorubicin results at each QC level were very close to the label claim of 1.5–2.5% free doxorubicin (Table 6).

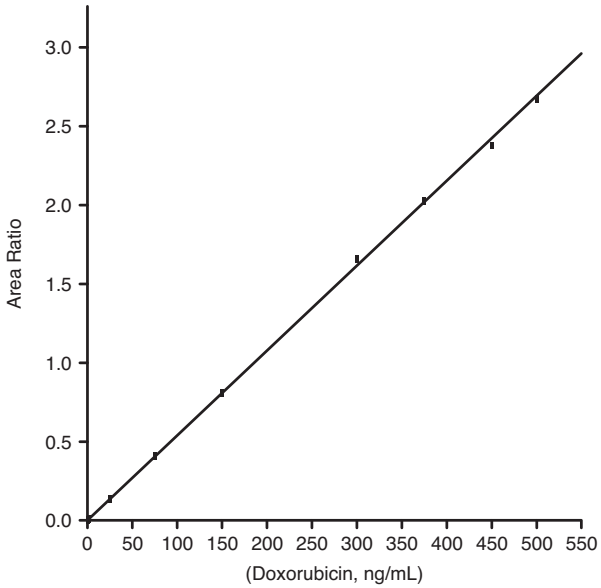

Figure 3: Doxorubicin linearity over the dynamic range 1–500 ng/mL

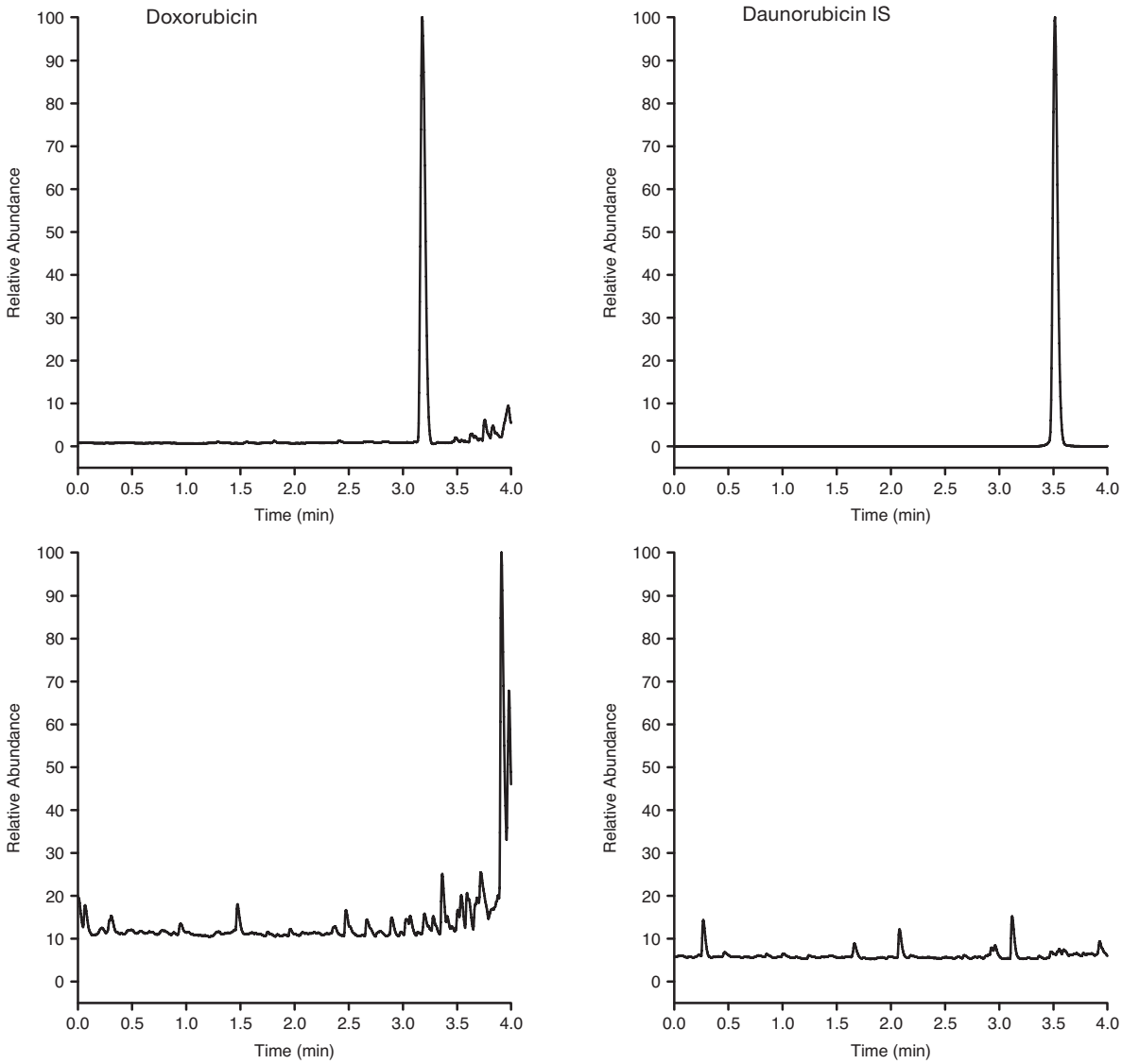

Figure 4: Representative chromatograms of doxorubicin SRM, extracted from human plasma (at 1 ng/mL top left, blank bottom left) and daunorubicin IS (top right, blank bottom right)

Accuracy and Precision

Standard	Specified Concentration [doxorubicin] (ng/mL)	Calculated Concentration [doxorubicin] (ng/mL)	% Diff
S1	1	1.0	-0.6
S2	2	1.9	-4.8
S3	25	25.7	2.9
S4	75	76.3	1.7
S5	150	150.6	0.4
S6	300	307.7	2.6
S7	375	376.1	0.3
S8	450	441.7	-1.8
S9	500	496.9	-0.6

Table 3. Accuracy data for extracted standards over the linear range 1–500 ng/mL

Standard	Concentration (ng/mL)	Number of Samples (N)	Peak Area Ratio (%RSD)	Analyte Peak Area (%RSD)
QCL	3	6	7.3	9.5
QCM	200	6	2.2	4.1
QCH	400	6	2.9	3.8

Table 4. Average precision data for six replicate QCs for doxorubicin

Recovery

Standard	Response	% Recovery at Each Level	% Matrix Interference at Each Level
Average QCL response ratio	0.018	89.8	2.5
Average overspike response ratio	0.021		
Average aqueous response ratio	0.020		
Average QCM response ratio	1.053	96.6	3.0
Average overspike response ratio	1.090		
Average aqueous response ratio	1.058		
Average QCH response ratio	2.186	102.5	-1.5
Average overspike response ratio	2.133		
Average aqueous response ratio	2.166		

Table 5: Recovery and matrix interference data for doxorubicin

Standard	Concentration (ng/mL)	Number of Samples (N)	Calculated Liposomal Doxorubicin (ng/mL) (%RSD)	Calculated Non-liposomal (Free) Doxorubicin (ng/mL) (%RSD)	Calculated % of Non-liposomal (Free) Doxorubicin	Calculated Total Doxorubicin (% recovery)
QCL	3,000	6	3,100 (2.2)	140 (14.4)	4.3	108.0
QCM	30,000	6	31,909 (3.4)	827 (8.7)	2.5	109.1
QCH	60,000	6	61,364 (3.7)	1574 (14.1)	2.5	104.9

Table 6: Average precision data for six replicate QCs for doxorubicin hydrochloride liposome injection (pegylated liposomal)

Conclusion

- SOLA HRP SPE cartridges and Accucore C18 HPLC columns used with the TSQ Vantage mass spectrometer allow for simple and effective extraction, separation, and quantification of liposomal (encapsulated) and non-liposomal (free) doxorubicin from human plasma.
- The method exhibited good linearity (r^2 = 0.999) for concentrations of doxorubicin in the range 1–500 ng/mL.
- A limit of quantitation of 1 ng/mL doxorubicin in plasma was achieved.
- Extraction recovery and matrix interference were found within the limits of acceptance generally applied to bioanalytical methods.
- Good accuracy and precision with and without IS correction were observed for both liposomal (encapsulated) and non-liposomal (free) doxorubicin at each QC level (Table 4). This highlights the benefit of the SOLA design in facilitating robust analytical workflows.

Reference

[1] http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf

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