Quantification of Doxorubicinol with the Presence of Excessive Doxorubicin in Human Plasma by Column Switching and LC/MS/MS Techniques

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Introduction

Doxorubicin is an anthracycline glycoside antibiotic used in cancer chemotherapy. Its major metabolite, doxorubicinol (Figure 2), is cytotoxic and may be responsible for some of the major adverse effects. The selective detection of doxorubicinol using LC-MS/MS techniques has been dependent upon the chromatographic separation of doxorubicinol from doxorubicin. However, the separation becomes complicated by the presence of excessive levels of doxorubicin. Excessive doxorubicin affected detection of doxorubicinol through its isotopical pattern because the molecular weight of doxorubicinol is only two Daltons higher than that of doxorubicin (Figure 1). Doxorubicin levels can easily exceed 10,000 times doxorubicinol levels. With a conventional method using one column, the peak of doxorubicinol could not be well separated from the huge background of doxorubicin. A column switching technique using two different columns was developed in order to achieve effective separation of doxorubicinol from excessive doxorubicin often observed in liposome formulations such as Doxil®.

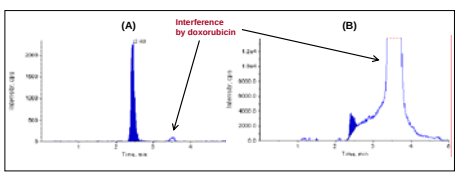

Figure 1. Chromatogram of doxorubicinol in MRM mode (546→399) using a single column. Doxorubicinol/Doxorubicin : (A) 1.0/1.0 ng/mL; (B) 1.5/20,000 ng/mL.

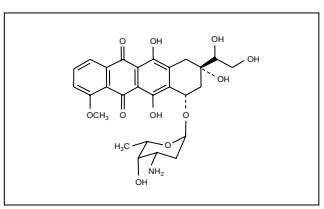

Figure 2. Structure of Doxorubicinol (MW 545.5)

Method

The plasma samples were processed using protein precipitation. The samples were injected on a C8 column with a flow rate of 0.35 mL/min using a two step gradient. The eluate containing the doxorubicinol peak was switched to a second column (C18) using six-port valve switching system. A single step gradient program was employed for the second column to achieve a baseline separation between doxorubicin and doxorubicinol.

Liquid Chromatography

Shimadzu 10ADvp HPLC system
Autosampler : PE 200
Column A : C8, Column B : C18
Mobile Phase A : ACN/H_2O/Formic Acid mixture
Mobile Phase C : Formic Acid in D.I. water
Mobile Phase B & D : Organic mixture

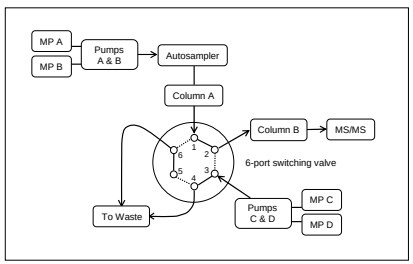

Figure 3. Diagram of LC/MS instruments.

Mass Spectrometry

Applied Biosystem/MDS-Sciex API 4000
Polarity : positive
Scan type : Multiple Reaction Monitoring
Doxorubicinol : 546.4 → 399.0
Doxorubicinol-^{13}C-d_3 (I.S.) : 550.4 → 403.0

Extraction: Protein Precipitation Method

Results

The method was fully validated over **a range of 0.5 to 256 ng/mL** with linear regression (1/x^2). The correlation coefficients for first three validation batches were 0.993 or better (Figure 4).

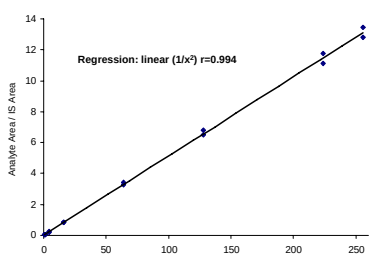

Figure 4. Calibration curve for 0.5 to 256 ng/mL Doxorubicinol

Figures 5 through 7 show typical chromatograms of extracted lower and upper limits of quatitation (LLOQ 0.5 ng/mL; ULOQ 256 ng/mL), and an extracted blank matrix injected after the ULOQ. At the LLOQ, the signal-to-noise ratio was greater than 50. **Figure 8** shows chromatogram of low QC sample in the presence of high concentration of doxorubicin (20,000 ng/mL).

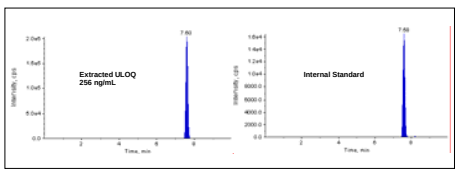

Figure 5. Chromatogram of an extracted ULOQ sample (256 ng/mL).

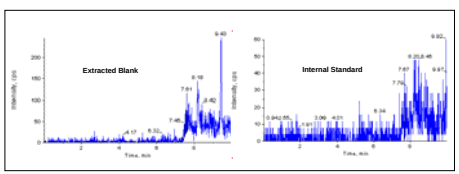

Figure 6. Chromatogram of an extracted blank sample injected after ULOQ.

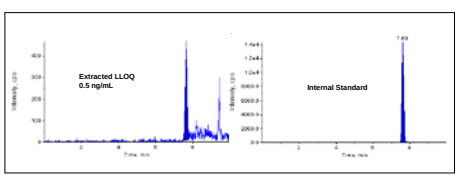

Figure 7. Chromatogram of an extracted LLOQ sample (0.5 ng/mL).

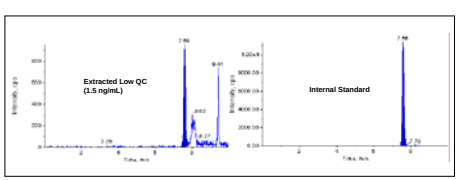

Figure 8. Chromatogram of an extracted Low QC sample (1.5 ng/mL) in Doxil® sample (20,000 ng/mL of doxorubicin).

Table 1 summarize result of inter-batch validation batch. All concentration levels demonstrate excellent precision and accuracy. **Table 2** indicates that Doxil® 20,000 ng/mL in human plasma did not show effect on the recovery of Doxorubicinol. The chromatographic separation provided good precision and accuracy for the low QC.

Table 1. Precision and Accuracy Results (inter-batch) for Doxorubicinol Quality Control Samples.

	0.5 ng/mL (LLOQ)	1.50 ng/mL (Low)	96.0 ng/mL (Mid)	192 ng/mL (High)
n	17	18	18	18
Mean	0.481	1.52	96.0	190
Precision (cv %)	12.1	6.4	2.7	2.5
Accuracy (bias %)	-3.9	1.3	0.0	-0.9

Table 2. Doxil® (20,000 ng/mL) Interference Check.

Sample name	Peak Area		Cal. Conc. (ng/mL)	% Bias
	Analyte	I.S.		
VAL AR 1.5 (Doxil® 20,000 ng/mL Doxorubicinol 1.50 ng/mL)	7553	90218	1.47	-2.2
	7115	84506	1.48	-1.6
	6305	76345	1.44	-3.7
	7115	89252	1.39	-7.4
	7657	92443	1.45	-3.4
	7324	85385	1.51	0.4
mean			1.46	
sd			0.039	
cv (%)			2.7	
bias (%)			-3.0	

Selectivity was tested by determining precision of low-level QCs (1.5 ng/mL) in six different lots of blank matrix (**Table 3**). No interference or matrix effects were observed.

Table 3. Matrix Effect for Doxorubicinol in Human Plasma.

	Avg drug area	Avg IS area	Avg area ratio
ME 1	10232.7	138560.5	0.0739
ME 2	10111.2	134371.2	0.0753
ME 3	9885.8	134077.2	0.0737
ME 4	10178.5	138136.3	0.0737
ME 5	9961.8	135038.8	0.0738
ME 6	10185.8	136910.5	0.0744
mean	**10092.6**	**136182.4**	**0.0741**
n	**6**	**6**	**6**
SD	**138.5**	**1950.6**	**0.000614**
%CV	**1.4**	**1.4**	**0.8**

Conclusion

A robust LC/MS/MS method for the determination of doxorubicinol with the presence of excessive doxorubicin in human plasma was developed and fully validated. This method showed acceptable accuracy, selectivity, and reproducibility, and was successfully applied to the analysis of clinical samples.