

## 마우스 혈장과 조직에서의 doxorubicin 측정 HPLC-MS/MS 방법

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## Validation of a HPLC MS/MS Method for Determination of Doxorubicin in Mouse Serum and its Small Tissues

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Doxorubicin (DXR) is a type of anti-cancer drug called an "anthracycline glycoside". It works by impairing DNA synthesis, a crucial feature of cell division, and thus is able to target rapidly dividing cells. Doxorubicin is a very serious anti-cancer medication with definite potential to do great harm as well as great good. A liquid chromatography-tandem mass spectroscopy (LC-MS/MS) method was developed to identify and quantify DXR in small-volume biological samples. After the addition of internal standard (IS, 5 µL of 1 µM/ml daunorubicin methanol solution) into the serum sample, the drug and IS were extracted by methanol. Following vortex for a 1 min and centrifugation at 15,000g for 10 min the organic phase was transferred and evaporated under a vacuum. The residue was reconstituted with 350 µL of mobile phase and 10 µL was injected into C18 column with mobile phase composed of 0.05 M ammonium acetate (0.1 M acetic acid adjusted to pH 3.5) and acetonitrile (40:60, v/v). The flow rate was kept constant at 350 µL/min. The ions were quantified in the multiple reaction mode (MRM), using positive ions, on a triple quadrupole mass spectrometer. The lower limits of quantification for Doxorubicin in plasma and small tissues were approximately 0.5 ng/mL and 0.5 ng/mL respectively. Intra- and inter-assay accuracy (% of nominal concentration) and precision (% CV) for all analytes were within 15%, respectively.

□ **Key words** - doxorubicin, daunorubicin, HPLC-MS/MS, validation

DXR is an anthracycline antibiotic that possesses broad spectrum antineoplastic activity, and is one of the most important anticancer agents in use<sup>1-5</sup>). However, clinical utility is hampered by cumulative, dose-limiting cardiotoxicity, myelosuppression, and the development of drug resistance.

DXR is composed of an aglycone backbone linked to a daunosamine sugar through an *O*-glycosidic bond at carbon 7. A variety of procedures to extract, resolve,

and quantify DXR has been published<sup>6-10</sup>) (Table 1). Many of these techniques are laborious and necessitate long analytical run times to achieve sufficient peak resolution, owing to the spectral and structural similarities of DXR<sup>11</sup>). Among the techniques, LC-MS/MS can permit and exact and efficient determination of doxorubicin. In this study, a simplified, rapid extraction procedure and a liquid chromatography-tandem mass spectroscopy (LC-MS/MS) method were developed to identify and quantify DXR and metabolites in plasma and tissue extracts. Tandem mass spectroscopy can permit the determination of DXR analysis and quantification in complex biological samples.

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**Table 1. The selected examples of extraction procedures and analytical techniques for quantitative analysis of DXR in plasma or tissue samples**

Species/samples	Extraction method	Recovery (%)	Detection	LOQ or LOD(nM)	Ref.
Murin plasma and tissues	Homogenised in PBS,	66-98	HPLC-Fluor	367	6
	Liquid/liquid extraction (chloroform/methanol,4:1(v/v))				7
Murin plasma and lung	Homogenised in methanol and Tris buffer(p,H 8.5), deproteinized with ACN	60-98	HPLC-Fluor	15	8
Rat, serum and tissues	Homogenized in phosphated buffer, deproteinized and extracted in methanol and ZnSO <sub>4</sub>	94-115	HPLC-Fluor	9-18	9
Human serum	Solid phase extraction (SPE)	97-105	HPLC-ES/MS	4.6	10

## Materials and Methods

Doxorubicin (>99% purity) were provided by Sigma (Chatham, NJ, USA). Daunorubicin (DNR) was purchased from Sigma (St. Louis, MO, USA); Water, acetonitrile, and methanol were from Burdick & Jackson (Muskegon, MI, USA). Ammonium acetate was from J.T. Baker (Phillipsburgh, NJ, USA). All chemicals and solvents were analytical or HPLC grades unless otherwise stated.

### Preparation of Samples

ICR 37 male mice were each given three times intravenous injection of doxorubicin total dose of 15 mg/kg. Blood samples were collected in heparinized tubes and cooled on ice at 0, 1, 2, 16, 24, 168 hour after 8-day post dose. Shortly after blood collection, serum was centrifuged at 4 and stored at -70°C until analysis. Tissues were collected at 8 days after doxorubicin administration. All samples were stored at -70°C. Before the analysis, all samples were prepared by extraction method, as described previously<sup>12)</sup>.

### LC/MS/MS Analysis

The analysis of DXR was performed using Applied Biosystems/MDS Sciex API 4000 triple quadrupole tandem mass spectrometers (ABI, Inc., Foster City, CA, USA) following chromatographic separation using an HPLC system consisting of an autosampler and dual pumps. HPLC system with a model 1100 Agilent (Palo Alto, CA, USA) was used. Separation was achieved by automated injection of 10 µL samples onto a reversed

phase analytical column (an Agilent Zorbax Extend Rapid Resolution 4.6 mm×50 mm 3.5 µm) under isocratic conditions at a flow rate of 400 µL/min.

### Determination of DXR Levels

The solvent for DXR extraction from plasma consisted of 60% acetonitrile (ACN) and 40% 0.05 M ammonium acetate, pH 3.5 and (v/v). Four hundred microliters of extraction solvent were added to 100 µL of plasma to achieve a final concentration of 20%. Standards and QC samples were prepared by serial dilution of DXR in extraction solvent prior to the addition of blank plasma. Ten microliters of DNR, the internal standard, was transferred into each sample immediately after the addition of extraction solvent to plasma, thus achieving a final concentration of 200 nM DNR. Samples were mixed, cooled in an ice water bath for 10 mins, and centrifuged for 10 mins at 15,000 g at 4°C. The deproteinized supernatant was recovered and analyzed immediately.

Frozen tissue samples were pulverized under liquid nitrogen using a mortar and pestle immersed in liquid nitrogen. Each tissue powders were weighed. A sufficient volume of mobile phase was added to each tube to achieve the desired tissue concentration. Ten microliters of the DNR standard was added per 10 microliters of DNR aliquot of tissue homogenate, producing a final concentration of 100 nM DNR. Samples were mixed briefly (<1 min) using a homogenizer (Tekmar, Cincinnati, OH, USA), cooled in an ice water bath, and centrifuged for 10 mins at 15,000g at 4°C. The deproteinized supernatant was recovered and analyzed immediately.

**Table 2. Compound-dependent instrumental parameters for doxorubicin and IS**

Compound	Retention time(min)	Selected ion pairs(m/z)	Declustering potential	Entrance Potential	Collision energy	Collision exit potential
Doxorubicin	1.19	544/361	103	9.86	19.5	11.1
Daunorubicin	1.46	528/321	76	9.7	22.5	10

### Method Validation

Extraction efficiencies for plasma and each tissue were determined by comparing the peak area of DXR standards extracted from tissue samples ( $n=5$ ) with DXR standards prepared in mobile phase. No statistical differences in peak area were observed for plasma or for each of the tissues examined. Standard and quality control samples were prepared by extracting tissues obtained from untreated animals. Serial dilutions of DXR was added to blank tissue homogenates and processed as described above.

### Results and Discussion

A sensitive and reliable HPLC-MS/MS method has been developed and validated for the simultaneous quantification of doxorubicin in mouse plasma and its small tissues. The respective retention times for Doxorubicin and the IS (Daunorubicin) were approximately 1.19 min and 1.46 min (Table 2). The quantification limit for doxorubicin in mouse plasma was 0.5 ng/mL. Standard and quality control samples were prepared by extracting tissues obtained from untreated animals. Serial dilutions of DXR were added to blank tissue homogenates and processed as described above.

To determine the intra-day and inter-day accuracy and precision, we analyzed three quality control (QC) samples prepared at nominal concentrations of 0.5, 2 and 20 ng/mL for doxorubicin. Quantification was based on the ratios of the peak areas of each analyte. Validation was performed through establishing inter- and intra-day accuracy and precision of the method on QC samples. Intra-day variability was tested on three QC samples using the same calibration curve, and inter-day variability was tested on three different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as a coefficient

of variation (CV) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was determined as the ratio (percentage) of the mean with reference to the true value.

The precision and accuracy data for the assay are presented in Table 3. The intra-day precision of the assay was less than 8.3% for each of the three concentrations of the QC samples. The inter-day precision of the assay was less than 8.8% for all the QC samples. At these concentrations, the intra- and inter-day CVs were determined to be <8.8%. All mice were each given three times intravenous injection of doxorubicin total dose of 15 mg/kg. Samples were collected 8-day post dose. Mean brain concentration of DXR is 1.23, heart is 3.19, liver is 293, spleen is 455 and lung is 141 ng/mL (Table 4). Figure 2 shows that the mean of plasma concentration of DXR.

As a result, reliable quantification of doxorubicin can

**Table 3. Results of the precision and accuracy studies for doxorubicinone in serum and tissue samples**

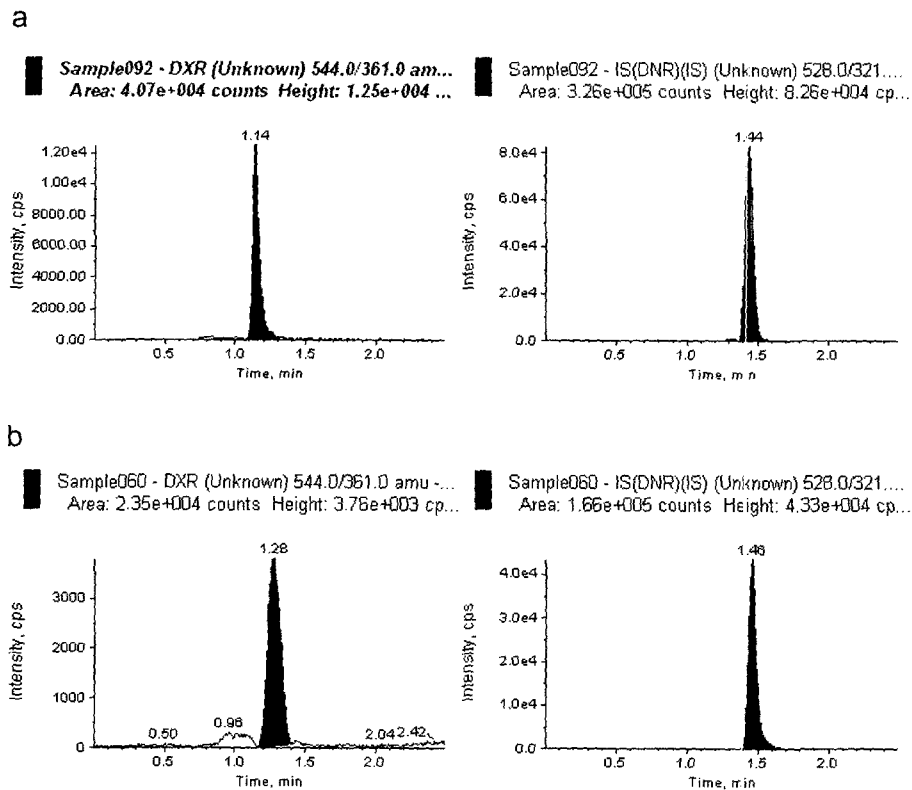
Concentration (ng/ml)	Recovery (% , mean $\pm$ S.D., n=3)				
	Serum	Heart	Liver	Spleen	Lung
0.5	92.8 $\pm$ 8.6	91.5 $\pm$ 0.2	96.2 $\pm$ 6.4	94.7 $\pm$ 3.1	92.5 $\pm$ 3.6
2	100.3 $\pm$ 0.6	97.2 $\pm$ 1.3	98.2 $\pm$ 1.0	97.2 $\pm$ 1.3	99.2 $\pm$ 2.0
20	92.8 $\pm$ 0.1	99.9 $\pm$ 0.1	100.0 $\pm$ 0.1	96.2 $\pm$ 6.4	100.0 $\pm$ 0.1

Quality control solution of DXR was prepared in blank plasma and tissues homogenates at known concentrations (v/v) or (w/v), extracted and analyzed by LC-MS/MS. The intra- (n=3) and inter-day (n=3) assay performance was determined.

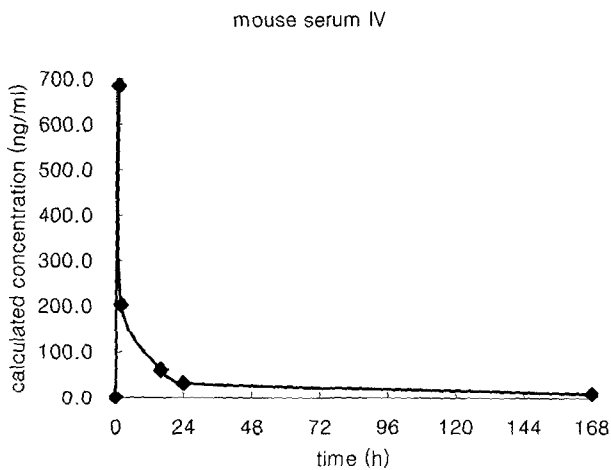
**Table 4. Mean tissues concentration of DXR in mice treated intravenously with total injection amount of 15 mg/kg DXR**

Sample	Brain	Heart	Liver	Spleen	Lung
Mean of concentration (ng/ml)	1.23	3.19	293	455	141

All mice were each given three times intravenous injection of doxorubicin total dose of 15mg/kg. Samples were collected 8-day post dose.



**Fig 1.** MRM chromatograms of (a) plasma standard 2ng/ml (left: doxorubicin (DXR), right: daunorubicin (DNR) and (b) DXR and DNR in heart samples)



**Fig 2.** Mean plasma concentration of DXR in mice treated intravenously with total injection amount of 15 mg/kg DXR.

be achieved over the range of 0.5 ng/mL (plasma) and 0.5 ng/mL (tissue), respectively. This method has been successfully used to study the pharmacokinetic profiles and biliary excretion of doxorubicin in mouse after a dose administration of doxorubicin. As this study of the

determination of doxorubicin in mouse, a simple and reproducible method to avoid extended homogenization steps that may promote degradation. It provides a significant increase in assay sensitivity and a considerable reduction in time of analysis. Given the structural similarities among the clinically-used anthracyclines, this assay and extraction procedure may be easily adapted for use with other such agents.

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