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# A Simple HPLC Method for Doxorubicin in Plasma and Tissues of Nude Mice

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Doxorubicin is a cytotoxic anthracycline that has been used for the treatment of several malignancies. Several HPLC methods have been reported for the quantification of doxorubicin in biological samples. Tissue matrix effect and sample size requirements, however, have been remaining issues for simple and easy-to-adapt analytical methods in small animal experiments. The present study established a simple HPLC method for doxorubicin in plasma and tissues (tumor, heart, spleen, liver, gastrointestinal tract, brain, lung, and kidney) of nude mice. Our method required a small sample volume (100 μL plasma and 10 mg tissue), which made it possible to use each blank tissue for calibration curves. The limit of quantification was 25 ng/mL in plasma and 0.1 to 0.4 μg/mg in other tissues with recovery rates ranging from 52.4 to 95.2%. The linearity, accuracy and precision in all tissues, except gastrointestinal tract (GIT), were found to be acceptable in the range of 25-2000 ng/mL plasma and 0.1-4 ng/mg tissue. This method was used successfully to determine the drug concentration in plasma and tissues of human tumor xenograft-bearing nude mice given intratumoral doxorubicin in a polymeric drug delivery system designed for sustained release. In conclusion, the present method may be useful as a simple and easy-to-adapt, yet, sensitive analytical method of doxorubicin for plasma and tissue pharmacokinetic studies in small animals such as nude mice.

**Key words:** Doxorubicin, HPLC, Plasma, Tissue, Nude mice, Human tumor xenograft

## INTRODUCTION

Doxorubicin (DOX) is a cytotoxic anthracycline antibiotic originally isolated from *Streptomyces peucetius* var. *caesius* and has been used for the past 30 years in the treatment of several hematologic as well as non-hematologic malignancies. DOX, similar to other anti-cancer drugs, shows unique cardiotoxicity as well as other general toxicities, such as myelosuppression (Arcamone et al., 1969; Hortobagyi, 1997; Lown, 1993; Licata et al., 2000). The pharmacokinetics of DOX determines its anti-tumor activity, as well as its toxicity profile. For better efficacy and toxicity profile, various drug delivery systems (DDS) and administration routes have been developed, including local delivery systems (Bagalkot et al., 2006; Weinberg et al., 2007; Gao et al., 2005). These DDSs, in the form of sustained release formulation, have presented a need for analytical methods with high sensitivity and specificity in various tissues of experimental animals (Loadman and Calabrese, 2001; Kummerle et al., 2003; Arnold et al., 2004; van Asperen et al., 1998).

Many methods have been reported for the analysis of DOX in biological samples using HPLC systems (Table I) (Loadman and Calabrese, 2001). Many detection methods have been employed, including UV (Tassin et al., 1997), electrochemical detector (Ricciarello et al., 1998), tandem mass spectroscopy (Arnold et al., 2004), and the use of radioactive isotopes (Behnia and Boroujerdi, 1999), however, most of the methods utilized fluorescent detection due to the strong fluorescence properties of the anthracyc-

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lines (van Asperen et al., 1998; de Bruijn et al., 1999). The HPLC/fluorescent detection method for analysis of DOX in biological matrices is easily adaptable, with sensitivity and selectivity comparable to other methods.

Sample pretreatment and extraction is considered very important steps that need improvement (Loadman and Calabrese, 2001). Compared to solid phase extraction (Ricciarello et al., 1998) or liquid-liquid extraction (Gilbert et al., 2005), a single step protein precipitation method is considered ideal for economic and time-saving purposes (Loadman and Calabrese, 2001). In addition, the limit of detection/quantification of DOX presents an issue for analysis of biological samples of small animals. This issue has been tackled by increasing the sample size (Chin et al., 2002; Bibby et al., 2005). Most of the analysis methods ignored or underestimated the matrix effect and used different matrix for calibration, which resulted in compromised sensitivity and specificity (Table I) (Alvarez-Cedron et al., 1999; van Asperen et al., 1998; Kummerle et al., 2003).

Herein, we developed and validated a simple, specific, and sensitive HPLC/fluorescence detection method for the analysis of DOX in plasma and tissues from nude mice. The method uses simple extraction procedures and a very small sample size (100 μL plasma and 10 mg tissue). We used respective calibration curves for each tissue and successfully applied the present method to study the plasma and tissue pharmacokinetics of DOX given as a thermo-sensitive polymeric DDS in tumor-bearing nude mice.

## MATERIALS AND METHODS

### Chemicals and reagents

Doxorubicin hydrochloride and daunorubicin hydrochloride (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $ZnSO_4$ was purchased from Merck Co. (Darmstadt, Germany). Heptanesulfonic acid sodium salt (HPLC-grade) was purchased from TCI Co. Ltd. (Tokyo, Japan). Acetone and other reagents, including solvents, were of the highest analytical grade and were purchased from Junsei Co. Ltd. (Tokyo, Japan). Deionized water (18.2 MΩ, NANOpure diamond™, Brandstead water purification system, Fistreem International Co. Ltd. Leicestershire, U.K.) was used throughout the experiment.

### Collection of murine specimens

Human gastric cancer cells, SNU-601, were obtained from Korean cell line bank (Seoul, Korea) and maintained in RPMI-1640 media supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin, and 10% heat-inactivated fetal bovine serum. The cultures were kept at 37ºC in a humidified atmosphere supplied with 5% $CO_2$. Female Balb/C-nu/nu mice were maintained in pathogen-free area with free access to food and water, in the animal facility of the Catholic Medical Center (Seoul, Korea). On the day of tumor induction, SNU-601 cells were harvested in serum-free media, and $1\times10^7$ cells were injected s.c. into the flank region of the mice. At the proper tumor size (300-500 $mm^3$), mice were anesthetized, and blood samples were collected by retro-orbital plexus puncture in heparinized tubes, and then centrifuged for plasma separation. Imme-

**Table I.** Recent representative methods of HPLC analysis for DOX quantification in biomatrices

| Author | Detection | Calibration matrix | Validation /sample matrix | Sample size | LOQ |
|---|---|---|---|---|---|
| van Asperen et al. | FL | Human plasma | Mouse plasma<br>Mouse tissues | 200 μL<br>40-70 mg | 1.3 ng/mL<br>96 ng/mg |
| Bruijin et al. | FL | Human plasma | Patient plasma | 1000 μL | 100 ng/mL |
| Alvarez-Cedron et al. | FL | Human plasma | Rat plasma<br>Rat tissues | 150 μL<br>7.5 mg | 5 ng/mL<br>100 ng/mg |
| Kummerle et al | FL | Human plasma | Pig/rat plasma<br>Pig/rat tissues | 500 μL<br>125 mg | 2 ng/mL<br>100 ng/mg |
| Ricciarello et al. | ECD | Human plasma | Human plasma | 200 μL | 1 ng/mL |
| Gilbert et al. | FL | Parrot plasma | Parrot plasma | 100 μL | 20 ng/mL |
| Hu et al. | FL | Rabbit tissues | Rabbit tissues | 100-200 mg | 30-50 ng/mL |
| Arnold et al. | MS/MS | Rat plasma<br>Rat tissues | Rat plasma<br>Rat tissues | 100 μL<br>30-100 mg | 0.2 ng/mL<br>0.15-15 ng/mg |

FL: Fluorescent

diately after blood sampling, animals were sacrificed by cervical dislocation. Organs (tumor, heart, lung, liver, kidney, brain, spleen, and GIT) were surgically removed within 20 min and 0.2 mg of tissue was homogenized with 3.8 mL of $KH_2PO_4$ 20 mM, (pH 3.8). The plasma and tissue homogenates were stored at -20°C until analysis.

For pharmacokinetic study, mice were given DOX intratumorally, when tumor size reached 300-500 $mm^3$. Drug was given either in 100 μL of PBS solution or 10% polyorganophosphazene gel solution (30 mg/kg). Polyorganophosphazene polymer was synthesized as previously described (Kang et al., 2006a). Plasma and various tissues were collected 1 h after drug administration as described above and stored until the day of analysis at -20°C. Tissue samples were processed in the same way, with blank tissues for HPLC analysis. The animal study protocol was approved by the Animal Committee of the Catholic University of Korea.

### Preparation of standards and controls

Stock aqueous solution of DOX (20, 1, and 0.1 μg/mL) and IS (50 μg/mL) were prepared in deionized water and stored at -70°C in polypropylene Eppendorf tubes. One hundred microliters of plasma or 200-500 μL of tissue homogenate were spiked with DOX and IS solution to prepare calibration standards (5-2000 ng/mL for plasma and 40-4000 ng/mg for tissue). Samples were incubated at 37°C for 15 min to allow protein binding equilibrium. For protein precipitation, samples were vortex-mixed with 250 μL acetone and 100 μL $ZnSO_4$ solutions (saturated) and re-incubated at 37°C for another 15 min. The supernatant was obtained after centrifugation and subjected to evaporation under a weak stream of nitrogen gas at room temperature. The dried residue was completely dissolved in 200 μL mobile phase and introduced into the HPLC system for analysis.

### Chromatographic equipment and conditions

The HPLC system consisted of solvent delivery pump model-306, 231XL auto-sampler, and spectrofluorometric detector model-122 (Gilson Corp., Middleton, WI, U.S.A.). Reversed phase separation was performed on Luna™ analytical column (150× 4.6 mm, C8, 5 μm spherical particles) with a guard column (Luna™ 2×4.6 mm, C8, Phenomenex, Torrance, CA, U.S.A.) at room temperature. The mobile phase was a mixture of acetonitrile: heptanesulfonic acid (0.2%, pH 4) at a ratio of 25:75, and gradient elution was utilized, i.e., 1 mL/min until 14 min, linear increase to 1.5 mL/min till 20 min, then maintained for 9 min (20-29 min) before returning back to 1 mL/min over 1 min. Detection was performed at $\lambda_{ex/em}$ 482/550 nm. Data acquisition was done using UniPoint ver. 5.11 software (Gilson Corp., Middleton, WI, U.S.A.). Calibration equations were obtained using least squares regression method on the nominal concentration versus the peak height ratio of DOX to the internal standard.

### Assay validation

At least three calibration curves were constructed for each tissue, and the precision and accuracy of the method were determined for the full range of the calibration curves (25-2000 ng/mL for plasma and 0.1-4 μg/g for tissue). Coefficients of variation (CV %) for three measurements and percentage ratio to nominal concentration were taken for precision and accuracy, respectively. LOQ is defined as the lowest concentration at which the precision and accuracy fall within 25% deviation, while the LOD is defined as the lowest concentration at S/N ratio above 5. The recovery of DOX and IS was determined from the ratio of the slope of the calibration curves obtained using extracted samples to that of diluted samples (n=3).

## RESULTS AND DISCUSSION

DOX has been a center of research interest in the development of drug delivery system (DDS) for systemic and local administration (Veronese et al., 2005; Alminana et al., 2004; Weinberg et al., 2007). Pharmacokinetic (PK) characterization of these DDS requires a robust, sensitive, yet simple HPLC method for DOX in biological matrices (Loadman and Calabrese, 2001). Only a few of the analytical methods for DOX are appropriate for PK studies using small experimental animals such as nude mice mainly due to small sample size. We developed a simple, yet sensitive, HPLC method for DOX then the method has been applied successfully for pharmacokinetic evaluation of DOX after administration in novel thermosensitive hydrogel DDS.

### Specificity and selectivity

DOX and IS were clearly resolved from interfering peaks in plasma, tumor, heart tissue (Fig. 1) and other tissues including spleen, liver, kidney, GIT, lung and brain tissue (data not shown). Although each tissue showed different interfering peaks, successful resolution has been obtained for all tissues under the same condition using linear gradient elution. Significant matrix effect has been reported

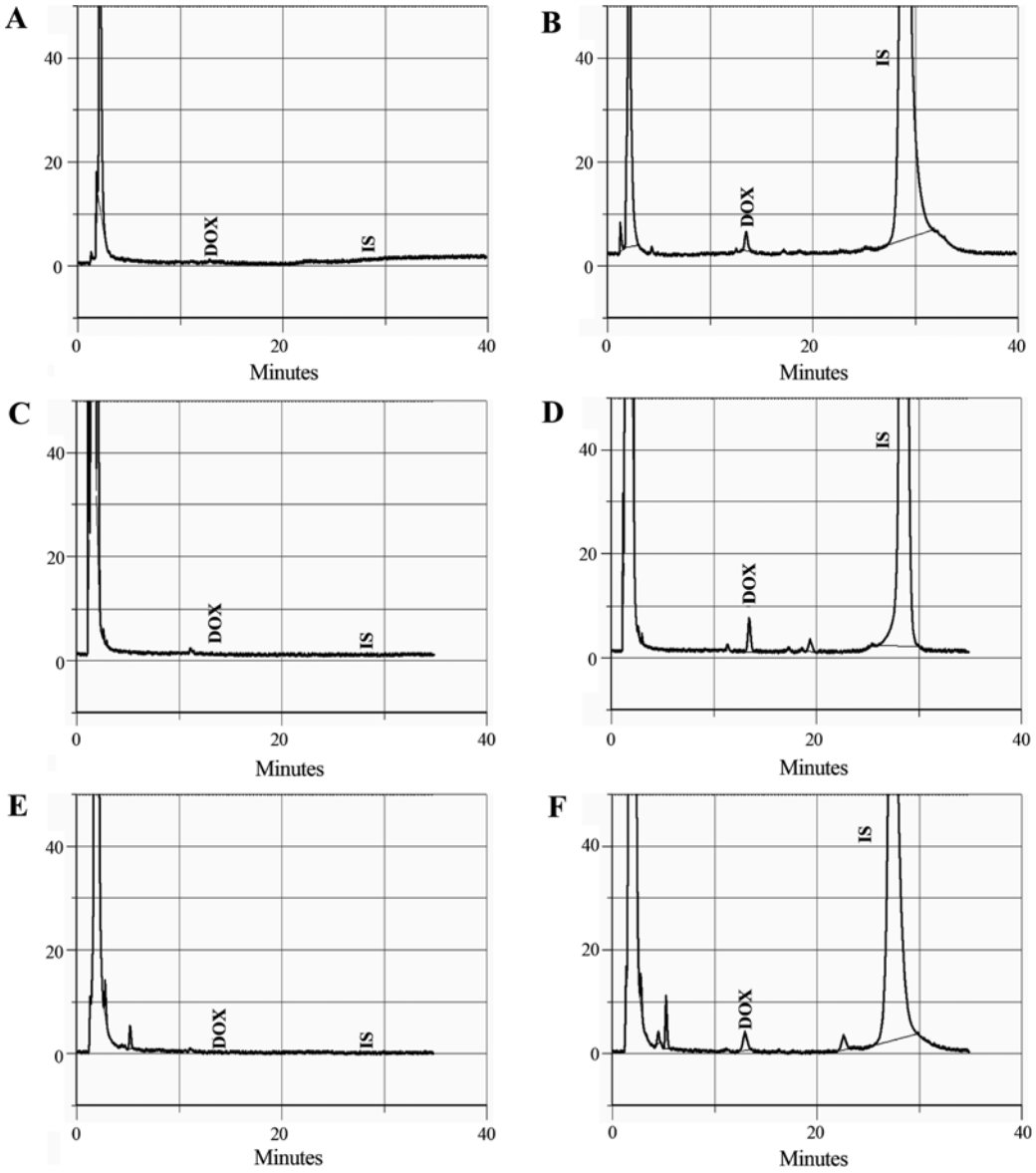


**Fig. 1.** Representative HPLC chromatograms for doxorubicin (DOX) showing specificity and resolution in plasma and tissues of nude mice. Chromatograms of blank and spiked samples at LOQ of DOX are shown for plasma (A & B), tumor tissue (C & D), and heart tissue (E & F), respectively.

to cause variation in DOX retention time in the plasma of different species (Alvarez-Cedron et al., 1999). Protein normalization dilution has been employed to minimize the matrix effect in previous reports (van Asperen et al., 1998; Kummerle et al., 2003; Arnold et al., 2004). We used plasma and each tissue of nude mice to construct respective calibration curves to avoid possible compromises in specificity and selectivity.

## Sample clean-up and recovery

Simple one-step protein precipitation using acetone/$ZnSO_4$ was used for all tissue tested in the current study. The percent recovery ranged from 52.4% to 95.2% (Table II), which is similar to the values reported in the literature (van Asperen et al., 1998). Protein precipitation, solid phase extraction, and liquid/liquid extraction are frequently used for sample clean-ups. Solid phase extraction is expensive and labor-intensive (Mou et al., 1997; Loadman and Calabrese, 2001); liquid/liquid extraction is not preferred due to use of organic solvents and emulsification problems (Hu et al., 2007). Protein precipitation is considered the method of choice for DOX extraction from bio-matrix (Loadman and Calabrese, 2001).

**Table II.** Recovery (%), LOQ and LOD of doxorubicin in plasma and tissues of nude mice

| Sample | Recovery % | Linearity ($R^2$) | LOQ* | LOD* |
|---|---|---|---|---|
| Plasma | 52.4 ± 4.1 | 0.9997 ± 0.0002 | 25 | 5 |
| Tumor | 71.4 ± 5.8 | 0.9994 ± 0.0003 | 0.1 | 0.02 |
| Heart | 88.1 ± 4.1 | 0.9986 ± 0.0007 | 0.1 | 0.02 |
| Spleen | 69.1 ± 8.2 | 0.9994 ± 0.0004 | 0.1 | 0.02 |
| GIT | 90.5 ±10.9 | 0.9989 ± 0.0012 | 0.4 | 0.02 |
| Brain | 73.8 ± 8.2 | 0.9998 ± 0.0003 | 0.1 | 0.02 |
| Lung | 95.2 ± 4.1 | 0.9984 ± 0.0025 | 0.1 | 0.02 |
| Kidney | 81 ± 4.1 | 0.9991 ± 0.0008 | 0.1 | 0.02 |
| Liver | 61.9 ± 4.1 | 0.9998 ± 0.0001 | 0.1 | 0.02 |

*LOQ and LOD concentration are in ng/mL for plasma and μg/g for the remaining tissues, respectively. Data are expressed as mean ± S.D. (n≥3).

**Table III.** Accuracy and precision (% C.V.) for doxorubicin analysis in nude mouse plasma

| Concentration (ng/mL) | Accuracy | Precision |
|---|---|---|
| 25 | 110.9 | 15.6 |
| 50 | 102.3 | 19.1 |
| 100 | 100.8 | 17.9 |
| 200 | 101.1 | 18.2 |
| 500 | 98.2 | 8.4 |
| 1000 | 98.5 | 12.7 |
| 2000 | 99.5 | 12.5 |

## Sensitivity and linearity

The LOD of DOX was determined as 5 ng/mL and 0.02 μg/mg in plasma and tissues, respectively (Table II). The LOQ of DOX in plasma samples were 25 ng/mL and LOQ in tissue samples were 0.1 μg/mg, except in GIT tissue, which showed 0.4 μg/mg (Table II).

The LOQ of DOX in the current method (equivalent to 5-25 ng/mL in the injection sample) was comparable to that of other studies in which fluorescent detector has been used (Alvarez-Cedron et al., 1999; Gilbert et al., 2005; Loadman and Calabrese, 2001). Greater sensitivity has been reported when ECD or MS/MS detection was used (Table I). Although higher sensitivity up 1.3 ng/mL has been reported for fluorescent detection, difficulty in method adaptation due to sample size was found (Bibby et al., 2005). Routinely used sample sizes for DOX analysis ranges from 100 to 1000 μL of plasma and 30 to 100 mg of tissue (Table I). Large injection volume and/or sample pooling method are used to improve the LOD/LOQ of DOX (Chin et al., 2002; Kummerle et al., 2003).

We did not use either large injection volume or sample pooling technique, yet obtained comparable sensitivity which may be appropriate for small animal studies such as nude mice.

Each calibration curve showed good correlation co-efficient ($R^2$) ranging from 0.9984-0.9998 (Table II). Acceptable accuracy and precision were achieved over the concentration range of 25-2000 ng/mL of DOX in plasma, with error less than 20% and 25%, respectively (Table III). For tissues, similar accuracy and precision were obtained over the range of 0.1-4 μg/mg of tissue, except in GIT (Table IV and V). The precision in GIT tissue showed large deviation (30-40%) at 0.1 and 0.2 μg/mg tissue concentrations which may be due to the remaining GIT content.

Calibration equation of any particular tissue or pooled calibration equation did not show an acceptable level of accuracy or precision for all tissues in our study (data not shown).

## Application of the method

The present method was used to determine the systemic distribution of DOX after intratumoral administration in PBS solution or hydrogel mixture (sol state, 10% thermosensitive polyorganophosphazene in PBS) (Fig. 2). Local delivery using the thermosensitive hydrogel was to control drug retention in injection site and minimize its distribution to normal tissues (Kang et al., 2006b). No interfering peaks have been detected from hydrogel formulation. The maximum concentration ($C_{max}$) in plasma and tissues was identified at 1 h after administration (first sampling time point). Plasma $C_{max}$ was

**Table IV.** Accuracy (%) of doxorubicin analysis in nude mouse tissue matrices

| Concentration (μg/mg) | Tumor (n=4) | Heart (n=3) | Spleen (n=3) | GIT (n=3) | Brain (n=3) | Lung (n=3) | Kidney (n=3) | Liver (n=3) |
|---|---|---|---|---|---|---|---|---|
| 0.1 | 106.3 | 102.6 | 105.5 | 92.9 | 96.4 | 94.7 | 104.9 | 95.5 |
| 0.2 | 107.2 | 102.4 | 103.3 | 111.7 | 99.6 | 98.5 | 96.4 | 100.2 |
| 0.4 | 107.1 | 100. | 98.8 | 99.4 | 110.7 | 98.1 | 93.3 | 102.4 |
| 0.8 | 97.1 | 89.2 | 98.2 | 96.3 | 95.5 | 88.2 | 96.7 | 101.1 |
| 2 | 102. | 104.2 | 103.6 | 100.5 | 104 | 103.8 | 101.6 | 98 |
| 4 | 102.2 | 99.6 | 101 | 101.7 | 102.1 | 99.4 | 98.5 | 99.4 |

**Table V.** Precision (% C.V.) of doxorubicin analysis in nude mouse tissue matrices

| Concentration (μg/mg) | Tumor (n=4) | Heart (n=3) | Spleen (n=3) | GIT (n=3) | Brain (n=3) | Lung (n=3) | Kidney (n=3) | Liver (n=3) |
|---|---|---|---|---|---|---|---|---|
| 0.1 | 14.8 | 19.9 | 11.6 | 31.2 | 12.6 | 3.3 | 4.7 | 0.9 |
| 0.2 | 5.6 | 6.2 | 11.9 | 39.6 | 0.3 | 3 | 6.3 | 1.6 |
| 0.4 | 2.2 | 2.6 | 11.1 | 13.9 | 2.2 | 4.1 | 5.0 | 3.8 |
| 0.8 | 6.8 | 4.39 | 8.3 | 13.1 | 5.9 | 13.8 | 10.3 | 1.1 |
| 2 | 10.8 | 5.4 | 7.6 | 10.6 | 3.47 | 2 | 7.8 | 3.8 |
| 4 | 7.4 | 7.1 | 12.2 | 10.5 | 2 | 3.8 | 4.8 | 5.1 |

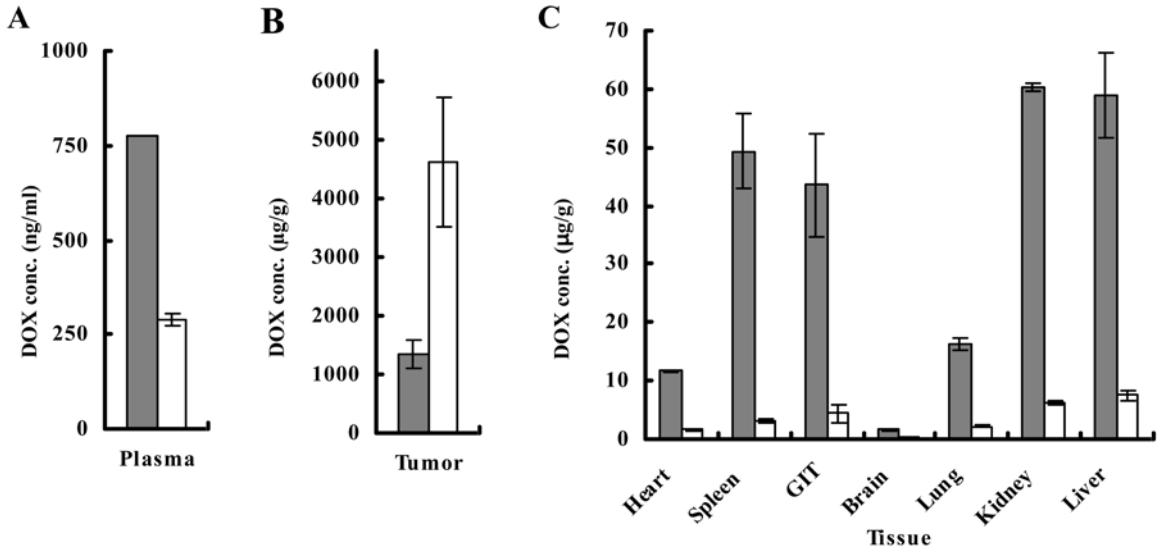


**Fig. 2.** Tissue distribution of DOX in tumor-bearing nude mice after intratumoral administration. DOX was administered at 30 mg/kg in 100 μL of drug solution (open bars) or drug-polyphosphazene hydrogel mixture (solid bars). DOX concentration was determined in plasma (A), tumor (B), and other tissues (C) at 1 h after drug administration. Data are presented as means ± SEM.

found to be 773 ng/mL and 300 ng/mL (Fig. 2A) and tumor $C_{max}$ was 1400 μg/mg and 4240 μg/mg when given as solution and hydrogel, respectively (Fig. 2B). Lower plasma concentration with higher concentration in tumor indicated successful retention of DOX in injection site and lower spill-over to systemic circulation for hydrogel administration compared to solution administration. Similar to plasma concentration, $C_{max}$ in other tissues were 7- to 16-fold higher for solution administration compared to hydrogel.

## CONCLUSION

In summary, a simple and specific HPLC method was developed for the analysis of DOX in plasma and various tissues of nude mice. The present method showed good specificity, accuracy and precision, without compromise in a small sample size or injection volume. The present method was successfully applied for the determination of low drug concentration in plasma and tissues after intratumoral administration of DOX in tumor-bearing nude mice, indicating suitability of the method for pharmacokinetic studies using small animal models such as nude mice.

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