A Sensitive High Performance Liquid Chromatography (HPLC) Assay for the Quantification of Doxorubicin Bound to DNA

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Abstract

Doxorubicin, a widely used anticancer agent, exhibits antitumor activity against a wide variety of malignancies. The drug exerts its cytotoxic effects by binding to and intercalating within the DNA of tumor and tissue cells. However, current assays are unable to accurately determine the concentration of intracellular active form of doxorubicin. Thus, we have developed a high performance liquid chromatography (HPLC) methodology in order to quantify the concentrations of doxorubicin that are bound to DNA in tumors and tissues as an intracellular cytotoxic measure of doxorubicin exposure after administration of small molecule and nanoparticle formulations of doxorubicin. The assay uses daunorubicin as an internal standard; liquid-liquid phase extraction to isolate bound drug; a Shimadzu HPLC with fluorescence detection equipped with a Phenomenex Luna C18 (2 um, 2.0 x 100 mm) analytical column; and a gradient mobile phase of 0.1% formic acid in water and acetonitrile. The assay has a lower limit of quantification (LLOQ) of 10 ng/mL and is shown to be linear up to 3,000 ng/mL. We demonstrated the suitability of this assay for doxorubicin bound to DNA in vivo by using it to quantify the doxorubicin concentration within tumor samples from SKOV3 and HEC1A mice obtained 72 hours after administering PEGylated liposomal doxorubicin (Doxil®; PLD) IV at 6 mg/kg.

This HPLC assay allows for a sensitive and simple intracellular quantification of doxorubicin as compared to other methods and will be an important tool for future studies evaluating intracellular pharmacokinetics of doxorubicin and various nanoparticle carriers.

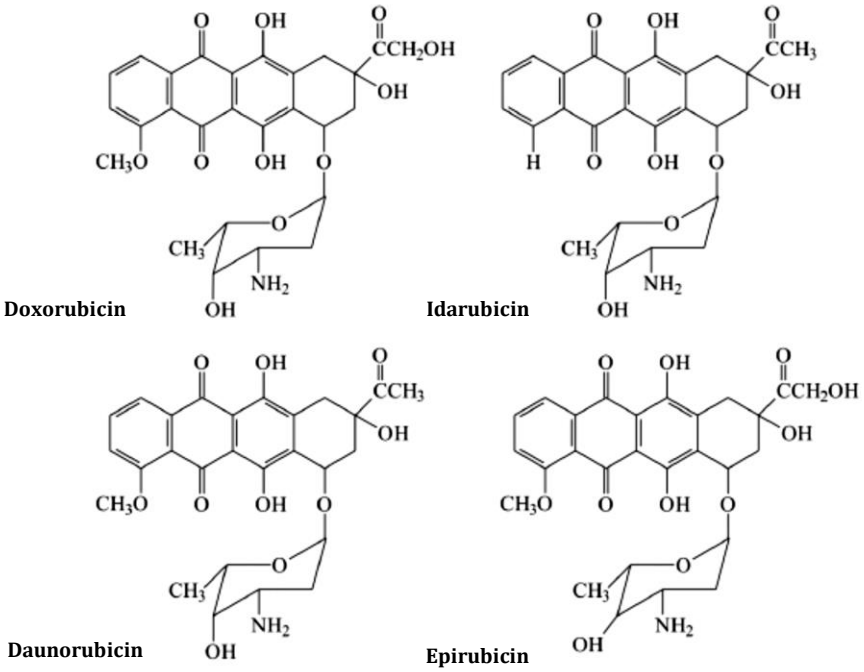
Keywords: HPLC, doxorubicin, intracellular pharmacokinetics, doxorubicin-DNA adducts

1: Introduction

1.1 Doxorubicin and PEGylated liposomal doxorubicin (Doxil)

The anthracycline class of anticancer agents consists of a tetracyclic ring system bound to an aminoglycoside. Of the four most common compounds found in this class, the most widely used is doxorubicin (Figure 1). Doxorubicin is a widely used cytotoxic anthracycline antibiotic that has been used in the treatment of a range of malignant tumors, including leukemia, lymphoma, stomach cancer, bone cancer, multiple myeloma, ovarian cancer and breast cancer, and has been an important chemotherapeutic agent since approval in 1974[1]. These drugs are typically used in combination with other groups of drugs, each exhibiting a different mechanism of action, to increase tumor death and to minimize resistance. However, the clinical use of doxorubicin is limited by a cumulative dose-dependent cardiomyopathy, which can eventually lead to heart failure, ranging from 5-48% depending on dose[2], and carries a mortality rate of 20–40%[3]. Such toxicities can be reduced or avoided though an administration schedule that produces low peak plasma drug concentrations. Despite enormous efforts in creating derivatives that are more efficacious and less cardiotoxic, doxorubicin remains a cornerstone anticancer agent[4].

Figure 1: Structure of anthracyclines in current clinical use for anticancer therapy.



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Doxorubicin exerts its effect when it is taken up into the nucleus of cells, where it binds with high affinity to DNA via intercalation between base pairs[5]. There is good evidence to support that the mechanism of action of doxorubicin as a topoisomerase II inhibitor. Once doxorubicin is intercalated into DNA, it perturbs the re-ligation step of topoisomerase II, resulting in the formation of the 'cleavable complex'[6-7]. Failure to repair DNA double strand breaks results in an apoptotic

response. Other cellular responses to doxorubicin have also emerged, including the formation of doxorubicin-DNA adducts[8] and the inhibition of the DNA methyltransferase[9]. A range of several other diverse effects have also been mentioned, though the method of cell death remains unclear.

In general, long-circulating PEGylated liposomal doxorubicin (PLD) formulations (such as Doxil©) offer distinct advantages over conventional doxorubicin, including reducing cardiac toxicity and increasing tolerability and efficacy in solid tumors[10-11]. The most significant advantage of PLD over non-PEGylated liposomal products is its much longer circulation, which results in greater uptake by tumor tissue (due to the leaky vasculature of tumor vessels, PLD preferentially distributes to tumors more than normal tissue)[13-14]. The toxicity profiles of both Doxil© and conventional doxorubicin have been thoroughly reviewed[11] and the incidence of heart failure was shown to be lower with Doxil compared with conventional doxorubicin[11-12].

1.2 Doxorubicin-DNA adducts

The history of discovery of anthracycline-DNA adducts has been previously reviewed in the literature[15-16]. Despite a large body of evidence that doxorubicin acts predominantly via intercalation, the lack of understanding of the full mechanism of action has hindered efforts to produce newer derivatives with higher activity and reduced side effects. Adducts were initially characterized in a cell-free environment, where doxorubicin-induced transcriptional blockages were observed at 5' GpC sequences[17]. Further research in this cell-free environment revealed that formaldehyde was a byproduct of the reaction conditions, suggesting that formaldehyde was necessary for the covalent linkage with guanine[18] (Figure 3). Many studies have since demonstrated the requirement for formaldehyde for activation of anthracyclines to form adducts in vitro[18-22].

This led to the structure of these adducts to be resolved by NMR and mass spectrometry[18-21] (Figure 2). The drug is linked by a single aminal covalent bond (N-C-N) to only one strand of DNA, using strong hydrogen-bonding interactions on the opposite strand to stabilize the duplex, from the 3' amino of daunosamine to the exocyclic 2-NH_2 amino of guanine[18-19,21] (Figure 2A). This adduct stabilizes the local DNA region to such an extent that the adducts can even be detected by classical denaturation-based crosslinking assays[23-24]. These adducts have now been detected *in vivo* in tumor cells in culture using several methods - the most direct using C^{14}-labelled doxorubicin to yield C^{14}-labelled doxorubicin-DNA complexes[25-26]. Increased cellular levels of formaldehyde have been detected in tumor cells (1.5-4.0uM) compared to normal cells[28-29], suggesting the increased formation of adducts within tumor cells. These adducts have now been shown to be substantially more cytotoxic than lesions induced by topoisomerase II lesions[27].

The characteristics of adducts derived within the cell have not been chemically characterized, but some information exists from *in vitro* studies. These adducts are intrinsically unstable, demonstrating the reversibility of Schiff base complexes. Due to the aminal linkage, adducts are both heat and alkali labile, exhibiting a half-life of 5-40 hours *in vitro* at 37°C depending upon the site of adduct formation[17,23-24,30]. These adducts can be maintained for extended periods of time (several months) at 4°C and can remain almost indefinitely if kept in equilibrium with sufficient free drug at 37°C[19]. The conditions required for adduct formation *in vitro* have been examined in

Figure 2: Structure of doxorubicin-DNA adducts.

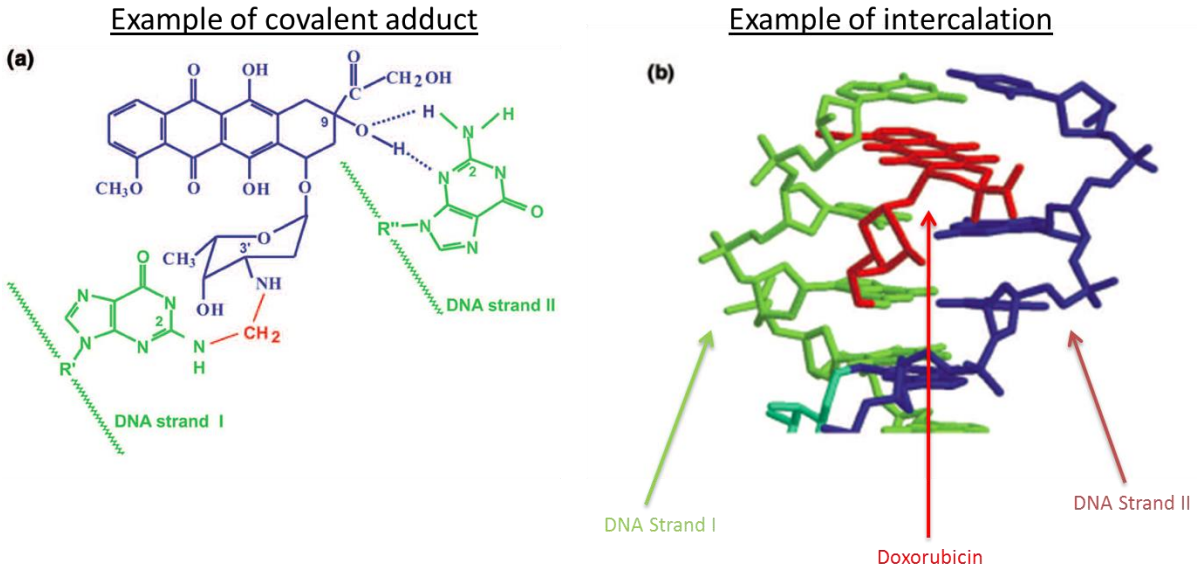

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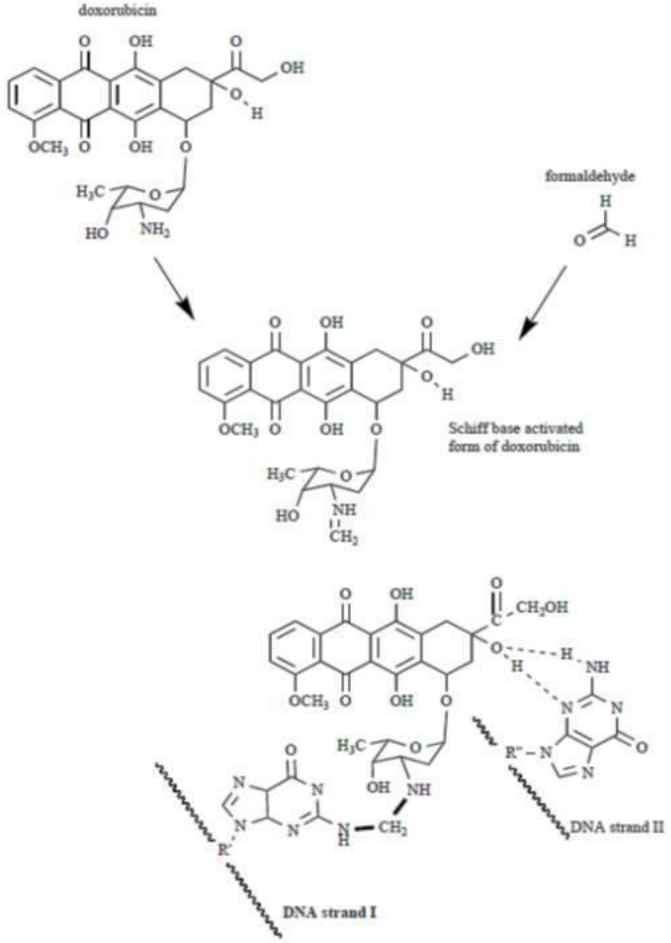
Figure 3: Schiff base reaction of doxorubicin with DNA to form doxorubicin-DNA adducts.



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several studies[18-19,21,32]: optimal formation at pH 7, double stranded DNA (dsDNA) is required, and the extent of formation dependent on both DNA and formaldehyde concentration. The overall half-life reported recently for doxorubicin-DNA adducts in tumor cells in culture is 13 hours[31].

1.3 Previous concerns with methods of detection

There are several publications that have reported methods of determining doxorubicin, and some of its metabolites[33]: capillary electrophoresis, laser-induced fluorescence detection, radioimmunoassay, high performance liquid chromatography (HPLC), fluorescence detection, chemiluminescence detection, electrochemical detection, and mass spectrometric detection (Table 1). These methods each utilize a variety of pre-treatment procedures for samples, some of which are time-consuming solid-phase extractions. However, many of these methods also lack sensitivity and selectivity and require high expense or increased technical experience and capabilities. Further, efforts have also been hampered due to the failure to achieve chromatographic resolution of peaks and the high affinity of anthracyclines for cellular constituents[34]. The majority of these methods also obtain their samples from cultured cells versus from whole tissue or tumor. Therefore, it would be beneficial to research and develop a cost-effective, in-house, timely method of quantification of anthracyclines from a realistic sample from pharmacokinetic studies.

1.4 Purpose of method development

Many studies have measured total drug levels in solid tumors following administration of liposomal drugs[42-46]. However, most of these foundational studies do not distinguish between encapsulated and released (bioavailable) drug within the tumor tissue. Since only released drug is available for biological activity, the therapeutic effects of nanoparticle-containing doxorubicin can be correlated to the levels of bioavailable drug in tumor tissue versus levels of total drug measured within the tumor tissue. Thus, knowing the levels and rate of bioavailable drug will help in the design and

Table 1: Quantitative techniques used to detect doxorubicin–DNA adducts

Methodology	Minimum Doxorubicin Dose	Adducts per 10^7 bp DNA	Sample Source	Reference
Chromatography specific for covalently bound doxorubicin using an intercalator affinity column	50 uM	~ 1220	Cultured cells	35
Gene-specific and cross-linking assays	7.5 uM	100	Cultured Cells	36-38
^{32}P DNA post-labelling	0.27 uM / 2.5 gm tumor	10	Intra-tumoral injection into rat mammary carcinoma	39
^{14}C-Doxorubicin bound to cellular DNA detected by decay counting	1 umol / 200 gm rat 1 uM	70 1	Rat liver Cultured cells	8, 27, 40-41

***Adapted from Reference 25*

comparison of improved nanoparticle formulations for doxorubicin-containing anticancer drugs. As nuclear DNA serves as the final site of action, the measurement of doxorubicin bound to DNA provides a good estimate of bioavailable levels of drug *in vivo*. Thus, we have developed a high performance liquid chromatography (HPLC) methodology in order to quantify the concentrations of doxorubicin that are bound to DNA in tumors and tissues as an intracellular cytotoxic measure of doxorubicin exposure after administration of small molecule and nanoparticle formulations of doxorubicin.

2: Materials and Methods

2.1 Materials

The following chemicals and reagents were purchased from Sigma Aldrich (St. Louis, Missouri): doxorubicin, daunorubicin (internal standard), acetonitrile, methanol, ethanol, genomic DNA from calf thymus, collagenase (type I), ammonium sulfate, and calcium chloride (≥93.0%, anhydrous, granular). Acetonitrile and methanol were of HPLC grade; ethanol was of analytical grade.

The following chemicals and reagents were purchased from Fisher Scientific (Waltham, Massachusetts): Fermentas DNAseI enzyme and 10x reaction buffer, lambda DNA, and formaldehyde. Formaldehyde was of molecular biology grade.

Ambion TE Buffer (pH 8.0) and Quant-iT PicoGreen dsDNA reagent were purchased from Life Technologies (Grand Island, New York). Qiagen DNeasy Blood & Tissue Kit for DNA extraction was purchased directly from Qiagen (Valencia, California).

2.2 Procedure

2.2.1 Standard & quality control stock preparation

Stock solutions of doxorubicin (1 mg/mL) and daunorubicin (1 mg/mL) were solubilized using methanol and agitated for five minutes prior to initial use. Stock solutions were stored at -80°C until ready for use. Calf thymus DNA was solubilized into TE buffer (pH 8) for four hours to a final concentration of 0.5 mg/mL. Stock DNA was stored in aliquots at -20°C until ready for use.

2.2.2 Standard & quality control sample preparation

Standard curve reactions were made in duplicate and quality control (QC) reactions were made in sextuplets per each doxorubicin concentration level. Standards were completed utilizing eight levels, ranging from 10 to 3000 ng/mL. Quality controls were run at the following levels: 20 (LLOQ), 30, 500, and 2500 ng/mL. Each 0.5 mL reaction contained 30 ug calf thymus DNA and doxorubicin (by adding appropriate methanolic spiking solution) in TE buffer (pH 8).

2.2.3 Mock sample preparation

Reactions were made in triplicate per each doxorubicin concentration and run in parallel. Each 0.5 mL reaction contained 30 ug calf thymus DNA, doxorubicin (ranging between 5 ng to 1500 ng), and 0.37% formaldehyde in TE buffer (pH 8). Doxorubicin was added by adding appropriate methanolic spiking solution. Reactions were run at 4°C for four hours to allow for 100% conversion of all doxorubicin binding to DNA.

2.2.4 Tissue preparation & digestion

Frozen tissue samples were removed and weighed into reinforced homogenization tubes filled with zirconium oxide beads. All samples were kept on ice during preparation. Tubes were spiked with PBS (pH 7.24) and homogenized using a Bertin Precellys 24 at 4°C at 5,000 rpm for no more than 20 seconds.

A 2 mg/mL collagenase suspension was created using 0.22 mg/mL calcium chloride dissolved in PBS (pH 7.24). Using a ratio of 1 uL of collagenase solution per 1 mg tissue, collagenase was added to each homogenized tissue sample. Samples were placed in a 37°C incubator with agitation at 250 rpm for one hour.

2.2.5 Doxorubicin-DNA extraction & quantification

Digested tissue homogenate underwent DNA extraction using a DNeasy Blood & Tissue Kit utilizing a modified, optimized protocol based on the manufacturer's instructions and those found within the literature. Final DNA samples were eluted into 300 uL TE buffer. DNA was quantified utilizing PicoGreen dsDNA fluorescent reagent using a Tecan infinite 200 fluorometer and a standard curve based on lambda DNA concentrations verified by microdot UV detection at 280 nm.

2.2.6 Extraction Procedure

Extracted DNA samples (0.5 mL) were combined with 50 units DNAseI and 10x Reaction Buffer, vortexing to mix completely. Samples were placed in a 37°C incubator with agitation at 250 rpm for fifteen minutes. Afterwards, we used liquid-liquid extraction with acetonitrile in a 2:1 ratio (1000 uL ACN:500 uL aqueous), vortexing for five minutes to ensure adequate separation. Sufficient ammonium sulfate was added to each sample to saturate the solution before centrifuging to separate aqueous and organic layers.

An aliquot of the upper layer was transferred into another tube and evaporated under nitrogen at 45°C. The residue was reconstituted into 250 uL of Reconstitution Buffer (85:50 mobile phase A:B – see 2.2.8), and 10 uL of this solution was injected into the HPLC.

2.2.7 HPLC instrumentation

Analysis by HPLC, all from Shimadzu, was performed using a LC-20AB pump, an automatic sample injection system (SIL-20AC HT), and a stainless steel Phenomenex Luna C18 (2 um, 2.0 x 100 mm) analytical column contained in an oven (CTO-20A). The column effluent was monitored using a variable wavelength fluorescence detector (RF-10A XL) operated at excitation of 490 nm and emission of 590 nm.

2.2.8 HPLC system conditions

A gradient mobile phase of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was filtered through a membrane filter (0.2 um). The oven temperature was 32°C, and the flow rate was 0.35 mL/min.

2.2.9 Data analysis

The ratio of the peak area of doxorubicin to that of the internal standard (daunorubicin) was used as the assay parameter. Peak area ratios were plotted against analyte concentrations, and standard calibration curves were obtained from least-squares linear regression analysis of the data.

The linearity of the method was confirmed using classical statistical tests, that is, comparison of intercept with zero and correlation coefficients.

2.2.10 Stability study

Control mock samples were spiked to a standard concentration of doxorubicin (100 and 1000 ng/mL) in triplicate per time point (zero, 2, 4, and 6 weeks). All samples were stored at -80°C. For each time point, samples were immediately assayed according to the procedure given above.

2.2.11 Proof of concept study

Three female nu-nu mice (non-tumored) were obtained 24 hours after administering PEGylated liposomal doxorubicin once (Doxil®; PLD) at 10 mg/kg intravenously. The mice were euthanized and their organs (liver, spleen, kidneys, brain, plasma, lungs) harvested and flash frozen before being stored at -80°C. Liver tissue samples were assayed according to the procedure given above.

3: Results

3.1 Retention times and linearity

Observed retention times were 6.2 and 9.6 minutes for doxorubicin and daunorubicin (internal standard), respectively (Figure 4). The peak area ratio of doxorubicin over the internal standard varied linearly with concentration over the experimental range used (10-1000 ng/mL) (Figure 5). The correlation coefficients (r^2) for calibration curves were equal to or better than 0.995.

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. Furthermore, the relative standard deviation (or RSD%), computed by dividing the standard deviation by the mean value, ranged from roughly 0.6% to 11.4%.

Figure 4: HPLC chromatogram representation of samples.

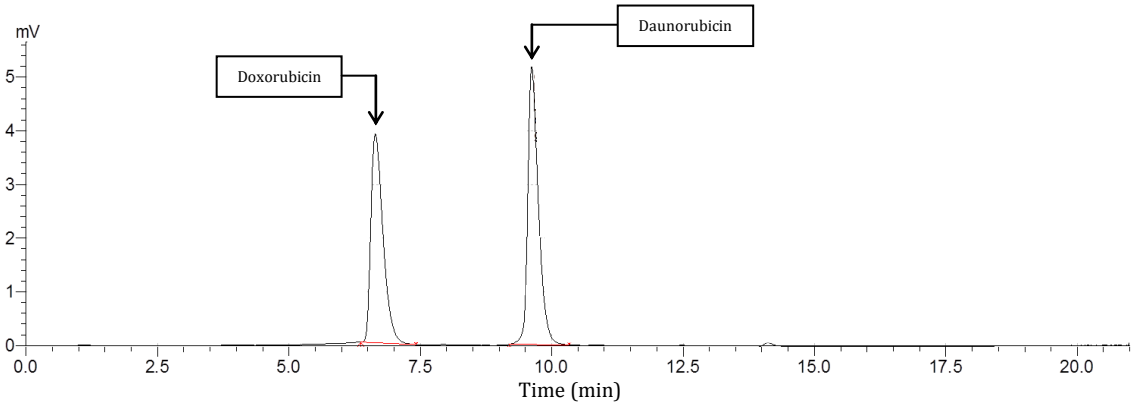
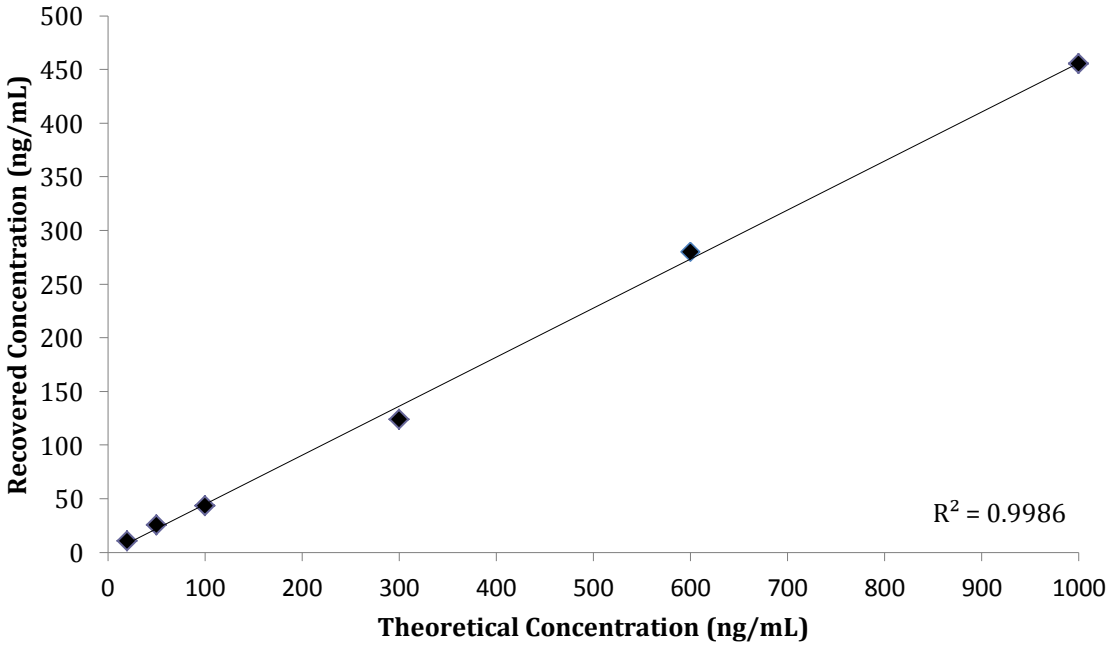


Figure 5: Extraction of doxorubicin is linear across experimental range.



3.2 Precision and accuracy

For each quality control point (30, 100, and 1000 ng/mL), each representing a low, moderate, and high concentration in our validation range, the relative standard deviation was calculated, ranging from 0.7% to 12.4% (overall bias% range represented in Table 2).

The intraday and between-day precision of the assay was assessed by performing three triplicate (n = 9) analyses of these same three quality control points above (30, 100, and 1000 ng/mL). Results, expressed as a coefficient of variation (CV%), are presented in Table 2, ranging between roughly 4.0% and 8.8%.

3.3 Recovery

The extraction efficiency (recovery) was determined by the extraction of doxorubicin from mock samples of known concentrations (30, 100, and 1000 ng/mL) in triplicate. After extraction, the internal standard was added. Recovered doxorubicin concentrations were compared against extracted external standards. The standards were prepared with addition of stock doxorubicin and internal standard in a sample matrix to give concentrations equivalent to 100% recovery. The mean recovery was determined to be 63% ± 1.7% (n = 27) across the validated range (Table 2).

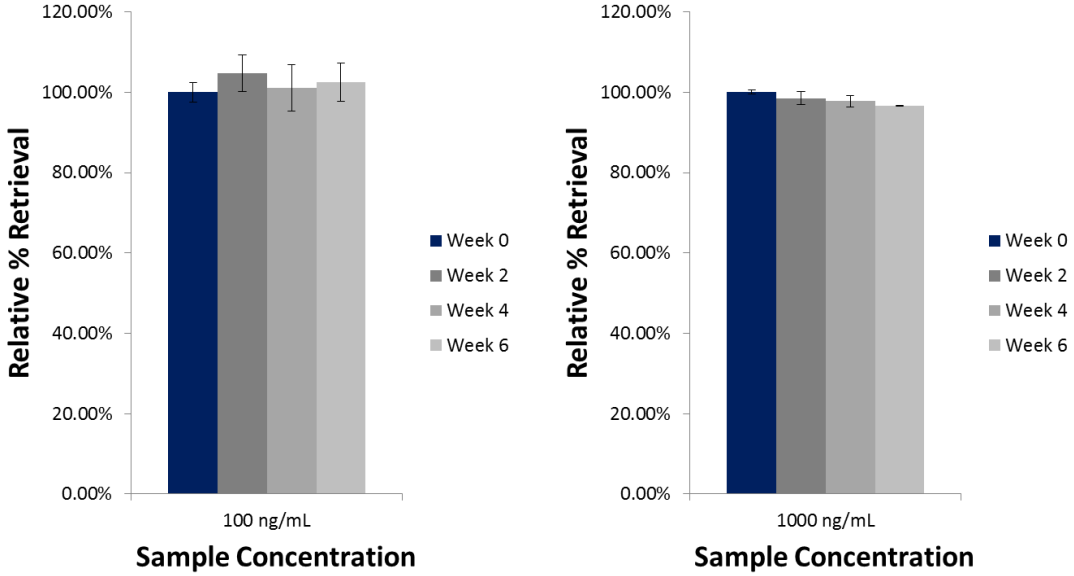
3.4 Limit of quantification and limit of detection

The limit of quantification was 10 ng/mL for doxorubicin. At this level, the error ranged from 4.3% to 12.1%. The limit of detection, representing a signal to noise ratio of 3:1, was 1.5 ng/mL for doxorubicin.

Table 2: Summary of quality control validation.

Sample Concentration	30 ng/mL	100 ng/mL	1000 ng/mL
n	9	9	9
Curve Linear Range	Fully validated over a range of 10 to 3000 ng/mL with linear regression ($1 / x^2$)		
Sample Stability (6 week)	-----	100 – 104.72%	96.60 – 100%
Mean Recovery	63.15 ± 5.56%	64.62 ± 2.59%	61.20 ± 3.89%
Precision of Recovery (CV%)	8.81%	4.01%	6.36%
Accuracy (Bias %)	87.9 – 111.4%	87.6 – 98.5%	85.7 – 100.7%
Max Difference	13.45%	7.91%	10.70%

Figure 6: Stability of Doxorubicin-DNA Samples Stored at -80°C.



3.5 Stability

The stability of doxorubicin-DNA adduct samples stored at -80°C as mock samples was determined at two concentrations on either end of the validation range (100 and 1000 ng/mL). The concentrations were expressed as a percent as compared with the concentration measured at time zero (relative percent retrieval). For doxorubicin-DNA adduct samples, no significant difference was observed between time zero and six weeks in storage at both concentrations tested (Figure 6).

3.6 Proof of concept

Based on the developed assay, true *in vivo* samples were tested, as opposed to prepared mock samples, in order to demonstrate the application of this method in future pharmacokinetic studies. Liver tissue samples (n = 3; see 2.2.11 for treatment description) were first used to evaluate intra-sample variation (Table 3). Samples were processed in tandem from the same liver homogenate and, after correcting the doxorubicin concentration for recovery and normalizing the data based on the amount of DNA extracted in the sample, showed limited sample variability (1.506 ng/ug ± 0.440 ng/ug; CV% 29.2). Liver tissue samples (n = 3) were then used to evaluate inter-sample variation. Tissues samples run in parallel from the same liver showed that a minimum of 100 mg of tissue is required to limit the variability presented (1.416 ng/ug ± 0.308 ng/ug; CV% 21.7) (Table 4).

Table 3: Intra-sample evaluation of doxorubicin-DNA concentration in liver of a female nu-nu mouse administered Doxil at 6 mg/kg IV x1 after 24 hours.

	Measured Dox Conc. on DNA (ng)	Corrected Dox Conc. on DNA (ng)	DNA Conc. (ug)	Normalized Conc. (ng/ug)
Aliquot 1	12.921	21.535	12.940	1.664
Aliquot 2	15.233	25.388	13.746	1.846
Aliquot 3	10.285	17.141	16.977	1.009
				1.506 ± 0.440

Table 4: Inter-sample evaluation of doxorubicin-DNA concentration in liver of a female nu-nu mouse administered Doxil at 6 mg/kg IV x1 after 24 hours.

	Measured Dox Conc. on DNA (ng)	Corrected Dox Conc. on DNA (ng)	DNA Conc. (ug)	Normalized Conc. (ng/ug)
10 mg	Non-detectable			
25 mg	Non-detectable			
50 mg (Sample 1)	4.090	6.817	1.09	6.254
50 mg (Sample 2)	7.695	12.825	4.73	2.711
50 mg (Sample 3)	4.130	6.883	10.79	0.638
100 mg (Sample 1)	8.345	13.908	12.91	1.077
100 mg (Sample 2)	10.890	18.150	10.81	1.679
100 mg (Sample 3)	8.575	14.291	9.59	1.490
				1.416 ± 0.308

Table 5: Doxorubicin-DNA tumor concentration at 72 hours in female SCID mice bearing orthotopic SKOV3 ovarian tumors administered Doxil at 6 mg/kg IV x1.

	Measured Dox Conc. on DNA (ng)	Corrected Dox Conc. on DNA (ng)	DNA Conc. (ug)	Normalized Conc. (ng/ug)
Sample 1	29.357	46.598	24.44	1.907
Sample 2	26.110	41.444	19.68	2.106
Sample 3	27.394	43.483	19.03	2.285
				2.099 ± 0.189

Table 6: Doxorubicin-DNA tumor concentration at 72 hours in female SCID mice bearing orthotopic HEC1A ovarian tumors administered Doxil at 6 mg/kg IV x1.

	Measured Dox Conc. on DNA (ng)	Corrected Dox Conc. on DNA (ng)	DNA Conc. (ug)	Normalized Conc. (ng/ug)
Sample 1	18.625	29.564	20.87	1.416
Sample 2	22.293	35.386	28.36	1.247
Sample 3	21.710	34.460	21.88	1.574
				1.413 ± 0.164

3.7 Applications in pharmacokinetic studies

The HPLC method developed here for quantification of doxorubicin bound to DNA from tissue samples is suitable for the analysis of samples during pre-clinical pharmacokinetic studies within animal models. Table 5 and 6 illustrates a mock pharmacokinetic study utilizing our developed method to quantify doxorubicin concentrations from Doxil dosed (6 mg/kg) female SKOV3 and NEC1A mice after 72 hours and greater than 72 hours respectively. We observed a normalized intracellular concentration of 1.413 ± 0.164 ng/ug within SKOV3 tumor tissue samples, and 2.099 ± 0.189 ng/ug within the HEC1A tumor tissue samples.

4: Discussion & Conclusions

With the advent of nanotechnologies, the pharmaceutical market has seen a vast growth in new nanoparticle formulations for chemotherapeutic agents, all with the promise that they afford an active targeting of tumor cells to release a chemotherapeutic payload into individual cells, as compared to other nanoparticles that utilize a passive targeting mechanism (such as used by Doxil©). While the concentration of doxorubicin has been measured by a wide variety of methods, few of these methods afford the knowledge of knowing how much drug has successfully entered the individual cells. To determine levels of the cytotoxic active form of doxorubicin, defined as the drug that successfully integrated with DNA to exert a mechanism of cell death, would allow for a method of comparison between these different nanoparticle formulations (Figure 7). Specifically, it would allow for the removal of additional sources of doxorubicin from a tissue sample that could contaminate a true intracellular sample, such as remaining blood, drug sequestered in intravascular spaces, and entrapped unreleased drug from its respective nanoparticle formulation.

This HPLC assay allows for a sensitive and simple intracellular quantification of doxorubicin as compared to other methods and will be an important tool for future studies evaluating intracellular pharmacokinetics of doxorubicin and various nanoparticle carriers. This method has been used to successfully determine the levels of doxorubicin bound to DNA from organ tissue of a Doxil© dosed mouse (see section 3.6), proving its potential use in future pharmacokinetic studies.

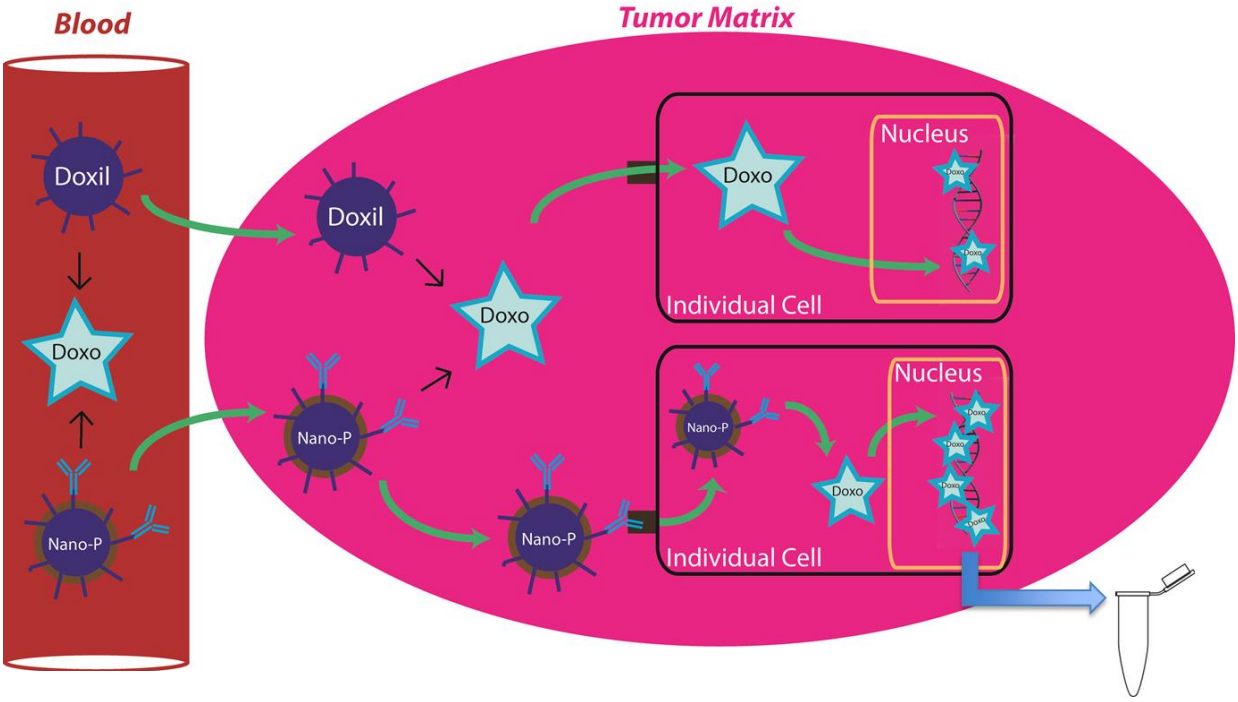
This method affords several advantages, including reproducibility and accuracy of the processed samples. The materials and automation required to perform this assay are found in most molecular biology and analytical chemistry facilities; as such, the entire process can be performed in-house without additional specific training. This process also allows for the processing of a great number of tubes simultaneously (up to 48 samples with current equipment). Furthermore, the stability of the doxorubicin-DNA samples proved to be resilient during storage (lasting at least six weeks), ensuring sample integrity during an extended period of time after tissue harvesting.

However, like all methodologies, some limitations do exist for this assay. A correction factor of 0.63, based on the observed mean recovery, is utilized in order to correct for the loss of recovered material, and as such, reported values are best given as theoretical ranges. Also, due to the sensitive nature of both the doxorubicin's stability and the aminal linkage that forms adducts, changes in the sample environment can interact significantly to change drug stability and recovery. The integrity of the DNA, and thus potential for doxorubicin quantification, that is able to be purified can also be affected based on the quality of the original tissue sample.

In conclusion, a sensitive HPLC method has been developed and validated to quantify the levels of doxorubicin bound to DNA from a tissue sample. This method was based on the need to accurately determine the intracellular kinetics, and provide a comparison, for various nanoparticle formulations of doxorubicin. Furthermore, this proposed method has successfully applied to a realistic example study tissue sample from a mouse-based pharmacokinetic study.

Figure 7: Differences in Distribution within Tumor Tissue.

As compared to other nanoparticles that utilize a passive targeting mechanism (such as used by Doxil©), new nanoparticle formulations ('Nano-P') for chemotherapeutic agents potentially afford an active targeting of tumor cells to release a chemotherapeutic payload into individual cells. This increased payload to individual cells would provide increased drug bound to DNA, which we can then measure using our proposed method, along with traditional pharmacokinetic measurements (plasma and tissue matrix).



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A.T.L developed and performed all experimentation associated with the methodology and wrote the manuscript. W.C.Z. and S.K.O. supervised and provided various coordination during the project. C.M.S. coordinated all mouse-related experimentation, care, and harvesting. T.F.W. provided access to tumor tissue samples for the final proof of concept.

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Supplemental Figure 1: Schematic of developed HPLC assay for quantification of doxorubicin bound to DNA from tissue samples.