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A sensitive high performance liquid chromatography assay for the quantification of doxorubicin associated with DNA in tumor and tissues

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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 the intracellular active form of doxorubicin. Thus, the development of a sample processing method and a high-performance liquid chromatography (HPLC) methodology was performed in order to quantify doxorubicin that is associated with DNA in tumors and tissues, which provided 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 drug associated with DNA; a Shimadzu HPLC with fluorescence detection equipped with a Phenomenex Luna C18 (2 μm, 2.0 × 100 mm) analytical column and a gradient mobile phase of 0.1% formic acid in water or acetonitrile for separation and quantification. The assay has a lower limit of detection (LLOQ) of 10 ng/mL and is shown to be linear up to 3000 ng/mL. The intra- and inter-day precision of the assay expressed as a coefficient of variation (CV%) ranged from 4.01 to 8.81%. Furthermore, the suitability of this assay for measuring doxorubicin associated with DNA *in vivo* was demonstrated by using it to quantify the doxorubicin concentration within tumor samples from SKOV3 and HEC1A mice obtained 72 h after administration of PEGylated liposomal doxorubicin (Doxil®; PLD) at 6 mg/kg IV x 1. This HPLC assay allows for sensitive intracellular quantification of doxorubicin and will be an important tool for future studies evaluating intracellular pharmacokinetics of doxorubicin and various nanoparticle formulations of doxorubicin.

1. Introduction

The anthracycline class of anticancer agents is characterized by a tetracyclic ring system bound to an aminoglycoside. 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. Of the 4 most common compounds found in this class, the most widely used is doxorubicin. A potent cytotoxic antibiotic gaining FDA approval in 1974 [1], doxorubicin has a relatively wide spectrum of activity [2], as it has been used in the treatment of a range of malignant tumors,

Abbreviations: CV%, coefficient of variation; DNA, deoxyribonucleic acid; Dox, doxorubicin; dsDNA, double-stranded DNA; HPLC, high performance liquid chromatography; IV, intravenous; LLOQ, lower limit of quantification; NMR, nuclear magnetic resonance; PK, pharmacokinetics; PLD, PEGylated liposomal doxorubicin; RSD%, relative standard deviation.

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including leukemia, lymphoma, stomach cancer, bone cancer, multiple myeloma, ovarian cancer, and breast cancer. However, the clinical use of doxorubicin, similarly with all anthracyclines, is limited by cumulative dose-dependent cardiomyopathy, which can eventually lead to heart failure, presentation ranging from 5 to 48% depending on dose [2,3], and carries a mortality rate of 20–40% [4]. Such toxicities can be reduced or avoided via an administration schedule that produces low peak plasma drug concentrations. Unfortunately, this precaution reduces drug efficacy. Despite enormous efforts in creating derivatives that are more efficacious and less cardiotoxic, doxorubicin-containing agents remain a cornerstone of cancer treatment [2].

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 doxorubicin's mechanism of action as a topoisomerase II inhibitor [6]. Once doxorubicin is intercalated into DNA, it perturbs the re-ligation step of topoisomerase II, resulting in the formation of the 'cleavable complex', eventually resulting in double-strand DNA cleavage. Failure to repair DNA double-strand breaks results in an apoptotic response. Other cellular responses to doxorubicin include the formation of doxorubicin-DNA adducts [7] and the inhibition of DNA methyltransferase [8]. A range of several other diverse effects also have been mentioned, though the method of cell death remains unclear.

The most well-known improvement of a formulation using doxorubicin has been its incorporation into a liposome. The most frequently used liposomal formulation of doxorubicin is a PEGylated liposomal doxorubicin (PLD) known as Doxil®. In general, PEGylation of the liposome affords distinct advantages over conventional doxorubicin, including a longer circulation time, increased stability, and reduction of cardiac toxicity [9,10]. The toxicity profiles of both PLD and conventional doxorubicin have been reviewed thoroughly [9], and the incidence of heart failure has been shown to be lower with PLD as compared with conventional small-molecule doxorubicin [9,10]. The most significant advantage of PLD over non-PEGylated liposomal products is its much longer circulation, which results in greater uptake by tumor tissue [11]. This is primarily due to the enhanced permeability and retention (EPR) effect—the leaky vasculature of tumor vessels, which preferentially distributes PLD to tumors relative to normal tissues. All of these characteristics lead to an increase in drug tolerability and efficacy in solid tumors.

The history of discovery of anthracycline-DNA adducts and their biophysical characterization has been previously reviewed [12]. Despite a large body of evidence suggesting that doxorubicin acts predominantly via intercalation, the lack of understanding of the full mechanism of its action has hindered efforts to produce newer derivatives with increased antitumor activity and reduced side effects. DNA adducts were characterized previously in a cell-free environment, where doxorubicin-induced transcriptional blockages were observed at 5′ GpC sequences [13]. 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 [14]. Many studies have since demonstrated the requirement of formaldehyde for activation of anthracyclines to form adducts *in vitro* [14–18].

The structures of these adducts have also been resolved by NMR and mass spectrometry [12,14–17]. The drug is proposed to be 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, from the 3′ amino of daunosamine to the exocyclic 2-NH_2 amino of guanine [14,15,17]. This adduct stabilizes the local DNA region to such an extent that the adducts can be detected by classical denaturation-based crosslinking assays [19].

The characteristics of adducts formed within the cell have not been extensively characterized, but some information exists from *in vitro* studies. These adducts are intrinsically unstable, demonstrating the reversibility of Schiff base complexes [12]. Due to the aminal linkage, adducts are both heat and alkali labile, exhibiting a half-life of 5–40 h *in vitro* at 37 °C, depending upon the site of adduct formation [13,19,20]. 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 [15]. The conditions required for adduct formation *in vitro* have been examined in several studies [14,15,17,21]; optimal formation occurs at pH 7, double-stranded DNA (dsDNA) is required, and the extent of formation is dependent on both DNA and formaldehyde concentration. The overall half-life reported recently for doxorubicin-DNA adducts in tumor cells in culture is 13 h [22].

Adducts have been detected *in vivo* in tumor cells in culture using several methods, the most direct using ^{14}C-labelled doxorubicin to yield ^{14}C -labelled doxorubicin-DNA complexes [22,23]. These adducts have been shown to be substantially more cytotoxic than lesions induced by topoisomerase II [24]. Encouragingly, increased cellular levels of formaldehyde have been detected in tumor cells (1.5–4.0 μM) compared to normal cells [25,26], suggesting the increased formation of adducts within tumor cells.

Several publications have reported methods for determining concentrations of doxorubicin and its adducts [22,27] utilizing capillary electrophoresis, laser-induced fluorescence detection, radioimmunoassay, high performance liquid chromatography (HPLC), fluorescence detection, chemiluminescence detection, electrochemical detection, or mass spectrometric detection (representative examples are outlined in Table 1). Each method utilizes a variety of pre-treatment procedures for samples, some of which are time-consuming solid-phase extractions. In addition, some of these techniques are laborious, expensive, and require significant technical experience which necessitates long processing and analytical run times. Additionally, many of these methods lack sensitivity and selectivity. For instance, a standard UV absorption detector has a high detection limit (μM levels or higher), imparting a handicap to this common detection technique. Similarly, HPLC with fluorimetric detection is a reliable and specific method, but it is relatively slow and sometimes lacks sensitivity. While the problem of resolving low concentrations has partially been resolved through the use of laser-induced fluorescence, some methods achieve lower LLOQ by large sample injections (50–70 μL). 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 [28]. The instability of doxorubicin also limits the utility of otherwise promising extraction strategies and would implicate a simple and rapid sample pre-treatment procedure is desirable. The majority of the methods used to quantify either nuclear fractions or adducts also obtain their samples from cultured cells *versus* from whole tissue or tumor, providing less relevant and simplified data than that obtained from *in vivo* preclinical and clinical studies. However, the predominant use *in vitro* studies to evaluate these effects is primarily due to the DNA requirements for detection which range from 1 to 2000 μg DNA required for sample analysis. Therefore, it would be beneficial to research and develop a cost-effective and timely method for quantification of anthracyclines from actual biological samples, such as those obtained from pharmacokinetic studies in animal models.

Many studies have measured total drug levels in solid tumors following administration of liposomal drugs [29,30]. Given that only drug released from liposomes into cells is available for biological activity, it would be advantageous to correlate: (1) the therapeutic effect of nanoparticles containing doxorubicin to (2) the levels of biologically active drug in tumor tissue *versus* (3) levels of total drug (encapsulated plus released) measured within the