REVIEW

Nanotherapeutics With Anthracyclines: Methods of Determination and Quantification of Anthracyclines in Biological Samples

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Summary

Anthracyclines, e.g. doxorubicin, pirarubicin, are widely used as cytostatic agents in the polymer nanotherapeutics designed for the highly effective antitumor therapy with reduced side effects. However, their precise dosage scheme needs to be optimized, which requires an accurate method for their quantification on the cellular level *in vitro* during nanocarrier development and in body fluids and tissues during testing *in vivo*. Various methods detecting the anthracycline content in biological samples have already been designed. Most of them are highly demanding and they differ in exactness and reproducibility. The cellular uptake and localization is predominantly observed and determined by microscopy techniques, the anthracycline content is usually quantified by chromatographic analysis using fluorescence detection. We reviewed and compared published methods concerning the detection of anthracycline nanocarriers.

Key words

Anthracycline • Nanocarrier • Quantification

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Introduction

Anthracyclines belong to the most potent cytostatics ever developed and various types of cancer are successfully treated by them. However, their administration may lead to cardiomyopathy and congestive heart failure. The first anthracycline, daunorubicin (DNR), was isolated from the *Streptomyces peucetius* in the 1960s and it is currently used in the treatment of acute leukemia (Coombs *et al*. 2015). The most common anthracycline, doxorubicin (DOX), which is believed to be one of the most powerful cytostatic agents, was developed shortly after. Until now, several new anthracyclines, e.g. epirubicin (EPI), pirarubicin (PIR), with enhanced antitumor activity and improved pharmacological profile have been synthesized and introduced into clinical practice (Minotti *et al*. 2004).

Nowadays, modern medicine requires targeted therapy, especially in cancer treatment. Over the past few decades, the targeted therapy using drug nanocarriers has been studied in detail. Anthracyclines, especially DOX, have served as active agents of various nanocarriers in cancer therapy (Wicki *et al*. 2015). The drug nanocarriers are accumulated in the solid tumor tissue due to the Enhanced Permeability and Retention effect (Matsumura and Maeda 1986) resulting in increased antitumor activity when compared with the free drug. The majority of the DOX-bearing nanocarriers utilize physical entrapment of the hydrophobic DOX molecules into the hydrophobic core (Deepa *et al*. 2014). However, the premature drug release from these nanocarriers in blood may lead to severe toxic effects on the healthy tissue and to decreased antitumor activity.

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The polymer conjugates with anthracyclines bound *via* biodegradable spacers enable the stimuli-sensitive release in the target tissue and prevent the premature drug release during blood circulation. Their benefits in cancer therapy have been proved *in vivo* and also in clinical trials. Unfortunately, their introduction into clinical practice is still awaited.

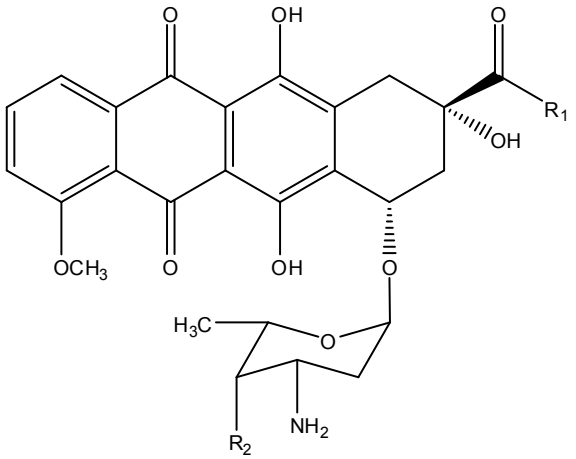
The clinical efficiency of anthracyclines has been proved to be related to their actual concentration in the tumor tissues (Speth *et al*. 1988). Moreover, their overall toxicity is dependent on the plasmatic concentration profile. Various analytical methods for the determination and quantification of free anthracyclines in the biological samples have been reviewed (Loadman and Calabrese 2001, Zagotto *et al*. 2001). Although anthracyclines are frequently used in diverse nanocarriers, the reviews dealing with the methods of the determination and quantification of the anthracycline nanocarriers are rare. The majority of the published methods analyzing these nanocarriers determine the total drug amount, both released and not released. Here, we summarize the methods for the determination of free and nanocarrier bound anthracyclines.

Anthracyclines and their nanocarriers

Since DOX is the most frequently used anthracycline in the clinics and in designing the new drug delivery systems, numerous methods of its determination in biological samples have been published. Its biodistribution has been compared with the biodistribution of other anthracyclines, e.g. DNR, idarubicin (IDA), EPI, PIR (Nagasawa *et al*. 1996, Pérez-Ruiz *et al*. 2001) or with its active metabolites (Anderson *et al*. 2002).

Numerous nanocarriers with anthracyclines have been synthesized and tested *in vivo*. Their biodistribution is crucial for the enhanced antitumor activity and for the minimized side effects. The biodistribution of the released drug and the drug carrier has been determined for the polymer-coated liposomes (Goren *et al.* 2000, Xiong *et al*. 2005, Li *et al*. 2009), among others, Doxil (Uster *et al*. 1998, Laginha *et al.* 2005); micelle-forming block polymers (Yoo and Park 2001, Shuai *et al.* 2004, Dai *et al.* 2008, Cuong *et al*. 2010, Wu *et al.* 2012); nanoparticles (Janes *et al*. 2001, Park *et al.* 2009, Jin *et al*. 2012); nanoparticles codelivering DNA or RNA (Chen *et al*. 2010, Meng *et al.* 2010); modified polypeptides (Yang *et al*. 2006) with physically entrapped anthracyclines. The physical entrapment of the drug cannot prevent the drug leakage during blood circulation and during the processing of the biological sample in the course of the drug content quantification. The attention should be paid to the used procedure, its duration and the applied excipients.

The nanocarrier with the anthracycline covalently attached *via* a spacer degradable in the tumor tissue or cells can decrease the drug leakage from the nanocarrier during blood transportation and enable the drug release in the target site (Fraier *et al*. 1995, Etrych *et al*. 2011). The carrier structure can be based on the water-soluble and biocompatible polymer, e.g. copolymers of *N*-(2-hydroxypropyl)-methacrylamide (HPMA) (Fraier *et al*. 1995, Etrych *et al*. 2011) or polyethylene glycol (PEG) (Rodrigues *et al*. 1999), proteins (Kratz *et al*. 1998, Beyer *et al*. 2001) and synthetic polypeptides (Dreher *et al*. 2003).



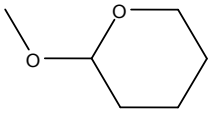
	R1	R2
Daunorubicin (DNR)	$-CH_3$	-OH
Doxorubicin (DOX)	$-CH_2OH$	-OH
Pirarubicin (PIR)	$-CH_2OH$	

Fig. 1. The chemical structures of anthracyclines.

Methods of anthracycline determination and quantification

Modern techniques enable the highly sensitive anthracycline detection in strongly diluted samples. However, these samples require very careful handling. Thus, prior to the review of the detection techniques, the anthracycline characteristics and possible complications concerning sample handling and pretreatment will be discussed.

Anthracycline characteristics important for their determination

The anthracycline structure consists of the tetracyclic aglycone (adriamycinone) and the sugar moiety, usually daunosamine (Fig. 1). The aglycone serves as the chromophore (light absorption at λ = 400-500 nm) and fluorescent moiety (light emission at λ = 500-600 nm upon excitation). Their chemical properties, e.g. fluorescence, enable their quantification in biological samples. However, their interaction with DNA leading to the fluorescence quenching complicates the direct determination of the anthracycline level in the cells (Rizzo *et al*. 1989). The anthracycline binding to plasma proteins can also lead to the imprecise results (Chassany *et al*. 1996).

Sample handling

For the precise quantification it is crucial to handle the samples containing anthracyclines with care, including used labware, their exposure to light and their stability in aqueous solutions.

Firstly, the anthracyclines sorb strongly to a variety of materials. Polypropylene labware and containers should be preferred to glass (Tomlinson and Malspeis 1982). Secondly, they are photolabile and thermolabile (Wood *et al.* 1990). Therefore, their exposure to light and high temperature should be minimized. Finally, the stability of anthracyclines and their metabolites in aqueous solutions, concerning biological samples, is relatively low (Beijnen *et al.* 1986). Thus, the biological samples have to be stored at –20 °C in the dark.

Not only is the active agent sensitive, but also the anthracycline nanocarriers themselves might be susceptible to the rapid degradation and the subsequent undesired drug leakage during sample preparation or storage under certain conditions. The nanocarriers based on self-assembled structures like liposome membranes or polymeric micelles can be disrupted by reagents or solvents routinely used during the drug determination and quantification methods. For example, the detergents, e.g. Triton X 100, interact with the liposome membranes (López *et al.* 1998). The micelle-based nanocarriers are highly sensitive to the change of pH, ionic strength, dilution etc. which can occur during the sample preparation, e.g. its deproteinization (Owen *et al*. 2012).

The drug leakage from the nanocarrier structure should not occur when the anthracycline is covalently bound to the system structure. Nevertheless, the attention should be paid to the nature of the spacer and its degradation mechanisms. The nanocarriers with the drugs bound *via* pH-sensitive spacers, e.g. hydrazone bonds, should be kept in the aqueous solution of neutral or slightly basic pH (Etrych *et al*. 2002). The addition of the non-selective proteases, e.g. trypsin, should be avoided when handling with the spacers degradable by lysosomal or other proteases (Ulbrich *et al.* 1981, Šubr *et al*. 1992). Therefore, the application of the published methods for the new nanocarriers should be considered in detail.

Sample preparation and anthracycline isolation

The majority of the instrumental methods determining the drug presence or content in biological samples require the sample pretreatment which consists of four major strategies – cell disruption, deproteinization, liquid-liquid extraction (LLE) or solid phase extraction (SPE) and their combination.

Intracellularly localized anthracyclines may be inaccessible for quantification. The analysis methods, e.g. HPLC, utilize analyte solutions, therefore the drug entrapped in precipitated cells will not be determined. In addition, the DNA-anthracycline complex formation can lead to the deceptive results as well. Several additives and mechanical techniques have been applied to disrupt the cellular membrane and DNA-drug complexes. The partial disruption of the cellular membranes occurs after adding the deproteinization agents and organic solvents during LLE. The enhanced cellular disruption can be achieved by detergents (Triton X-100), proteinases, ultrasound or their combination (Andersen *et al.* 1994). The DNA-anthracycline complex dissociates upon the exposure to sodium dodecyl sulphate (Rizzo *et al.* 1989) or DNAse (Sakai-Kato *et al.* 2010).

The biological fluids are directly deproteinized using organic solvents (acetonitrile), metal ions (zinc sulphate), acid (perchloric acid) or salt (ammonium sulphate). When analyzing human blood the choice of the anticoagulant is essential, since heparin has been proved

to interfere with the DOX measurements (Kümmerle *et al*. 2003). The tissue samples are generally mechanically homogenized, diluted with a particular buffer and deproteinized subsequently (Maudens *et al*. 2011). After deproteinization and centrifugation the samples can be directly analyzed (Álvarez-Cedrón *et al*. 1999).

Another approach consists of LLE (with or without prior deproteinization) using immiscible organic solvents, mainly halogenated solvents (Etrych *et al*. 2011). At first the sample pH is adjusted to 8.5-9.5 in order to convert anthracycline salts to the hydrophobic anthracycline bases. The extraction is followed either by organic phase evaporation and dilution of the evaporate in smaller volumes (Wall *et al.* 2007) or by back-extraction into the small volume of the aqueous solution (Gilbert *et al*. 2005). The redissolved analyte is predominantly analyzed using HPLC with the fluorescence detector (Xiong *et al*. 2005). LLE is widely used for the sample preparation. The sample pH adjustment, the choice of the organic solvent suitable for the analyte and extraction conditions lead to high extraction coefficients. However, the simultaneous determination of diverse anthracyclines and their metabolites with different hydro-lipophilicity may provide misleading results since some analytes will be extracted in the higher or lower amount.

The anthracyclines loaded to the nanocarriers physically are likely to be transferred to the organic phase with the released drug. Therefore, the total amount of the anthracycline can be determined in the biological samples using LLE (Li *et al*. 2009). The anthracyclines covalently bound to the hydrophilic polymers stay in the aqueous phase. Firstly, the released drug is extracted from the sample whose pH is adjusted to mild basic. Then, the polymer bound and free anthracyclines are hydrolyzed to the aglycone and the total drug amount is determined (Fraier *et al*. 1995).

During solid phase extraction the aqueous analyte solutions from biological samples are applied on the solid phase, usually reversed-phase C18 (Lachatre *et al*. 2000), C8 (Nicholls *et al.* 1992), C2 (Buehler *et al*. 1999) sorbents or polymeric Oasis HLB (Difrancesco *et al.* 2007) and MCX (Krogh-Madsen *et al*. 2010) sorbents. The analyte is retained on the solid phase and the biological impurities are washed out using water or buffers (Maudens *et al.* 2011). The analyte is subsequently eluted by organic solvents, e.g. acetonitrile, chloroform or their combination, and the eluate is usually analyzed. The online elution by HPLC mobile phase can be performed (Rudolphi *et al.* 1995).

Instrumental analysis

Various instrumental techniques have been utilized to determine the presence of anthracyclines in cells, e.g. flow cytometry (FC), fluorescence microscopy (FM), laser scanning microscopy (LSCM), spectrofluorometry and chromatographic methods. These techniques are usually based on the anthracycline fluorescent properties. FM, LSCM and FC are widely used methods which offer *in situ* determination of drug fluorescence and its intracellular localization in „samples without pretreatment". These approaches are combined in many studies or supplemented with quantitative anthracycline determination, e.g. by HPLC.

Fluorescent microscopy and confocal laser scanning microscopy techniques

Microscopic methods offer the detailed view of anthracycline uptake and intracellular localization after administration of free drugs or drug nanocarriers. The LSCM is a preferable method in comparison with FC, because it provides high resolution images of the anthracycline subcellular distribution in the cell lines (Coley *et al*. 1993). LSCM is a common method for evaluation of the intracellular localization of various types of anthracycline nanocarriers administered *in vitro* (Janes *et al*. 2001, Luo *et al.* 2002, Dreher *et al.* 2003, Cuong *et al*. 2010).

The drug distribution within cellular organelles can vary when the free drug or the drug bearing nanocarrier, e.g. protein conjugates with DOX, is added to the cell culture. The precise determination of the drug localization helps to clarify the mechanisms of action of these drug carriers. Using specific markers, e.g. for lysosomes, mitochondria, and the Golgi apparatus, it has been demonstrated that DOX from the protein conjugates was accumulated in the mitochondria and Golgi compartments, but not in the lysosomes, whereas free DOX showed a time-dependent intracellular shift from the nucleus to mitochondria and the Golgi apparatus (Beyer *et al*. 2001).

In many cases the samples were chemically pretreated before testing. Their fixation can keep samples preserved for later observation; on the other hand, changes of the cell morphology, drug fluorescence or drug localization in cell compartments can occur. The most usual fixation of cells is with 2-4 % paraformaldehyde (Jin *et al*. 2012, Wu *et al*. 2012).

The drug quantification by these techniques is insufficient and can be influenced by many factors, e.g. the

fluorescent signal quenching after binding of anthracyclines to DNA. Nevertheless, the attempt to quantify the drug using LCSM, which consists in quantifying fluorescent signal intensity from microscopy pictures by Image J software (Meng *et al.* 2010), was proposed.

FLIM

Fluorescent life time microscopy (FLIM) is also used for the documentation of the anthracycline presence inside the cells. The method can detect subcellular localization of different forms of anthracyclines or their derivatives using measurement of lifetime distribution, which can differ for free or nanocarrier bound anthracyclines in diverse parts of cells (Dai *et al.* 2008). For example, FLIM was used to elucidate intracellular localization of polymeric conjugates based on HPMA copolymers containing DOX bound through a biodegradable spacers (Říhová *et al.* 2010).

Flow cytometry

Microscopic techniques are often combined with FC enabling determination of fluorescence intensity and thus providing information about the drug content. With this technique, the drug fluorescence can be also evaluated *in situ* (Meng *et al.* 2010). The precise drug quantification is complicated by the fact that binding of anthracyclines to DNA causes quenching of fluorescence emission as mentioned above. Therefore, it is questionable whether the fluorescence which the *in situ* assays are based on is linearly related to the drug content of cells. For this reason the combination of detection techniques is recommended. Nevertheless, some studies were focused on the verification of the applicability of FC for anthracycline quantification. The DNR content in rat bone marrow cells was determined after incubation *in vitro* by FC and measurement of [^{3}H]DNR radioactivity. They showed a linear relationship between drug-related radioactivity and fluorescence intensity over the measured drug concentration range (0.1 to 3 μg/ml) (Nooter *et al.* 1983).

In some studies utilizing FC the sample fixation was also used (Xiong *et al.* 2005, Li *et al*. 2009). However, the fixation is less frequent than for microscopic techniques.

Liquid chromatographic analysis

Chromatographic analysis with the fluorescence detector is the dominant method of anthracycline quantification. The analysis requires sample pretreatment by deproteinization, LLE or SPE. The fluorescence detection is highly sensitive and enables analysis of subnanogram drug amounts (Rossi *et al.* 1993).

In most cases, the reversed-phase C18 stacionary phase is utilized, although C8, cyano and phenyl stacionary phase have been used, too. Isocratic or gradient mobile phases of various compositions have been published and reviewed previously (Chen *et al*. 2001, Maudens *et al.* 2011). The composition of the mobile phase for DOX, EPI and their metabolites was optimized using the solvent selectivity triangle (Nicholls *et al.* 1992).

Moreover, HPLC separation enables to quantify several anthracyclines or other drugs at the same time (Alhareth *et al.* 2012). The samples with various metabolites can be analyzed using HPLC separation coupled with electrospray tandem mass spectroscopy which can identify the metabolite chemical structure and quantify very low drug concentrations (Liu *et al.* 2008).

The anthracyclines separated by HPLC can be detected also by UV/VIS-spectrophotometrically (Chin *et al.* 2002), electrochemically (Ricciarello *et al.* 1998), chemiluminescently (Ahmed *et al.* 2009). Although the electrochemical or chemiluminescent detection of anthracyclines is sensitive, these methods are rarely used. UV spectrometrical analysis of anthracyclines is less sensitive.

In our experience, HPLC separation and subsequent fluorescence detection with precedent LLE is a highly reproducible, selective and sensitive method of quantifying the released and not released drug from the anthracycline nanocarriers (Šťastný *et al.* 2002, Chytil *et al.* 2008).

Other instrumental methods

When one anthracycline is analyzed, the overall fluorescence of the analyte solution can be measured fluorimetrically, e.g. using the microplate reader (Meng *et al.* 2010). Other instrumental methods have been used to analyze the drug content, e.g. Rayleigh scattering spectrometry (Liu *et al.* 2007), capillary electrophoresis with UV absorption detection (Gavenda *et al.* 2001), with laser-induced fluorescence detection (Lu *et al.* 2009) or with amperometric detection (Hu *et al.* 2000). However, the majority of these studies concerned the free anthracyclines.

In vitro and in vivo models used in the anthracycline biodistribution studies

In vitro

The broad spectrum of *in vitro* models, mainly based on the mouse and human cell cultures, are used to

study cellular penetration of free anthracyclines, their subcellular distribution and content in specific cell types. The *in vitro* models are also used to study cell uptake, release and subcellular localization of anthracyclines from nanocarriers in order to obtain preliminary data about their cytotoxicity potential and their possible application for cancer treatment. The *in vitro* models were often used to study the ability of various nanocarrier systems to overcome the drug resistance of cancer cells (Meng *et al.* 2010, Jin *et al.* 2012). The *in vitro* models are suitable for the detailed study of subcellular distribution – cytoplasmic, nuclear or membrane binding of the drug after the nanocarrier treatment (Rodrigues *et al.* 1999, Beyer *et al.* 2001).

In vivo

The anthracycline quantification in tumor tissue or other organs and body fluids after *in vivo* administration to animals is usually performed after dissection and *ex vivo* detection, e.g. by chromatographic analysis using fluorescence detection with drug isolation by LLE (Xiong *et al.* 2005, Li *et al.* 2009) or by the measurement of radioactively labeled nanocarriers as in the case of detecting the HPMA enzymatically bound Dox in body fluids and other organs (Jelinkova *et al.* 2003). Drug accumulation in tissues can be evaluated by FM of dissected organs or prepared organ sections (Shen *et al.* 2008, Chen *et al.* 2010). The direct *in vivo* visualization of the anthracycline biodistribution using fluorescence imaging is complicated due to absorption of the fluorescence signal from the anthracycline by tissues.

Anthracycline nanocarriers in clinical trials

The similar approaches of drug quantification described above were applied in several clinical studies. The anthracycline nanocarriers investigated in clinical trials or already approved for treatment of humans, e.g. PEGylated liposome with entrapped DOX (Doxil – Caelyx), were reviewed here (Minotti *et al.* 2004). The method using LLE and subsequent HPLC analysis to quantify released and not released DOX from HPMA-based conjugates in human samples was developed (Fraier *et al.* 1995, Říhová *et al.* 2003). The HPLC analysis of DOX content in human plasma and urine was combined with ^{123}I-based imaging of HPMA-based conjugate in clinical trials concerning patients with hepatoma (Julyan *et al.* 1999). Indeed, the anthracycline content in human samples can be measured using other methods, HPLC coupled with mass spectroscopy for simultaneous determination of anthracyclines and their metabolites (Lachatre *et al.* 2000) or LSCM imaging of DOX distribution in slices from tumor biopsy (Lankelma *et al.* 1999). However, these methods concern free anthracyclines.

Conclusions

We reviewed the recent approaches of qualitative and quantitative determination of anthracycline nanocarriers. The precise and reproducible method determining the anthracycline content in biological samples is one of the important requirements of successful introduction of novel anthracycline nanocarriers into experimental trials and into clinical practice in the future. We concentrated on the instrumental methods enabling anthracycline determination in biological samples with respect to sample handling and pretreatment. In summary, the real-time visualization of the cellular fate of the anthracycline carriers is usually performed by microscopy techniques and FACS. However, the precise quantification of the drug content within the cells using these techniques is complicated. The accurate quantification of the drug content is usually executed using HPLC devices with the fluorescence detectors after anthracycline isolation from the biological sample by deproteinization, LLE or SPE.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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