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Optimization of LC method for the quantification of doxorubicin in plasma and urine samples in view of pharmacokinetic, biomedical and drug monitoring therapy studies

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ABSTRACT

A simple, rapid, reliable and sensitive method based on liquid chromatography with fluorescence detection (LC-FL) for the quantification of doxorubicin (DOX) in human plasma and urine samples was developed. The assay was carried out after the solid-phase extraction procedure (SPE) with hydrophilic-lipophilic balance (HLB) cartridges, and with daunorubicin hydrochloride (DAU) used as the internal standard. Chromatographic separation was performed on a Discovery HS C18 column in isocratic elution mode, and the detection of the analytes set at excitation and emission wavelengths of 487 and 555 nm, respectively. The developed LC-FL method has been validated for accuracy, precision, selectivity, linearity, recovery and stability. The limits of detection and quantification for DOX were 0.5 and 1 ng/mL in both biological fluids, respectively. Linearity was confirmed in the range of 1–1000 ng/mL and 0.001–25 μg/mL in plasma and urine samples, respectively, with a correlation coefficient greater than 0.9994. The proposed LC-FL method is selective, precise and accurate, and has been successfully applied for drug monitoring in pediatric cancer patients treated with DOX as a component of OEPA (Oncovin (Vincristine)-Etoposide-Prednisone-Adriamycin) and IOA (Ifosfamide-Oncovin-Adriamycin) chemotherapeutic schemes. Moreover, real exposure of hospital personnel to the anthracycline drugs in plasma and urine was evaluated in clinical practice.

1. Introduction

Doxorubicin, 14-hydroxydaunomycin (DOX), simultaneously called Adriamycin, is an anthracycline, cytotoxic antibiotic obtained from the bacterial strain *Streptomyces peucetius* var. *caesius* [1]. Due to its broad spectrum of antineoplastic action, doxorubicin is currently used for the treatment of acute leukemia, Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL), soft tissue sarcomas (including rhabdomyosarcoma, RMS), neuroblastoma, Wilms tumor, breast carcinoma, hepatocarcinoma, ovarian carcinoma and many others [2–4]. Despite their broad clinical usage, the mechanism of action of anthracyclines is not fully explained and remains unclear [5]. However, there are several suggested mechanisms by which DOX causes the death of tumor cells. Some of them include the intercalation or alkylation of DNA and disruption of DNA repair via topoisomerase II. This leads to the inhibition of DNA and RNA synthesis. Another way is the formation of free radicals, which can react with the cell membrane and damage its function [6]. The resulting free radicals also respond to acute cardiotoxicity [7]. Symptoms such as hypotension, arthritis, or myocarditis are reversible and are not a basis for the discontinuation of therapy. In turn, chronic myocardial infarction is a far greater risk for patients treated with this antibiotic, which can subsequently lead to congestive heart failure, with an ultimate mortality rate of 20–40% [8]. This is a very serious health complication, which can appear many years after the end of treatment. The other side effects caused by DOX include: vomiting, nausea, myelosuppression and mucositis [9]. This drug is mainly metabolized by the liver with biliary excretion and fecal elimination; however, a

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small amount is excreted through the kidneys as an unmetabolized drug [10].

DOX belongs to a class of anticancer drugs widely used in chemotherapy with both pediatric and adults patients. It can be used alone or in combination with other anticancer drugs in many cancer therapeutic protocols for adults and children e.g. GPOH-HD 2002, 2000 ALL, NOPHO AML – 92 protocol, (NOPHO) AML – 93, ALL – BFM 95, NHL – BFM 95 protocol, NOPHO 92 SR/IR (Table S1) [2,4,11–16]. Another example of such a therapeutic protocol is EurNet-PHL-C1 including OEPA (Oncovin (Vincristine)-Etoposide-Prednisone-Adriamycin) cycles, which is commonly used to treat the classic form of HL in children and adolescents as well as the international protocol CWS, including IOA (Ifosfamide-Oncovin-Adriamycin) cycles, used for the treatment of soft tissue sarcomas in children with RMS [3]. Unfortunately, both mono- and multi-drug therapeutic protocols based on DOX administration can be ineffective because of individual differences in the patient's genotype and the possibility of interactions between the used drugs. Thus, the monitoring of DOX in biological fluids is very important in clinical practice, since optimizing the dose to the individual needs of the patient can improve the effectiveness of chemotherapy and may decrease the severity of adverse effects, including also the risk of cardiomyopathy with congestive heart failure. It is particularly important in children for whom the risk of an inappropriate dosage adjustment can be higher because of limited numbers of clinical reports published in the world literature. To the best of our knowledge, only one paper has described the plasma and urine profiles of DOX in the OEPA scheme in children [11], while there is no information about DOX profiles in the RMS treatment regimen for children. On the other hand, methodologies used for drug monitoring therapy should guarantee precise and accurate drug determination in a relatively low sample volume, be fast and simple as well as be based on the analytical apparatuses commonly used in many laboratories.

In the world scientific literature, there are a lot of analytical methods for the determination of DOX in different biological matrices, including plasma [15,17–24], urine [23,25,26] and animal serum and tissue samples [27,28] (Table S2). These methodologies were based on liquid chromatography (LC) [15,19,23,25–28], ultra-high performance liquid chromatography (UHPLC) [17,18,20,24] and capillary electrophoresis (CE) [21,22]. For them, different detection modes like ultraviolet (UV) [18,22,28], fluorescence (FL) [15,21,23,24,27], electrochemical detection [19], and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [17,20,25,26] were applied. It is the fact that the chemical structure of DOX allows the use of different detection modes, but these detectors are able to monitor DOX with a different sensitivity and selectivity. For example, the previous LC-UV and CE-UV methods were carried out at $\lambda = 254$ nm [18] and 234 nm [28], and they allowed DOX to be monitored with an limit of quantification (LOQ) of $\geq$ 30 ng/mL. The advantage of UV detection was its simplicity, low cost of the apparatuses and the fact that this detector belongs to the most popular detectors used in many pharmaceutical and clinical laboratories. On the other hand, a serious disadvantage is its relatively low sensitivity compared to FL and MS which may be not enough for many clinical and pharmacological studies.

FL detection is more attractive for DOX determination in biological matrices because of its higher selectivity and sensitivity (about 30 x higher) than offered by UV. Additionally, this anticancer drug possesses natural florescence activity, which reduces the necessity of the derivatization steps before chromatographic/electrophoretic separation. According to the literature data, DOX quantifications in biological samples based on FL detection were carried out at the excitation wavelength ranging from 470 to 480 nm, while the emission wavelength was from 548 to 560 nm [15,21,23,24,27]. Those methodologies were able to detect DOX with LOQ parameters from 0.31 to 10 ng/mL. Compared to MS, the FL detector is easy to use and cheap. Moreover, FL apparatuses belong to the basic equipment of many laboratories.

For DOX quantification in biological samples, MS detection has also been used [17,20,25,26]. This type of detection possesses many advantages such as high selectivity (the mass spectrum is specific for each compound) and sensitivity, which in the case of electrospray ionization (ESI) coupled to MS detection for DOX is almost 2200 and more than 72 times higher in comparison to UV and FL, respectively. In fact, lower LOQ parameters were calculated for previously reported LC–MS methodologies (Table S2). However, MS apparatuses because of their high costs and the need to employ highly qualified personnel are not commonly used in clinical laboratories. In consequence, despite many benefits of MS detection, the application of separation methods based on MS can be problematic in clinical practice.

It should be noted that sample preparation procedures can decide about the success or failure of the developed method for DOX quantification. According to the literature data, the extraction of this anthracycline from biological matrices has been based on protein precipitation with methanol (MeOH) [15,17,27], a mixture of acetonitrile (ACN)/MeOH [18], trichloroacetic acid [21] and MeOH/water with the addition of zinci sulfas [24]. Moreover, liquid-liquid extraction (LLE) using different mixtures of organic solvents such as chloroform with the addition of ACN and phosphate buffer [22], and isopropanol/chloroform [23] as well as solid-phase extraction (SPE) with C18 [25,26,28] and HLB cartridges [19,20] were applied. Unfortunately, many of these extraction procedures possess several limitations, like long sample preparation procedure [23], low extraction efficiency [20,22,23,25,26,28], high limits of detection (LOD) [15,18,21,24,27,28] or no data confirming their usefulness in clinical practice [18,19,21,26]. Moreover, the relatively large human plasma/urine volume of 1 mL was required per sample, which may be difficult to safely obtain for pediatric patients [20,23,25,26]. Therefore, the application of those methodologies for routine DOX determination in monitoring therapy may be problematic. Moreover, time-consuming chromatographic/electrophoretic separation ($\geq$ 15 min) may additionally decrease the utility of the earlier reported techniques for DOX quantification in clinical investigations [15,19,22,23,25–28].

The aim of the study was to develop a rapid, sensitive and precise LC method based on a detection mode widely used in many laboratories, simultaneously with an uncomplicated sample preparation procedure which could be commonly used in clinical practice for the quantification of DOX in human plasma and urine samples. Next, the developed and validated LC method was successfully used for the monitoring therapy of a child with HL and a pediatric patient with RMS. Finally, the plasma and urinary profiles of DOX in children were calculated and compared to the ones reported in the literature. Additionally, the exposure of hospital personnel to the anthracycline in plasma and urine was evaluated.

2. Materials and methods

2.1. Reagents

Doxorubicin hydrochloride (DOX) (>98% purity) and daunorubicin hydrochloride (DAU) used as the internal standard (I.S.) (>98% purity) were purchased from Tocris Bioscience (Bristol, United Kingdom). Hydrochloric acid (HCl) (36%), ethyl acetate, chloroform, 2-propanol and dichloromethane (DCHM) of analytical grade were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile (ACN) and methanol (MeOH) were provided by J.T. Baker (Phillipsburg, NJ, USA). Formic acid came from Sigma-Aldrich (St. Louis, MO, USA). The water used in the experiment was purified by the Milli-Q system (Molsheim, France). Supel Select HLB