A new validated method for the quantification of Doxorubicin in plasma by High Performance Liquid Chromatography (HPLC) with application in pharmacokinetic studies: quantification of Doxorubicin by HPLC in pharmacokinetic studies

DOI: 10.46981/sfjhv3n3-008

Received in: April 29th, 2022
Accepted in: June 30th, 2022

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ABSTRACT
Doxorubicin (Dox) is a drug widely used in cancer chemotherapy. Dox is essential in the treatment of pediatric patients diagnosed with different types of cancer, including osteosarcoma (OS). Dox has been used clinically for more than 3 decades, but recently it has been recognized that the cytotoxic effect occurs by multiple mechanisms that have not yet been conclusively identified. Known risk factors for Dox include cumulative dose, younger age, concomitant use of some other antineoplastic agents, female gender, and increased dose intensity. For these reasons, it is important to develop and improve plasma quantification methods for this antineoplastic drug, with the idea that they are more easily usable in clinical practice and thus seek to improve the treatment of patients using Dox. The objective of the present study was to develop an economical, precise, rapid, and exact analytical method by HPLC for the determination of Dox for pharmacokinetic studies in pediatric patients. This method was successfully applied to study Dox chronopharmacokinetics in pediatric patients with osteosarcoma and thus evaluate its chronotoxicity.

Keywords: chromatography, HPLC, doxorubicin, pharmacokinetic, osteosarcoma.

1 INTRODUCTION
Doxorubicin (Dox) is a drug widely used in cancer chemotherapy. It is an antibiotic of the anthracycline family, with a structure very similar to that of Daunorubicin (Dau), the second most widely used anthracycline in the treatment of cancer. (Zhuang et al., 2020) Dox is a DNA intercalary that prevents cell replication. (Agudelo et al., 2014; Heini et al, 2021)

Dox is essential in the treatment of pediatric patients diagnosed with different types of cancer, including acute lymphoblastic leukemia (ALL) and osteosarcoma (OS). (Kopp et al, 2019) Dox has a molecular structure made up of C27H29NO11, a molecular weight of 543.52 g / Mol and an elimination half-life: 21-30 hours. (Krischke et al, 2016) Dox has been used clinically for more than 3 decades, and only recently has it been recognized that the cytotoxic effect occurs at the cellular level by multiple mechanisms that have not yet been conclusively identified, the key factors being a combination of Dox-radical formation free induced due to metabolic activation, damaging actions at the membrane level and intercalation of drugs in DNA. (Renu & Arunachalam, 2018) The therapeutic window for Dox is narrow and several serious side effects are associated with this drug including myelosuppression, mucositis, and...
cardiotoxicity. (Danesi et al., 2002) Known risk factors for Dox include cumulative dose, younger age, concomitant use of some others antineoplastic agents, female gender, and higher dose intensity. (Cai et al., 2019; Upadhyay et al., 2021)

Some other analytical methods have been developed for Dox quantification with fluorescence or UV detection. One of the first methods was the one presented by Beijnen et al (Beijnen et al., 1991) who used fluorescence detection, in addition to a protein precipitation with propanol and a 5-micron column. Years later Lucas et al (Lucas et al., 2016) improved this technique by reducing retention times from 10 to 5 minutes, for which they used a fluorescence detector, a Phenomenex Luna C18 column (2 μm, 2.0 × 100 mm) and a mobile phase in a formic acid gradient 1% in water or acetonitrile for separation and quantification.

The HPLC methods prior to this one has the disadvantage that the retention time of the drug is a little longer or that the extraction and preparation process is more laborious. This new analytical method that is proposed uses a fluorescence detector, a column C18, and a method of precipitation of proteins with methanol that reduces the final time of the assay. The reduction of the time of the chromatographic method is essential in the use of this technique in clinical practice, especially when it is used to measure drugs with the potential to produce toxic effects in high-risk patients, as it was in this study in where it was used to measure Dox in pediatric patients with OS.

The objective of the present study was to develop an economical, precise, rapid, and exact analytical method by HPLC for the determination of Dox pharmacokinetic studies in pediatric patients with OS. Although Dox is widely used in the treatment of different types of cancer such as OS, and its interindividual variability is well known, the pharmacokinetics of this antineoplastic agent is not a common practice established in Mexico, it is necessary to develop studies like this one that can demonstrate the importance of the implementation in clinical practice of the pharmacokinetics of high-risk drugs such as Dox.

2 MATERIALS

The validation of the analytical technique for this work was part of a research protocol carried out in pediatric patients with ALL to assess cardiotoxicity by Dox, which was approved by the Research and Research Ethics Committees of the Hospital Materno Infantil de Durango, Mexico, with registration number 516 / 019, in accordance with the Declaration of Helsinki and the General Health Law of Mexico.

The pharmacokinetics of patients with OS was carried out at the request of the treating physician. Parents of patients were asked to give their informed consent in writing; In addition, the children were also asked to give their informed assent in writing.
2.1 PATIENTS

Two patients 9 and 13 years old, two girls with osteosarcoma (OS), treated with Dox received two intravenous infusions of Dox of 50 mg / m² for 48 hours. There was a period of two weeks between each infusion, maintaining the same duration of administration. Two pharmacokinetic studies of Dox were performed, one at each time of the two infusions.

2.2 CHROMATOGRAPHIC SYSTEM

An Agilent 1100 series HPLC equipment Wilmington, DE, USA (Agilent Technologies, Inc. 2002) was used, which consists of a quaternary pump, an automatic injection system, a variable wavelength fluorescence detector and a column oven. Chromatographic data was collected using Agilent Chem Station Software. (Software Agilent Chem Station, 2004) An Agilent Bonus-RP column (1.8um 4.6 x 50mm) (Agilent Bonus-RP) was used.

2.3 CHEMICALS AND REAGENTS

Doxorubicin Hydrochloride (D1515-10 MG) was obtained from Sigma-Aldrich (Sigma-Aldrich Products). Water (tri-distilled filtered with Milli-Q plus equipment with a 0.22µm filter with a resistivity of 18.5MΩ), acetonitrile, methanol, and formic acid (HPLC grade) were purchased from Sigma Aldrich.

3 METHODS

3.1 CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions were a mobile phase Acetonitrile/water (35:65 v/v) acidified with formic acid at 0.05% with a flow of 0.6 mL/min. The column temperature was controlled between 22 °C and 28 °C and the injection volume was 30 µL. The fluorescence detection wavelength was 480 nm of excitation and 560 of emission. (De Bruijn et al, 1999)

3.2 EXTRACTION PROCEDURE

The extraction procedure is based on the precipitation of proteins. (Alshammari, Al-Hassan, Hadda & Aljofan, 2016; Mohammed, Eissa, & Ahmed, 2017) To a 400 µL aliquot of plasma containing a known Dox concentration, 200 µL of mobile phase was added, then centrifuged at 8,000 rpm, the supernatant was taken and filtered with 13mm, 0.22um, Hydrophobic PTFE filters. from Thermo Scientific®.
3.3 PREPARATION OF THE INTERNAL STANDARD

Stock solutions were prepared for Dox (1 mg/mL) and for Dau (1 mg/mL), which was used as internal standard (IS). Compounds were dissolved in methanol and used to prepare plasma calibration curve concentrations with a concentration range of 10 to 500 ng/ml for each.

3.4 ASSAY VALIDATION

The validation of the analytical method was carried out in accordance with the guidelines published in the Official Mexican Standard NOM-177-SSA1-2013. (NORMA Oficial Mexicana, 2013) The plasma calibration curve was performed with the following concentrations: 10, 100, 200, 500, 1000, 1,500, 2,000 and 5,000 ng/mL. In each test, a blank plasma sample from healthy volunteers processed without the presence of the internal standard was analyzed to confirm the absence of interference.

The minimum concentration of Dau internal standard was 10 ng/mL. The intra- and inter-day variability parameters were determined to evaluate the precision and accuracy of the method.

Absolute recovery was determined as the percentage of drug extracting the Dox from the plasma and the resulting solution compared to the drug dissolved in methanol.

To evaluate the selectivity of the method, Dau, acetylsalicylic acid, and ondansetron were tested to detect possible interferences of the compounds under study and other drugs administered concomitantly. The latter was considered important as Dau and ondansetron are drugs commonly used in the treatment of ALL along with Dox.

3.5 PHARMACOKINETIC STUDY

As part of the chemotherapy protocol, two pediatric patients with OS were given two rounds of Dox 50 mg/m² intravenously over 48 hours, with 2 weeks between each of the two rounds of Dox. In addition, as a cardioprotective agent, 500 mg IV of cardioxane was administered in an infusion one hour before Dox. Two weeks later a second round of Dox was administered, at the same concentration as the previous one.

Blood samples were taken before and after Dox. The pharmacokinetic study along with its routine follow-up are shown in Table 1. The samples were extracted through an intravenous cannula previously made in one of the arms of the patient who received chemotherapy; blood samples were collected in plastic tubes with heparin as an anticoagulant (Sarstedt®). Biological samples were centrifuged for 10 min at 3,500 rpm and plasma was immediately processed for DOX quantification.
Table 1. - Sampling times for the two pharmacokinetic studies performed on the 2 patients with OS.

<table>
<thead>
<tr>
<th>Time / hour</th>
<th>Number of sample</th>
<th>Infusion 1</th>
<th>Infusion 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Before infusion</td>
<td>0</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>During the infusion</td>
<td>1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>After infusion</td>
<td>1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

3.6 STATISTICAL ANALYSIS

The validation of the analytical method was carried out based on the criteria of the Official Mexican Standard NOM-177-SSA1-2013. (NORMA Oficial Mexicana, 2013) Dox concentration-time data were analyzed using Monolix® (version 2020R1. Antony, France: Lixoft).

4 RESULTS

4.1 EXTRACTION PROCEDURE

The extraction procedure was carried out successfully. The yield for a 1 ml sample of plasma was 92%.

4.2 RETENTION TIMES AND LINEARITY

Figure 1 Shows a typical Dox plasma chromatogram of a sample obtained from the pharmacokinetic study. Figure 2 shows a plasma chromatogram with application of the internal standard (Dau), the retention times of Dox and Dau (internal standard) were 1,098 min and 3,125 min respectively. The total HPLC run time was 15 min. No interference of any kind was observed in the blank plasma samples. The calibration curves prepared for Dox in plasma were linear, Figure 3.

The limit of quantification was 10 ng/mL for Dox. The precision error ranged between 4.4% and 6.9%. The detection limit, representing a 3:1 signal-to-noise ratio, was 1.5 ng/mL (2.75 nM) for Dox. All the coefficients of determination (r2) for the calibration curves were equal to or greater than 0.999.
Figure 1.- Chromatogram of plasma with Dox without standard application of a sample obtained from a pharmacokinetic study

![Chromatogram of plasma with Dox without standard application](image1)

Figure 2.- Plasma chromatogram with Dox and application of internal Dau standard

![Plasma chromatogram with Dox and application of internal Dau standard](image2)

Figure 3.- Calibration curve for Dox in plasma.

![Calibration curve for Dox in plasma](image3)
4.3 PRECISION AND ACCURACY

For each concentration of Dox (10, 20, 50, 100, 1000, 1500, 2000 and 5000 ng / mL), they were prepared in bulk for individual injections between days.

The precision analyzes of the standards were renewed daily. The intraday and interday precision of the assay was evaluated by performing three analyzes in triplicate.

The precision results, expressed as coefficient of variation (CV%), are presented in Table 2 and range between 3.89% and 4.12%.

Table 2.- Summary of the validation test

<table>
<thead>
<tr>
<th>Curve range</th>
<th>Range de 10ng/ml a 5000ng/ml (with linear regression of 1 / x²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve</td>
<td>R²</td>
</tr>
<tr>
<td>Quality controls</td>
<td>Recovery accuracy (CV%)</td>
</tr>
<tr>
<td></td>
<td>Accuracy (bias %)</td>
</tr>
<tr>
<td>Stability</td>
<td>3 weeks (4-8°C)</td>
</tr>
</tbody>
</table>

4.4 RECOVERY

The efficiency of the extraction performed (recovery) was determined from the Dox extraction of simulated samples at known concentrations (100 and 1000 and 2000 ng/mL) in triplicate with the internal standard added after extraction. Recovered Dox concentrations were compared to extracted external standards, prepared by adding a stock solution of stock Dox and internal standard. The mean recovery of extracted doxorubicin from the sham samples was determined to be 76% ± 1.7% (n = 20).

4.5 PHARMACOKINETIC ANALYSIS

The sensitivity and specificity of the proposed method were found to be sufficient for accurate quantification of Dox in plasma from OS patients after a 50 mg / m² intravenous infusion dose of the drug. Therefore, we used the method to study the Dox pharmacokinetics in two patients. Figure 4 (A, and B) shows the Dox plasma concentration-time profile of the two infusions of two patients. Dox had a peak at 48 hrs in all 2 patients, at which point the infusion ended. A rapid elimination process is observed two hours post-infusion (hour 50), followed by a slower elimination phase until hour 20 post-infusion (hour 68). For the pharmacokinetics performed two weeks later, the pharmacokinetic profiles and parameters were similar in patients however, for patient 1 (figure 4 A) the pharmacokinetics were different, showing a very long distribution period and a delay in the elimination of Dox, this due to at the time of drug administration as described by Gándara-Mireles et al. (Gándara-Mireles et al, 2021)
Table 3 shows the pharmacokinetic parameters of Dox after its administration during the two infusions in the 2 patients with OS.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Infusion number</th>
<th>Clearance (L/hr)</th>
<th>Half-time γ(t1/2γ) (hr)</th>
<th>Area down the curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>87.17</td>
<td>19.71</td>
<td>1.391</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>56.75</td>
<td>9.35</td>
<td>1.626</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>108.8</td>
<td>20.94</td>
<td>1.352</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>96.09</td>
<td>18.51</td>
<td>1.425</td>
</tr>
</tbody>
</table>

5 DISCUSSION

In Mexico, Dox is used to treat different types of cancer, including; some types of lymphoma, leukemias and solid tumours like OS, therefore, the development of simple, cost-effective, accurate,
rapid, and sensitive HPLC methods is key to allow the routine measurement of plasma levels and the study of the pharmacokinetics of these drugs with a narrow therapeutic window in high-risk patients, to correlate its pharmacological effects with plasma levels and pharmacokinetic behavior.

As part of a study protocol in children with OS, a reliable, sensitive, and accurate HPLC method was designed for Dox determination in plasma and has the potential to be applied for Dox analysis in pharmacokinetic studies.

The determinations of the Dox concentrations were carried out using high-performance liquid chromatography based on the combination of two previously reported methods. (Lucas et al, 2016; Beijnen et al, 1991)

This new method has an important advantage over the other methods that have already been published; for example, with shorter retention times of Dox due to the type of column we have used (Agilent Bonus-RP (1.8um 4.6 x 50mm), with decreases of minutes per sample. Residual contaminants are easily washable from the column before each injection, and this method does not require pH adjustment of the samples.

In their work Lucas A.T. et al in 2016 (Lucas et al, 2016), reported a retention time of 6 minutes for the determination of Dox, in our work by using a column with smaller dimensions we increased the pressure and we managed to considerably reduce the retention time (1.07 minutes).

Several preliminary studies were carried out to optimize the chromatographic conditions for the quantification of Dox. One of the studies was for the optimization of the pH of the mobile phase. Jemal M. et al in 1998 (Jemal, Ouyang & Teitz, 1998), pioneered their research to study the effects of variable concentrations of additives in the mobile phase of high-performance liquid chromatography of acetonitrile/water, they made an important finding since they realized that adding the Formic acid caused less attenuation of the negative ion response in the run. Equally important was the fact that the addition of formic acid had the desirable effect of maintaining a reasonably high-capacity factor (k’) for the analyte even at a relatively high acetonitrile concentration. A concentration of 1 mM formic acid in the mobile phase was large enough to achieve the reproducible long retention time for the analyte, such that in our work we have used formic acid at 0.05% and this gave us a 29% reproducibility higher than when we use glacial acetic acid to acidify our mobile phase. On the other hand, Lucas A.T. et al. in 2016 (Lucas et al, 2016), used formic acid in its acetonitrile/water mobile phase, however, although they found very good results when using said the acid in percentages of 0.1% in the mobile phase, we found that in our work, by reducing this concentration to 0.05%, the separation of ionizable substances was better.

One of the critical points in the assembly of the analytical technique is to add the mobile phase in an isocratic way or by gradient, in that sense de Bruijn P., et al in 1999 (Bruijn et al, 1999) obtained very good results when developing their technique with an isocratic addition of mobile phase, this gave
him very good results. In our work, some mixtures of the mobile phase of acetonitrile and water were made with different pH and different flow rates. These gradient methods failed to produce symmetrical peaks, and the precision and sensitivity were not adequate for the proposed pharmacokinetic studies, so we tested isocratically and managed to obtain the best result.

Lucas A.T., et al in 2016 (Lucas et al, 2016), obtained an intraday and interday precision of the test expressed as a coefficient of variation (CV%) that ranged between 4.01% and 8.81%, in our work we have obtained CV% values of 3.89% and 4.12%, these values are adapted to what the Mexican Standard for the validation of analytical methods requires (NORMA Oficial Mexicana, 2013). On the other hand, de Bruijn P., et al in 1999 (de Bruijn et al, 1999) demonstrated excellent accuracy (91.0-106%) and precision (0.90-10.2%) in the values reported for their analytical technique, however, our technique proved to have very good accuracy values with mean triplicate values of 93.06-99.0%.

There are various works in the literature that speaks about the validation of analytical techniques for quantifying high-risk drugs in children with some type of cancer, however, there is little specifically described the validation of a Dox analytical technique with application in pharmacokinetic studies in pediatric patients with OS, even though osteosarcoma is the most common primary bone cancer in children and adolescents and represents approximately 400-500 new diagnoses annually, which represents up to 10% of new cancer diagnoses in the population in the USA. (Arndt & Crist, 1999; Brown, Tellez-Gabriel & Heymann 2017)

In addition to what was mentioned above about the high prevalence of OS, a study by Mirabello et al (Mirabello, Troisi & Savage, 2009) based on data from the National Cancer Institute's Surveillance, Epidemiology, and End Results Program indicates a higher incidence and slightly worse outcome of osteosarcoma in Hispanics compared to non-Hispanics including Mexico. These reports show the need for surveillance of patients with this disease.

In our work, it was found that the sensitivity and specificity of the proposed method were sufficient for the precise quantification of Dox in plasma of patients with OS, therefore, Dox pharmacokinetics were performed. The graphs presented of the pharmacokinetic studies of the two patients show that it is better described using a two-compartment model, this compartment description is very important in clinical practice, as mentioned by Bassingthwaighte, J. B., et al in 2012 (Bassingthwaighte, 2012), in whose article he mentions that in the pharmacokinetics of high-risk patients, compartmental models are widely used to describe concentration-time curves of the concentration of a drug after administration. This data provides a description of how many parameters such as how long it remains in the body and is a guide to define the dosage regimens, the method of administration, and the expectations of their effects.
Our pharmacokinetic findings agree with many published articles. For example, we report an average elimination gamma half-life ($\gamma(t_{1/2\gamma})$) (counting the two pharmacokinetic analyzes performed on each of the 2 patients) of 14.5 hrs for the first patient and 19.95 hrs for the second patient. These results are comparable to the results published by Krischke M. et al in 2016 (Krischke, 2016), they reported a Dox elimination half-life in four pediatric patients studied that ranged from 21 to 30 hours.

It is interesting to highlight the results obtained in patient number 2, whose results show a shorter elimination gamma half-life ($\gamma(t_{1/2\gamma})$), even though the second infusion of this patient shows a shorter elimination half-life than the first, we can observe that the second infusion has an area under the curve much larger than the first, we can see how during this infusion there is a post-infusion stage (2 hours after the infusion ended) where the drug was kept in high concentrations, this fact caused toxicity to patient number 2 due to the time of administration of Dox, this case report carried out by our workgroup with this patient has already been published due to its high importance where the effect of chronopharmacokinetics on the toxicity caused by Dox. (Gándara-Mireles et al, 2021)

The importance of pharmacokinetics as a complement to the treatment of patients under treatment with high-risk drugs such as Dox is evident. Coulson J. (Coulson, 2020) shows us in his work how the human body is affected by numerous interrelated pharmacokinetic factors and as to maximize the therapeutic benefit and guarantee patient safety, an optimal understanding of these factors is needed. Understanding the dose-concentration-effect relationship is a fundamental component of clinical pharmacology. (Standing, 2017; Srinivasan et al, 2020)

In our work, we present the validation of an analytical, fast, and effective method that was used to follow up two pediatric patients with OS during two infusions of their treatment with Dox, with the aim of helping to guarantee the safety and efficacy of the use of Dox.

As Vogt W. (Vogt, 2014) because of his pharmacokinetic study, he found that of the dosage regimens used for milrinone, none reached the therapeutic target range in the pediatric patients studied, for which reason dosage regimens were developed as a benefit. Optimized that considered pathophysiological and age-dependent differences.

In our work we were able to identify, thanks to the pharmacokinetic monitoring, an adverse effect from administering the Dox in the early morning, in this way the responsible physician can now consider this pharmacokinetic result and thus avoid this toxic effect in future patients.

6 CONCLUSION

This method of quantification of Dox by HPLC is reliable, fast, and specific for its application in pharmacokinetic studies and therapeutic follow-up in the pediatric area.
This analytical method can be used by pediatric or adult patients who are being treated with Dox against some type of cancer such as OS, acute lymphoblastic leukemia, and some type of lymphoma among others, which can be used in the clinical setting to monitor pharmacokinetic concentrations of Dox and designing or evaluating the doses of this drug.

This method was also successfully applied to the pharmacokinetic study of Dox in 2 pediatric patients with OS after an intravenous dose of 50 mg/m² for 48 hours.

The data could offer a valuable tool to assess the pharmacology and potential toxic effects of Dox.

CONFLICTS OF INTEREST
All authors report that there are no relevant conflicts of interest for this article.

FUNDING
To the Instituto Politécnico Nacional for project support: SIP 20200634.
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