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CLINICAL PHARMACOKINETIC OF DOXORUBICIN IN PATIENTS DIAGNOSED WITH NON-HODGKIN´S LYMPHOMA

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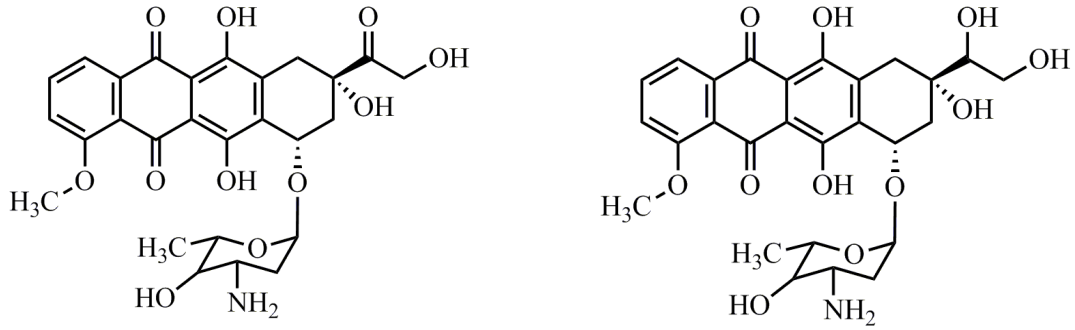
1.- INTRODUCTION

1.1. DOXORUBICIN

Doxorubicin (DOX) (Figure I-1) is widely used in clinical practice for the treatment of solid tumours and haematological malignancies. However, its clinical activity is limited, by its toxicity. Acute myelosuppression and chronic cardiomyopathy are dose-limiting adverse effects (1). DOX efficacy and toxicity show wide interindividual variability and knowing the pharmacokinetic-pharmacodynamic profile (PK/PD) has been suggested as an interesting approach in order to optimize the treatment with this drug. In fact, pharmacokinetic (PK) variability for DOX has been widely reported (2,3). Nevertheless, there are few data regarding the relationship between systemic exposure and clinical response, which is one of the most important prerequisites for conducting therapeutic drug monitoring (TDM) (4-7). In addition, quantifying the concentration of its main metabolite, doxorubicinol (DOXol) (Figure I-1), has been suggested due to its possible contribution to treatment efficacy and toxicity (7-11).



Doxorubicin Doxorubicinol

Figure I-1. Chemical structures of doxorubicin and doxorubicinol.

The spatial configuration of DOX facilitates its intercalation into the DNA. This intercalation induces modifications in DNA structure and allows the stabilization of the DNA-topoisomerase II. It leads to the DNA fragmentation, blockage of the synthesis of new genetic material as well as inhibition of its repair. Other mechanisms of action implied are the oxygen free radicals formation, alquilante effect and peroxidation of cellular lipids (12-14). The clinical use of DOX is limited by its important toxicity and side effects dose-dependent, the myelosuppression and cardiotoxicity being the most severe and relevant (1).

In the common range of dose administered, this antineoplastic shows a lineal kinetic. According to its complex distribution, the evolution of DOX plasma concentrations has been described in previous published articles with a two-compartment (15-20) or three-compartment model (2,3,18,21-28), thus compatible with a wide distribution into peripheral tissues (table I-1).

Table I-1. Pharmacokinetic parameters of DOX in adult population.

Structure model	*CL (L/h)*	*Q_2 (L/h)*	*Q_3 (L/h)*	*V_1 (L)*	*V_2 (L)*	*V_3 (L)*	*$t_{1/2_\alpha}$ (min)*	*$t_{1/2\beta}$ (h)*	*$t_{1/2\gamma}$ (h)*
Two compartments	48-62	60-112	NA	12-26	421-1130	NA	10-66	13-30	NA
Three compartments	54-62	56-86	22-36	18-22	1830-2360	72-106	5-12	1-3	19-30

CL: clearance; Q_n: intercompartmental clearance for the *n-th* compartment; V_n: volume of distribution for the *n-th* compartment; $t_{1/2n}$: half-life of distribution/elimination of the phase *n-th*; NA: not apply.

This antineoplastic drug is mainly metabolized in the liver by a NADPH-dependent aldoketo-reductase present in all the cell types and particularly in the erythrocytes, cells of the liver and kidney. Its main metabolite is DOXol, which activity is around 10 % of the DOX one (2). It has been reported that this metabolite could be implied in the DOX toxicity (7,9,29). The clearance of DOX (CL_{DOX}) has been previously reported around 60 L/h (2,5,15,16,18,23) being altered in elder patients, children (24,25), pregnant women (30), higher doses than 50 mg/m^2 (31), as well as obese patients (31,32), concomitant administration of P-glycoprotein inhibitors (2,33) or the cancer diagnostic (16,32). Around 50 % and 23 % of the dose are excreted in the bile as DOX and DOXol, respectively, and 10-20 % of DOX appear in faeces in 24 h.

Table I-2 shows the main population PK studies published until December 2015, with the parameters estimates values, the study characteristics (patients, diagnostic, treatment, etc.) and the covariates relationships found.

Table I-2. Description of the main population pharmacokinetic models of doxorubicin (1990 - 2015).

	Kontny (23)	Wilde (15)	Callies (2)	Thompson (25)	Joerger (22)	Escudero-Ortiz (3)	Völler (24)	Joerger (16)	Wong (17)
CL_{DOX} (L/h)	53.3 (31)	57.7 (8.1)	62.3 (20.5)	25.1 (-)	47.6 (24.6)	58 (19.7)	24.1 (30.7)	61.8 (14.3)	53.5 (15.4)
V_1 (L)	17.7 (19)	18.4 (147.5)	21.5 (-)	6.96 (-)	12.3 (11.8)	21.1 (-)	9.34 (26.7)	23.3 (44.7)	25.6 (15.8)
Q_2 (L/h)	58.7 (31)	73.2 (41.8)	85.8 (10.3)	24.8 (-)	60.3 (20.7)	84.2 (9.94)	26.8 (35.2)	112 (19.4)	55.9 (-)
V_2 (L)	1830 (20)	789.7 (0)	2360 (13.6)	557 (-)	421 (25)	2170 (7.26)	560 (-)	1130 (25.5)	446 (14.3)
Q_3 (L/h)	21.8 (29)	- (-)	35.6 (-)	6.59 (-)	- (-)	34.7 (-)	12.1 (-)	- (-)	- (-)
V_3 (L)	71.6 (-)	- (-)	104 (-)	16.5 (-)	- (-)	102 (-)	27.8 (-)	- (-)	- (-)
Sigma DOX (%)	23 (-)	44.7 (-)	22.5 (-)	- (-)	45.8 (-)	21.2 (-)	29.6 (-)	34.6 (-)	43.2 (-)
CL_{DOXol} (L/h)	31 (50)	34.7 (0)	143* (41.6)	50.2 (-)	108* (29.4*)	- (-)	42.5 (43)	- (-)	92.7 (23.8)
V_{DOXol} (L)	1150 (57)	477.1 (48.5)	3150* (47.7)	1100 (-)	1580* (51.3*)	- (-)	760 (48)	- (-)	1700 (35.4)
Sigma DOXol (%)	26 (-)	44.5 (-)	18.7 (-)	- (-)	40.3 (-)	- (-)	- (-)	36.2 (-)	38.9 (-)
Dose (mg/m^2)	(20 - 110)	25 (22-36)	60 (32-110)	(20-60)	60 (50-66)	41.6 (20-73)	25 (2-57)	20	75
Infusion (min)	(2 - 600)	30 (2-100)	30	(5-360)	15	(30-60)	235 (15-1440)	30	-
No. patients	82 (21 M)	30 (21 M)	36 (18 M)	22 (16 M)	59 (0 M)	33 (15 M)	94 (46 M)	7	44 (0 M)
No. samples DOX	934	258	350	326	232	205	630	91	264**
No. samples DOXol	935	252	357	326	196	-	588	91	264**
Max t sampling (h)	96	21	96	48	24	6	50	72	24
Diagnostic	-	HL	-	8 Ca	BC	9 Ca	5 Ca	KS-AIDS	BC
Protocol	-	BEACCOP	-	-	-	-	-	ABV	AT
Correlations	-	BSA-CL_{DOX}; BMI-V1; AGE-V_{DOXol}	BLR y AST - CL_{DOXol} y V_{DOXol}	BMI (>30) y % grasa - CL_{DOXol} y V_{DOXol}	BAS, AST, AGE - CL_{DOX}; CL_{CR} - CL_{DOXol}	-	AGE-CL_{DOX}	-	AGE-CL_{DOX}
Observations	1	2	3	4	5	6	7	8	9

The articles are identified with the first author name and the reference number signed between brackets (see references). Pharmacokinetics parameters expressed as the population estimate and the interindividual variability (IIV) in % between brackets. DOX: doxorubicin; DOXol: doxorubicinol; BSA: *body surface area;* BMI: *body mass index*; BLR: bilirubin; AST: aspartate aminotransferase; *: apparent parameters (divided by the metabolite conversion rate); **: six samples per patient (non specified value in the article); Dose and infusion: expressed as the mean value or standard according to the protocol administration, range of values registered between brackets; No. patients: number of patients enrolled in the study, number of males between brackets (females are the difference between the total patients and males); HL: Hodgkin´s lymphoma: Ca: cancer; KS-AIDS: Kaposi sarcoma associated with aids; BC: breast cancer; ABV: DOX, Bleomicina y vincristine; BEACOP: Bleomicina, etoposide, DOX, cyclophosphamide, vincristine, procarbazine, prednisone; AT: DOX and docetaxel. **Observations**: 1-Kontny: studied three sets of data (Callies, Thompson y Wilde) and it used the fourth one for external evaluation. Population estimates for an average individual of 1.8 m^2 of BSA. Dataset included 18 children between 3 and 18 years old. 2-Wilde: two-compartment model with DOXol. 3-Callies: estimates values of the pharmacokinetic parameters of metabolite are apparent (divided by the conversion rate to DOXol). It studies the P-glycoprotein inhibitor influence. Correlations between pharmacokinetic parameters of DOXol and the values of BLR and AST were found, but it were not in the final model. 4-Thompson: parameters are scaled linearly to BSA. 17 patients are younger than 18 years old. The influence of body composition of children on pharmacokinetic parameters has been evaluated. Relative standard erros have been reported, but not the IIV. 5-Joerger 2007: it is a two-compartment model of DOX in pregnant women. Developed a semiphisiological model for haematological toxicity. 6- Escudero-Ortiz: methodology article, it doesn´t study the covariates influence on pharmacokinetic parameters. 7-Völler: study in children, including younger than three years old. Parameters are scaled linearly to BSA. DOXol conversion rate fixed to 0.5. 8-Joerger 2005: two-compartment model for DOX including four different metabolites. F_{DOXol}/V_{DOXol} = 7.33 10^{-3}; K_{56}=2.18 h^{-1}. DOXol described as a two-compartment model. 9-Wong: Asiatic population. Two-compartment model for DOX. The haematology count has been done 15 days after the DOX administration.

Clinical response of DOX presents a wide IIV. Thereby an adequate PK/PD profile characterization in a specific population can be an interesting tool to prevent the toxicity and optimize efficacy. Even with the great experience with this antineoplastic, there are only a few articles interested in quantifying DOX and/or DOXol plasma concentrations in order to study its probable relationship with the efficacy and toxicity of this drug (5-11,18,33-35).

1.2. POPULATION PHARMACOKINETIC

The population PK is a methodology with a different point of view from the previous ones (Naïve Pooled Data Analysis, Standard Two Stage, etc.). It takes into account the characterization of the kinetic profile of the drug in the whole population instead of estimating the individual parameters. This methodology can be defined as the study of the inter- and intra-individual variabilities of the drug concentrations, when it has been administered at standard dose protocols in a group of patients with defined physiological and clinical characteristics.

Non linear mixed-effects modelling is the methodology most commonly used in population PK that presents two components:

- Structural model: defined by the fixed-effect parameters that relate the dependent variable (in PK it used to be the drug concentrations) with the independent ones (time and dose).
- Stochastic model: defined by the random effects parameters that evaluate the variability of the fixed-effects ones.

Different estimation methods of the parameters have been proposed. Classical ones are based on the minimization of the likelihood function value as for example the first order estimation method (FO), the first order conditional estimation with interaction (FOCEI) and the LAPLACIAN one (36). Furthermore, some algorithms based on two steps, expectation-maximization (EM) have been proposed: Iterative Two Stage (ITS), Monte Carlo Importance Sampling (IMP), Importance Sampling Assisted by Mode A Posteriori (IMPMAP), Stochastic Approximation Estimation Maximization (SAEM) all this ones being implemented in the software NONMEM v.7.3. (ICON Development Solutions, Hanover, EEUU) (37).

The model selection criteria is an important step in the model building process. The following criteria have to be taken into account together:

- Statistical criteria: minimization successful, likelihood ratio test (LRT), Akaike information criteria (AIC) and Bayesian information criteria (BIC).
- Estimation precision: relative standard error (RSE).
- Shrinkage: $\eta - shrinkage$ and $\varepsilon - shrinkage$.
- Outliers: weighted residual values (WRES).
- Graphical evaluation: goodness of fit plots (38-41).
- Plausibility and relevance of the results.

Simulation is a process consisting in generating data from a model. It can be develop in different stages of the population PK model building. This tool can be used, for example, to design a population PK study, for development and model evaluation (internally or externally), to select and evaluate dosing regimen schedules, etc.

The study of the covariates influence on the PK parameters is as well an important step in the model building process and it can explain a part of the variability in a more reliable way. The inclusion of covariates in the model has some advantages: to help the mechanistic interpretation of the model, to favour the hypothesis generation process, to decrease and explain the IIV, to identify subpopulations, to improve the predictive ability of the model, etc. Several covariates model building processes have been proposed in the literature among which we can highlight: Generalized Additive Modelling (GAM), Step-wise Covariate Modelling (SCM), Least Absolute Shrinkage and Selection Operator (LASSO), Full Fixed Effects Model Estimation (FFME) and Full Random Effects Model Estimation (FRME) (41-46).

The model evaluation is an essential step in the population PK model building. There are no clear recommendations to carry out this process, even there are building approach suggestions proposed by the regulatory agencies (38,47). Some of the most important parameters used to evaluate a model are: Median Prediction Error, Median Absolute Prediction Error, Root Mean Squared Prediction Error, WRES and conditional WRES (CWRES), prediction discrepancies (pd) and Normalised Predictions Distribution Errors (NPDE) (48-56).

The evaluation of the model can be internal or external accordingly to the dataset used in the process. When the same dataset is used to development and evaluates a model, the evaluation is defined as an internal one. On the other hand, when a different set of data is used for this process, it is called external evaluation. In addition to the model selection criteria, previously reported, the main methodologies for the internal evaluation are the following ones: data splitting, bootstrapping, cross validation, Monte Carlo simulations (visual predictive check, numerical predictive check, posterior predictive check) (40,41,51,56-67).

2.- OBJECTIVES

- Develop an analytical method of ultra high liquid chromatography (UHPLC) to quantify doxorubicin and doxorubicinol plasma concentrations and to allow its implementation in the clinical practice.

- Build a population pharmacokinetic model of doxorubicin and doxorubicinol in patients diagnosed of non-Hodgkin´s lymphoma.

- Evaluate the predictive and descriptive abilities of the population pharmacokinetic model developed according to internal evaluation techniques based on Monte Carlo simulations (Visual Predictive Check and Normalized Prediction Distribution Error) and bootstrap.

- Carry out a pharmacokinetic/pharmacodynamic analysis to study the link between the PK parameters of the drug and/or its main metabolite and the haematological toxicity parameters.

3.- MATERIAL AND METHODS

3.1. ANALYTICAL METHOD

Calibrators of DOX and DOXol were prepared in human plasma with concentration ranges of 8-3000 ng/mL and 3-150 ng/mL, respectively. All stock solutions and calibrators were frozen at -80°C until analysis.

As a precipitating agent, 20 % zinc sulphate in methanol/water (50:50 v/v) spiked with daunorubicin (DAU) (150 ng/mL) was selected. Samples preparation consisted in adding 100 µL of the precipitating agent to 100 µL of the calibrator or patient sample. This mixture was vortexed for 30 seconds and then centrifuged at 14000 rpm for five minutes; 10 µL of the supernatant were subsequently injected.

DAU, at a concentration of 150 ng/mL, was spiked into the precipitating agent solution as a quality control to detect analysis errors. Thus, the sample was reanalysed when the DAU peak height deviation was higher than 20 %.

Chromatographic separation was performed on a Kinetex® C_{18} UHPLC column (50 mm x 2.10 mm, particle size 1.7 µm, Phenomenex®). The mobile phase was composed of water (containing 0.4 % triethylamine and 0.4 % orthophosphoric acid)/acetonitrile (77:23, v/v). The mobile phase was filtered through a 0.2 µm filter. The flow rate was set to 0.500 mL/min, the column was maintained at 50 °C and wavelengths detection occurred at 470 nm (excitation) and 548 nm (emission).

Validation was accomplished following the FDA and EMA guidelines for bioanalytical methods (68,69). This method was validated for the requirements of linearity, sensitivity, selectivity, accuracy, intra-day and inter-day precision and stability. In addition, the carry-over and the lower limit of quantification were studied. The results were analysed with SPSS software v.21.0. (IBM Corporation, USA).

3.2. PATIENTS AND TREATMENT

The study has been carried out between June 2009 and June 2015 in patients diagnosed with non-Hodgkin´s lymphoma (NHL) treated with RCHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) every 21 days during 6 cycles. Prophylaxis of neutropenia with granulocyte-colony stimulating factor (GCSF) was performed as routine clinical practice. DOX was administered by continuous infusion of 30-60 min duration at 50 mg/m^2.

Information on the physical condition, the disease, the treatment and the patient was registered. Some of the covariates collected were: AGE, SEX, weight

(WGT), height (HGT), lean boy weight (LBW), body mass index (BMI), body surface area (BSA), albumin (ALB), bilirubin (BLR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA), clearance of creatinine (estimated with the Cockcroft-Gault formula) (CL_{CREA}), haemoglobin (HB), leukocyte count (LEU), neutrophil count (NEU), lymphocyte count (LIN), platelets count (PLA) and basal heart ejection fraction.

In order to estimate the best time points to characterize the PK of doxorubicin and doxorubicinol in the clinical routine, an analysis of the first partial derivate with respect to time was conducted with the software WinNonlin v.5.3. In addition, previous articles were taken into account for the choice of the sampling schedule (2,3,15,22,23). Accordingly to these considerations, the sampling times selected were: 0, 30, 90 and 180 min after the end of DOX infusion. Plasma was separated by centrifugation at 2500 rpm during 10 min from the blood and frozen at -80º C. Plasma samples were sent to the laboratory in less than five months for the DOX and DOXol quantification.

The study has been developed following the Helsinki Declaration (Seúl, October 2008). All patients were informed about the potential risks and benefits of their inclusion in the study. An informed consent was obtained for each participant. The table MM-1 shows the main characteristics of patients included in the study, as well as the characteristics of the DOX treatment administered.

Table MM-1. Characteristics of patients and treatment with doxorubicin.

Covariates (n=45)	Units	Mean (SD)	Range	Normal	Missing
Age	years	66 (15)	26 - 84	-	-
Weight	kg	71 (12)	43 - 110	-	-
Height	cm	164 (11)	143 - 192	-	-
Body Surface Area	m^2	1.8 (0.2)	1.3 - 2.3	-	-
Body Mass Index	kg/m^2	26.5 (3.9)	19.9 - 37.6	-	-
Lean Body Weight	kg	47.9 (10.0)	28.7 - 69.5	-	-
Treatment Characteristics					
Dose/Body Surface Area	mg/m^2	51 (7)	25 - 71	-	-
Dose	mg	89 (14)	53 - 130	-	-
Infusion duration	h	0.5 (0.2)	0.2 - 1.3	-	-
Infusion rate	mg/h	210 (119)	68 - 666	-	-
Biochemistry parameters					
Creatinine clearance	mL/min	91 (40)	40 - 201	90 - 140	2
Bilirubin	mg/dL	0.44 (0.19)	0.10 - 0.70	0.1 - 1.2	4
ALT	IU/L	23 (18)	7 - 88	1 - 37	3
AST	IU/L	25 (13)	12 - 64	1 - 41	3
Haematological parameters					
Haemoglobin	g/dl	11.6 (1.6)	8.5 - 15.2	13 - 18	2
Leukocytes	$x\ 10^9/L$	6.3 (2.7)	2.5 - 15.5	4.5 - 10.8	2
Neutrophils	$x\ 10^9/L$	4.1 (2.4)	1.0 - 14.1	1.4 - 6.5	3
Lymphocytes	$x\ 10^9/L$	1.4 (0.8)	0.3 - 4.0	1.2 - 3.5	2
Platelets	$x\ 10^9/L$	280 (118)	52 - 648	7.2 - 11.1	2

SD: standard deviation; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

3.3. POPULATION PHARMACOKINETIC ANALYSIS

The population PK analysis was developed following a non linear mixed-effects modelling methodology with NONMEM v.7.3. (ICON Development Solutions, Hanover, EEUU) and the FOCEI approximation. The evaluation, graphical representations and statistical analysis of the results were developed with the following softwares: PsN v.3.5.3. (70) available from http://psn.sourceforge.net/, R v.3.1.0, Xpose v.4.5.0. available from http://xpose.sourceforge.net, Pirana v.2.9.2. and RStudio® v.0.98.976. (RStudio Inc., Boston, EEUU).

The model selection criteria use throughout the whole model building process were as followed:

- Minimization successful.
- Decrease of the objective function value (OFV) higher than 3,84 ($p<0,05$) for one degree of freedom.
- RSE < 25 % for fixed effects parameters.
- RSE < 50 % for random effects parameters.
- η-*shrinkage* y ε-*shrinkage* < 50 %.
- Adequate GOF (PRED, IPRED, WRES, etc.).
- Plausibility and relevance of the results.
- Principle of parsimony.

Firstly, a model to describe DOX PK was built. When this model was completely established the DOXol data were added, and a joint model was developed. The population PK models of Wilde et al. (15) and Kontny et al. (23) were selected as best published models describing the evolution of DOX plasma concentrations, with two- and three-compartment model, respectively. The sparse data can lead to fix the values of some parameters, such as the volumes of distribution or the parameters related to the most extensive peripheral compartment. Both of these strategies were studied in two- and three-compartment models, by fixing the parameters values to previously published ones (15,23).

Random effects parameters relationships were studied as additive, exponential, proportional or mixed model. The shrinkage for the IIV and residual variability was estimated and reported as a percentage.

The influence on CL_{DOX} was evaluated for the following covariates: AGE, SEX, WGT, HGT, BMI, BSA, LBW, ALB, CREA, CL_{CREA}, BLR, AST, ALT, ECOG, international prognostic index (IPI), etc. Any possible relationship was studied graphically representing the IIV of CL_{DOX} vs. covariate. The selection criteria to keep the covariate in the model building process were to obtain a $r>0.2$ (Pearson product-moment correlation coefficient) or a $p\text{-value}<0.05$ (Kruskal-Wallis test) for the continuous and categorical ones, respectively. A GAM and SCM (forward inclusion: $p<0.05$; backward elimination: $p<0.01$) were carried out on CL_{DOX}, taking into account the results of this preliminary covariates selection. Individuals with samples associated to any value of |CWRES| higher than 4 were not taken into

account in the dataset (criterion more restrictive criteria than previously reported by Byon et al.) (41).

Following the development of an adequate model describing the DOX plasma concentrations in our population, the DOXol observations were add to the model following a one- or two-compartment model. All the previous steps reported for the DOX model building process were applied in the development of the final model, which included DOXol, the main active metabolite.

The final model was internally evaluated with the model selection criteria previously described (GOF, RSE, shrinkage, etc.). In addition, bootstraps, VPC and NPDE were computed for 1000 replicates in all these metrics. In addition, the number of replicates for the bootstrap was increased until no statistically significant differences ($p<0.05$) in the estimation population PK parameters were obtained (Student's t-test).

3.4. PK/PD ANALYSIS

The LEU, NEU and PLA were registered previously to all cycles of DOX treatment. These values were classified according to the Common Terminology Criteria for Adverse Events v3.0, CTCAE. Haematological toxicity was divided into two categories: toxicity (grade 3-4) and non toxicity (normal count, grade 1-2). The area under the curves of DOX and DOXol (AUC_{DOX} and AUC_{DOXol}) were estimated as the relationship between the dose administered and the CL of each entity (taking into account the DOXol conversion rate). DOX and DOXol exposure, the total AUC ($AUC_{total} = AUC_{DOX} + AUC_{DOXol}$) and the maximum concentration of the parent drug ($C_{max,DOX}$) and the one of its main metabolite ($C_{max,DOXol}$), were compared between the two groups (toxicity and non toxicity) with a Mann-Whitney test ($p>0,05$) and according to the different haematological information available (LEU, NEU, PLA). Treatment with GCSF between the two cycles studied was taken into account.

A correlation analysis between LEU, NEU or PLA and the PK parameters selected (AUC_{DOX}, AUC_{DOXol}, AUC_{total}, $C_{max,DOX}$ and $C_{max,DOXol}$) was carried out ($p<0.05$). Patients treated with GCSF between two cycles included in our study were removed from the analysis.

4.- RESULTS

4.1. ANALYTICAL METHOD

Linearity for the drug (8-3000 ng/mL) and the main metabolite concentrations (3-150 ng/mL) was observed ($r > 0.99$) and the maximum intra-day and inter-day precision coefficients of variation were less than 14 % for both. The lower limits of quantification (LLOQ) were 8 ng/mL (CV=10,9 %) for DOX and 3 ng/mL (CV=8,5 %) for DOXol, respectively. The data were weighted with the $1/x^2$ factor as a consequence of the wide range of concentrations. The recovery percentages, expressed as mean ± SD were 100.30 ± 8.10 % for DOX and 99.49 ± 6.42 % for DOXol. The carry-over was lower than 20 % of the LLOQ. Short term, post-preparative and freeze-thaw stabilities were shown for 6 h, 12 h and 3 cycles, respectively. Therefore, an UHPLC–fluorescence method for the quantification of DOX and its main metabolite, DOXol, was successfully developed and validated for criteria of linearity, selectivity, sensitivity, accuracy, precision and stability according to the specifications of the FDA and EMA guidelines for bioanalytical methods validation (68,69). This analytical method has been published in the Journal of Chromatography B (71).

4.2. PATIENTS AND TREATMENT

The study enrolled a total of 45 patients diagnosed with NHL and treated with DOX by intravenous infusion of 30-60 min duration every 21 days, until the administration of six complete cycles of treatment. The main diagnostic was diffuse large B-cell lymphoma (80 %). A total of 125 observations of DOX plasma concentrations and 120 of DOXol were analysed in a population PK model. Sampling times were performed at 0, 30, 90, and 180 min after the end of DOX infusion. In addition, three samples at 24 hours after drug administration were analysed.

4.3. POPULATION PHARMACOKINETIC ANALYSIS

The final population PK model of DOX and DOXol included three compartments for the parent drug and two for the metabolite, with first order distribution and elimination (figure R-1).

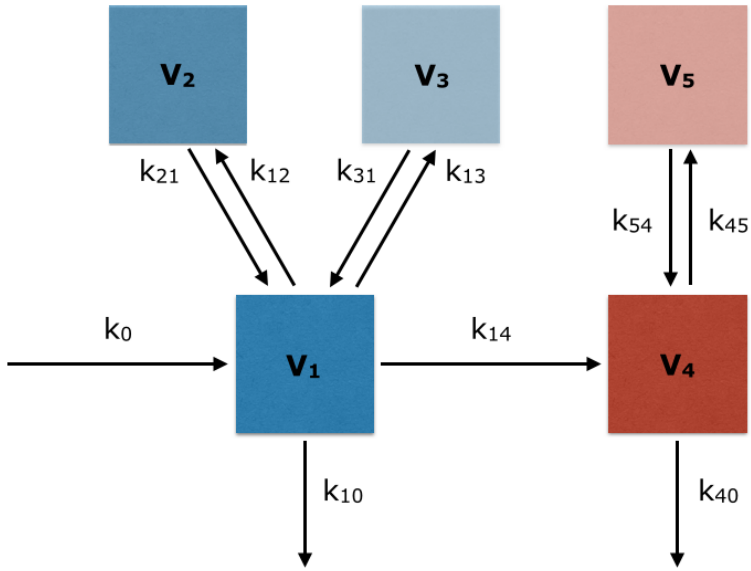


Figure R-1. Scheme of the structural model for doxorubicin (blue) and doxorubicinol (red).

V_n: volume of distribution of the compartment *n-th*; k_{ij}: constant rate between the compartment *i-th* and the *j-th*; k_0: is the drug incorporation rate; k_{10} y k_{40} are the elimination constants for the drug and metabolite, respectively.

The structural model of DOX and DOXol was described by the following equations:

$$\frac{dA(1)}{dt} = k_0 + k_{21} \cdot A(2) - k_{12} \cdot A(1) + k_{31} \cdot A(3) - k_{13} \cdot A(1) - k_{10} \cdot A(1) - k_{14} \cdot A(1) \quad \textit{Equation 1}$$

$$\frac{dA(2)}{dt} = k_{12} \cdot A(1) - k_{21} \cdot A(2) \quad \textit{Equation 2}$$

$$\frac{dA(3)}{dt} = k_{13} \cdot A(1) - k_{31} \cdot A(3) \quad \textit{Equation 3}$$

$$\frac{dA(4)}{dt} = k_{14} \cdot A(1) + k_{54} \cdot A(5) - k_{45} \cdot A(4) - k_{40} \cdot A(4) \quad \textit{Equation 4}$$

$$\frac{dA(5)}{dt} = k_{45} \cdot A(4) - k_{54} \cdot A(5) \quad \textit{Equation 5}$$

where A(n) is the quantity of drug or metabolite in the *n-th* compartment; k_{ij} are the constant rate between the compartment *i-th* and the *j-th; K_0: is the drug incorporation rate*; k_{10} y k_{40} are the elimination constants for the drug and metabolite, respectively.

The best variability models to describe the DOX and DOXol plasma concentrations were exponential for the interindividual variability (IIV) and the proportional for residual variability (in both entities). No correlation between IIV was found.

None of tested covariates have shown a statistically significant influence on CL_{DOX}. Then, the final model was the structural one presented in the figure R-1. One patient (ID=47) considered as outlier (WRES>4) was removed from the dataset . The adequate ability of the model proposed to describe the observed data

has been shown with the GOF for DOX (figure R-2) and DOXol (figure R-3). The evolution of the observed plasma concentrations, both for DOX and DOXol, was successful described by the individual and population predictions in the individual GOF.

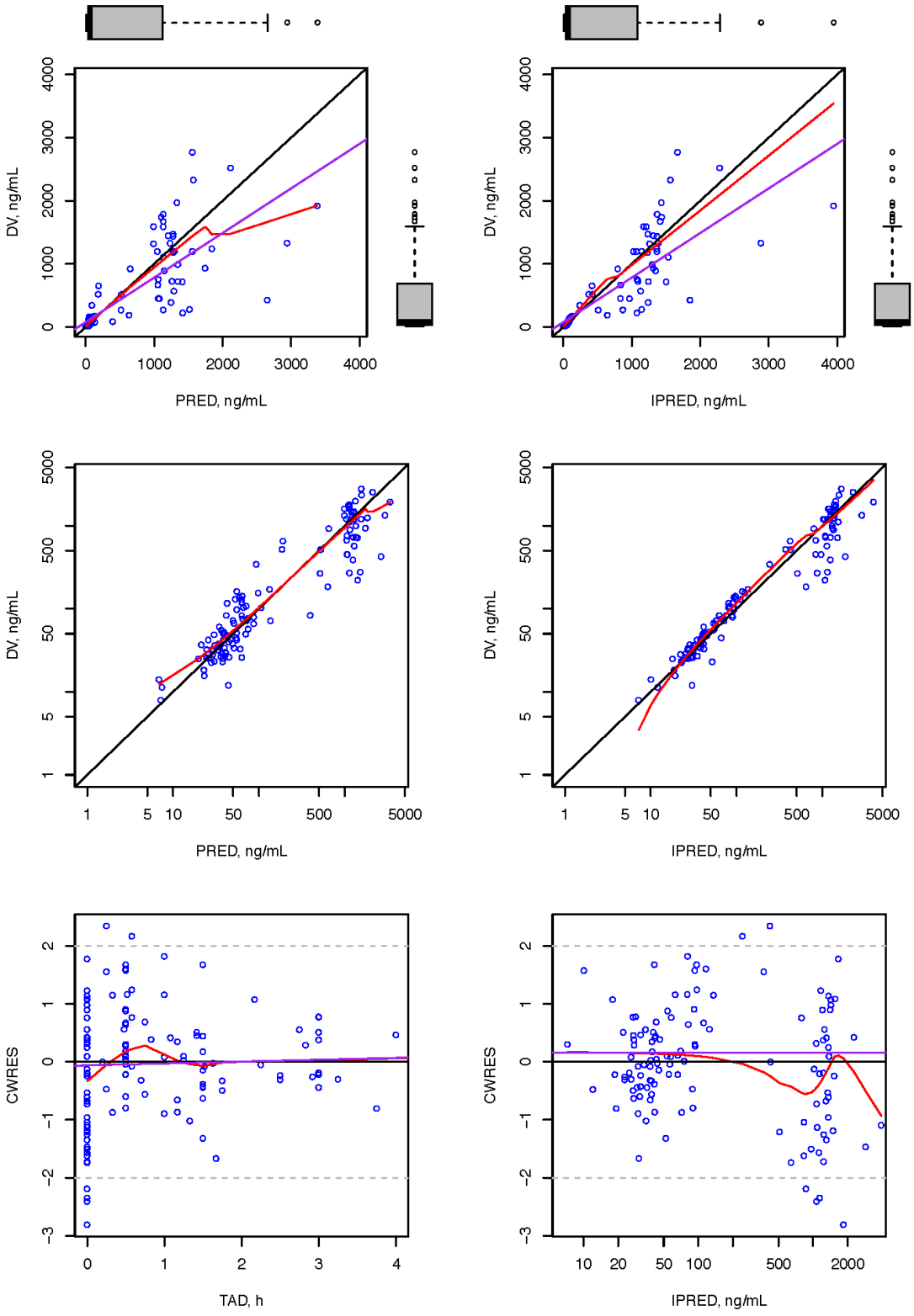


Figura R-2. Goodness of fit plot for doxorubicin with the final pharmacokinetic model.

DV: observed concentration of doxorubicin; PRED: population prediction; IPRED: individual prediction; CWRES: conditional weighted residuals; TAD: time after dose; —: lineal regresion; —: local regresion.

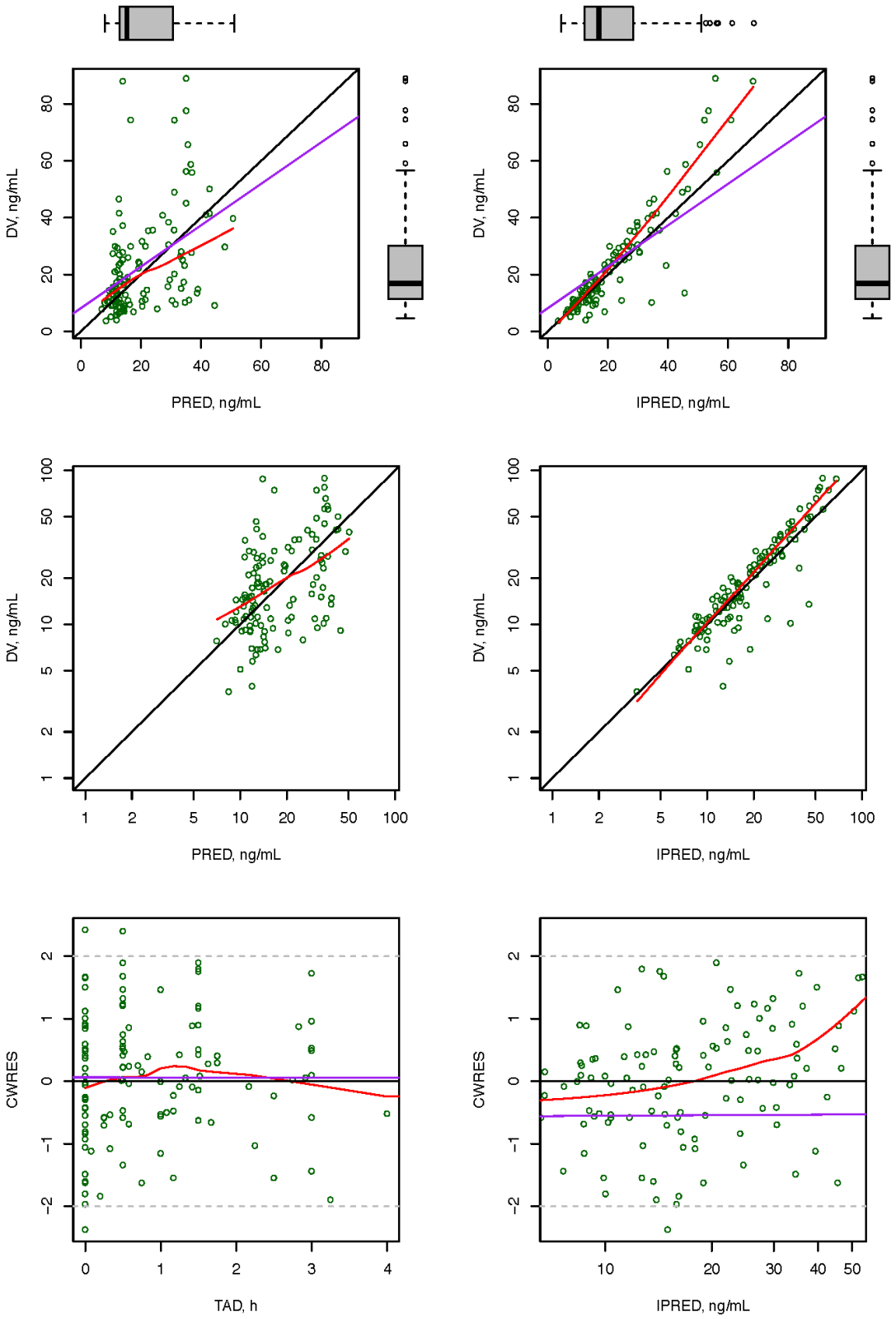


Figura R-3. Goodness of fit plot for doxorubicinol with the final pharmacokinetic model.

DV: observed concentration of doxorubicinol; PRED: population prediction; IPRED: individual prediction; CWRES: conditional weighted residuals; TAD: time after dose; —: lineal regresion; —: local regresion.

The results of the population PK of the final model, including DOX and DOXol, as well as the estimations obtained with the bootstrap methodology (n=1000) are shown in the table R-1. There were no statistically significant differences between the estimated parameters for a bootstrapping of a 1000 or 2500 replicates.

Table R-1. Pharmacokinetic parameters of the final model and results of the bootstrap.

	Final model (n=44)			*Bootstrap (n=1000)*		
Parameter	*Mean*	*RSE (%)*	*Shrinkage (%)*	*Mean*	*CV (%)*	*CI 95 %*
OFV	1844.0	-	-	1819.9	1.1	1691.3 - 1969.5
CL_{DOX}	62.4	11.5	-	63.4	1.7	50.3 - 79.1
V_1	**17.7**	**-**	**-**	**17.7**	**-**	**17.7 - 17.7**
Q_2	50.7	18.4	-	52.4	3.4	31.4 - 72.4
V_2	**1830**	**-**	**-**	**1830**	**-**	**1830 - 1830**
Q_3	28.4	13.5	-	29.9	5.4	21.9 - 44.8
V_3	**71**	**-**	**-**	**71.0**	**-**	**71.0 - 71.0**
V_4	**79.8**	**-**	**-**	**79.8**	**-**	**79.8 - 79.8**
CL_{DOXol}	26.8	42.9	-	37.0	38.1	14.0 - 88.2
F_{DOXol}	0.22	14.7	-	0.232	5.3	0.165 - 0.333
V_5	**653**	**-**	**-**	**653**	**-**	**653 - 653**
Q_5	424	18.0	-	468.6	10.5	309.0 - 694.3
$\eta_{CL,DOX}$	22.9	32.7	40	22.3	2.6	7.3 - 36.2
η_{Q2}	**64.1**	**-**	**-**	**64.1**	**-**	**64.1 - 64.1**
η_{Q3}	**28.2**	**-**	**-**	**28.2**	**-**	**28.2 - 28.2**
$\eta_{CL,DOXol}$	**47.2**	**-**	**-**	**47.2**	**-**	**47.2 - 47.2**
$\eta_{F,DOXol}$	41.7	19.6	22	39.4	5.5	16.7 - 58.2
η_{Q5}	58.9	39.4	35	82.6	40.2	15.7 - 162.8
ε_{DOX}	37.1	8.3	15	37.1	0.0	30.5 - 43.5
ε_{DOXol}	32.1	10.4	21	28.8	14.9	14.0 - 39.6

RSE: *relative standard error*; OFV: *objective function value;* CL_{DOX}: clearance of doxorubicin (DOX); V_n: volume of distribution of the *n-th* compartment; Q_n: intercompartmental clearance of the compartment *n-th;* CL_{DOXol}: clearance of doxorubicinol (DOXol); F_{DOXol}: conversion rate to DOXol; CV: coeficient of variation; CI: confidence interval; $\eta_{P:}$ interindividual variability of the *P* parameter: exponential error in all the cases; ε_{DOX} y ε_{DOXol}: residual variability of DOX y DOXol, respectively, proportional error model; The values of random effects parameters have been expressed as %; *Bootstrap* of 1000 replicates, where 484 had a successful minimization and a complete covariance step, these ones were taken into account to calculate the mean and 95 % CI of each parameter; **In bold letters: fixed parameters**.

The VPC generated from 1000 replicates performed with the final population PK model developed showed an adequate descriptive ability for DOX and DOXol observed plasma concentrations (figure R-4).

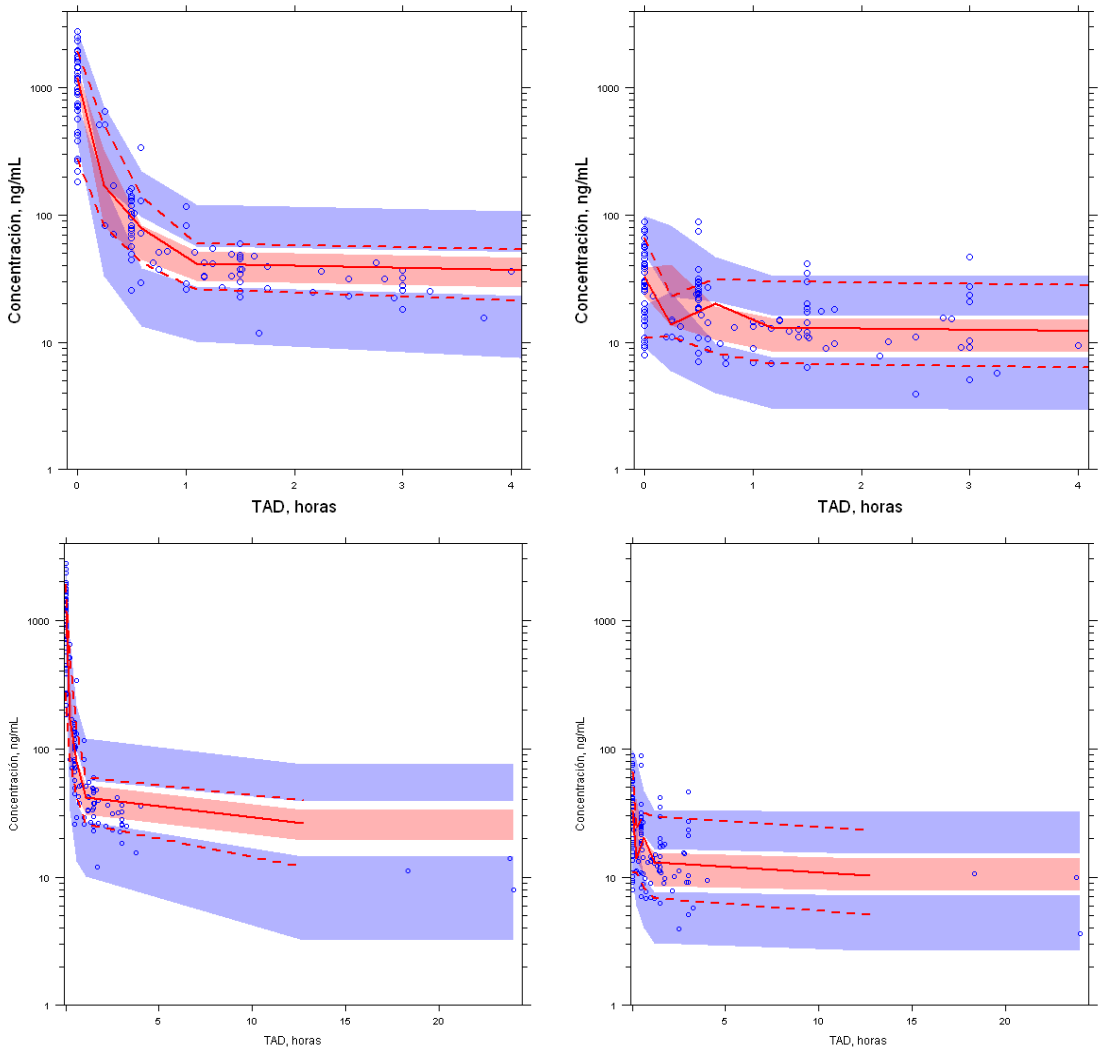


Figure R-4. Visual predictive check with final model for doxorubicin (left) and doxorubicinol (right).

◦: concentrations of doxorubicin (DOX); ■: 95 % confidence intervals for the percentiles 10 and 90 of the simulated data. ■: 95 % confidence intervals for the percentiles 50 of the simulated data. ---: percentiles 10 and 90 for the DOX observations; —: percentil 50 DOX observation; TAD: time after dose.

The statistical analysis of the NPDE showed, both for DOX and DOXol, that the mean and the variance were not significantly different from 0 and 1, respectively. Furthermore, their distribution was not different from a normal one,

4.4. PK/PD ANALYSIS

There were no patients classified in the toxicity group taking into account the platelets count. Then, this parameter was deleted of the PK/PD analysis. Six patients among the 44 included in the study did not have LEU or NEU values registration. Thus, they were removed from the dataset for the PK/PD analysis (n=38) Accordingly to leukopenia and neutropenia criteria, two and four patients were classified in toxicity group, respectively. Including the patients treated with GCSF in the toxicity group, the number of patients increased to 4 and 6, respectively.

Table R-2 shows the mean values of the different PK parameters categorized by the haematological toxicity group and differentiating when the patients treated with GCSF were included in the toxicity group or removed from the dataset.

Table R-3 shows the results of the correlation analysis between the previously mentioned PK parameters and the LEU or NEU reported just before the next DOX administration to the studied one.

Table R-2. Study of the relationship between pharmacokinetic and haematological parameters categorized by grade of toxicity.

		DOX						DOXol						DOX+DOXol		
	GCSF	*AUC (mg·h/L)*			*C_{max} (ng/mL)*			*AUC (mg·h/L)*			*C_{max} (ng/mL)*			*AUC total (mg·h/L)*		
		no tox	tox	p-valor	no tox	tox	p-valor	no tox	tox	p-valor	no tox	tox	p-valor	no tox	tox	p-valor
LEU	SI	1.44	1.57	0.366	1064	1212	0.461	0.75	0.60	0.284	34	22	0.289	2.18	2.17	0.801
NEU	SI	1.45	1.49	0.779	1052	1225	0.347	0.70	0.91	0.857	34	22	0.289	2.14	2.39	0.445
LEU	NO	1.44	1.78	0.120	1064	1186	0.604	0.75	0.63	0.604	34	28	0.742	2.18	2.41	0.419
NEU	NO	1.45	1.55	0.563	1052	1218	0.435	0.70	1.08	0.378	34	28	0.742	2.14	2.62	0.065

DOX: doxorubicin; DOXol: doxorubicinol; AUC: area under the curve; C_{max}: maximum concentration; LEU: leucocytes count; NEU: neutrophils count; GCSF: granulocyte-colony stimulating factor (taking into account or not the patients treated with GCSF); no tox: patients without haematological toxicity (mean of the parameter analysed in each study); tox: patients with haematological toxicity (mean of the parameter analysed in each study); p-value for the Mann-Whitney test.

Table R-3. Correlation analysis between pharmacokinetic and haematological parameters.

	Relationship	DOX				DOXol				DOX+DOXol	
		AUC (mg·h/L)		*C_{max} (ng/mL)*		*AUC (mg·h/L)*		*C_{max} (ng/mL)*		*AUC total (mg·h/L)*	
		r	p-valor	r	p-valor	r	p-valor	r	p-valor	r	p-valor
LEU	Exponential	-0.202	0.237	0.107	0.534	-0.100	0.562	0.109	0.544	-0.246	0.148
NEU	Exponential	-0.170	0.322	0.050	0.771	-0.182	0.288	0.079	0.663	-0.287	0.089
LEU	Lineal	-0.112	0.514	0.228	0.181	-0.051	0.769	0.202	0.259	-0.133	0.440
NEU	Lineal	-0.094	0.585	0.198	0.247	-0.089	0.606	0.175	0.330	-0.150	0.384

DOX: doxorubicin; DOXol: doxorubicinol; AUC: area under the curve; C_{max}: maximum concentration; LEU: leucocytes count; NEU: neutrophils count; r: Pearson product-moment correlation coefficient (with its p-value associated).

5.- DISCUSSION

In oncology, TDM remains a challenge and several difficulties are involved applying this approach in clinical routine. The availability of a suitable analytical method is one important and relevant limitation (72,73). Chromatographic techniques are time-consuming and require highly trained staff. Therefore the clinical implementation of these techniques is not always practical. Nevertheless, antineoplastic agents are usually measured with these techniques owing to their good specificity and the lack of alternatives.

More precise and accurate quantification methods for DOX and DOXol have been published. However, these analytical methods needed complex sample treatment or more sensitive detectors (MS/MS) than the ones proposed in our study. Thus, the UHPLC method developed required an easy sample pre-treatment and it allowed a clear chromatographic separation and a quantification of the drug and its main metabolite with short run times. Furthermore, the low cost of this technique as well as the LLOQ value were appropriate for TDM purpose (74-82). Its interest and relevance have been reinforced by its publication in the Journal of Chromatography B (71).

DOX PK has been widely studied (2,3,5,15-20,22-26,31,83). Firstly, two- or three-compartment models were evaluated in the DOX PK model building. Our data, obtained in the clinical routine, were sparse and suggested to use the easiest structural model possible. Nevertheless, a wrong decision in the structural model could conduct to wrong AUC estimations and mistakes in the dosage adjustment of this drug. Finally, a three-compartment model with volumes of distribution values fixed to the ones published by Kontny et al. (23), as well as a first order distribution and elimination showed the best fit for DOX plasma concentration observed.

Different strategies have been studied for DOX dosage optimization and other antineoplastic (33,84-88) concluding that in specific cases "it is totally erroneous to continue to use BSA alone for dose calculation" (85). According to this, the different measures of body size (weight, height, BMI, BSA, LBW) in addition to clinical variables (ECOG, IPI, etc.) and biochemistry covariates (BLR, AST, ALT, etc.) were evaluated to explain the CL_{DOX} variability. In general, previously published DOX population PK models have included few and different covariates: SEX (31,32), AGE (24), pregnancy (30), obesity (31,32), higher doses than 50 mg/m^2 (31) as well as concomitant administration of P-glycoprotein inhibitors (2,33) or cancer diagnostic (16,32). The covariates analyses tested (graphical, correlations, GAM and SCM) did not show any significant influence on the CL_{DOX}.

Thus, a five-compartment model, three for DOX and two for DOXol, with fixed values for the volumes of distribution was developed. The volumes of DOX were fixed, previously mentioned, to those established by Kontny et al. (23). DOXol

volumes of distribution were fixed to values resulting from a sensibility analysis carried out with our own data. The stochastic model, both for DOX and DOXol, was exponential error model for the IIV and proportional error relationship for the residual variability. Moreover, it was necessary to fix the IIV of the intercompartimental clearances of the parent drug (Q_2 and Q_3) and the clearance of the metabolite (CL_{DOXol}). Table R-1 shows the population PK parameters of the final model developed. All fixed effects parameters were estimated with a RSE lower than 25 % except for the CL_{DOXol} (42.9 %). The difficulty in estimating this parameter was due to the few data available. The RSE obtained for all random effects parameters and all shrinkage values were lower than 40 %, both results lower than the 50 % recommended.

The use of previous kinetic knowledge of the drug coupled with the observed data is another adequate strategy to develop a population PK model with sparse date to avoid over-parameterization. NONMEM software implements this analysis in the subroutine $PRIOR with the options NWPRI and TNPRI. This subroutine allows to introduce a penalty function for the estimation of the OFV, based on the prior distribution of the parameters previously established. This penalty function follows a normal distribution for fixed effects parameters in both option, NWPRI and TNPRI. For the random effects parameters, this function follows a normal distribution and an inverse Wishart for NWPRI and TRPRI, respectively. This methodology is useful to stabilize the parameters estimations when the available data are very sparse (89). However, it is very sensible to the prior information and it requires a detailed knowledge of the fixed and random effects parameters distributions in a similar population than the studied one. In addition, this methodology implies to assume the prior parameterization and structural model as the best one. This can affect the identifiability of the parameters (90) and can not allow evaluating different structural models than the previous described in the prior model. According to these reasons, we didn't investigate a prior approach, in order to have the opportunity to evaluate various reduced models. This way, we could remove or fix parameters and ensure the non over-parameterization of our model as well as the identifiably of the parameters. Moreover, we were able to evaluate various structural models, different from the one selected for the prior model.

The values estimated for DOX and DOXol PK parameters were similar to those previously reported in the literature (table I-2). In addition, the model was successfully internally evaluated by bootstraping, VPC and NPDE (n=1000 replicates). These results showed the adequate descriptive and predictive ability of the model developed for DOX and DOXol and support it as a useful and valid tool for TDM in the clinical suitability.

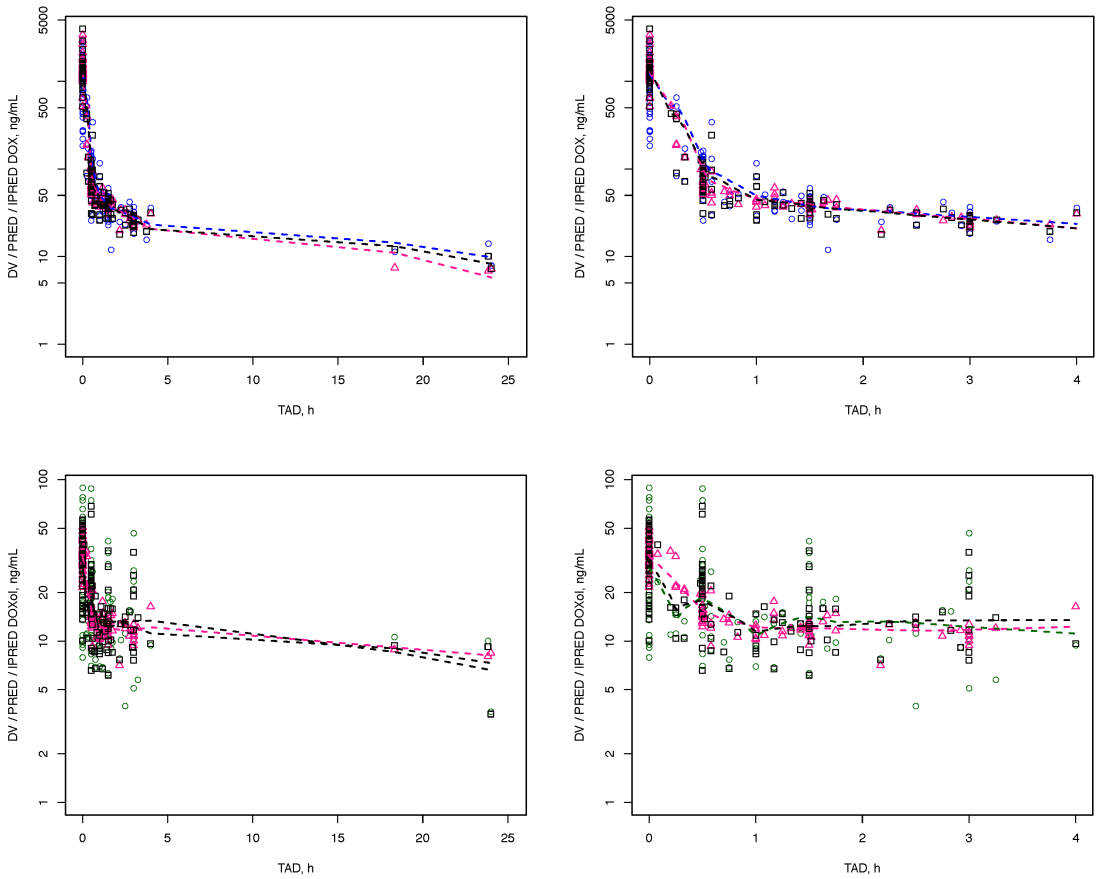


Figure D-1. Evolution of doxorubicin (top) and doxorubicinol (bottom) plasma concentrations observed with the population and individual predictions obtained with the final population pharmacokinetic model

DV: dependent variable (concentration); △PRED: population prediction; □ IPRED: individual prediction; DOX: doxorubicin; DOXol: doxorubicinol; o: observation of DOX; o: observation of DOXol; TAD: time after dose; ---: locally weighted polynomial regression (trend line) of DOX observations; ---: trend line of DOXol observations; ---: trend line of PRED; ---: trend line of IPRED.

Over 50 % of the patients treated with DOX recommended dosages suffers a dose-dependent neutropenia at the nadir, around two weeks after the drug administration or even after 4 weeks after. This pathological situation is frequently recovered before the following administration (1,18). In the non-recovery cases, an administration of GCSF or a delay for the following DOX administration is needed. The ability to predict this situation could be helpful for the early identification of the patients who would need an additional treatment by GCSF or a dosage adjustment in the next cycle administrated. Several relationships between DOX and/or DOXol plasma concentrations, at specific time points or at the steady state, and the bone marrow depletion at the nadir (LEU, NEU, PLA, survival factor, etc.) according to the classical exponential and sigmoidal maximum effect models have been established (18,33,91,92).

Unfortunately, from the PK/PD analysis carried out, no statistically significant relationship ($p>0.05$) between the PK parameters reflecting the drug or metabolite exposure (AUC_{DOX}, AUC_{DOX}, $C_{max,DOX}$, $C_{max,DOXol}$ y AUC_{total}) and the bone marrow depletion (LEU, NEU) have been established, according to lineal and exponential models. However, a trend between AUC_{total} and neutropenia, considering as a categorical (toxicity – non toxicity) ($p=0.065$) or continuous covariate ($p=0.089$), was observed (figure D-2).

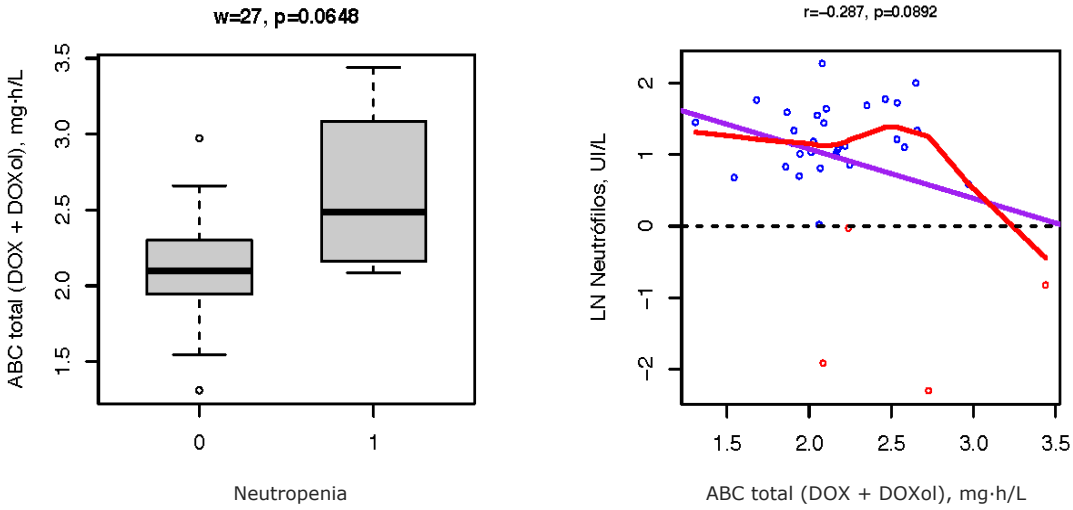


Figure D-2. Pharmacokinetic/pharmacodynamic analysis categorical (left) and continuous (right).

Neutropenia: 0 = neutropenia grade 1-2 and absence of toxicity, 1 is neutropenia grade 3-4; —: trend line; —: lineal regression; ---: value of neutropenia grade 3-4; ◦: values of neutrophils count with neutropenia grade 3-4.

In consequence, an UHPLC method to quantify DOX and DOXol plasma concentrations has been successfully developed and validated. Its clinical suitability has allowed establishing the PK profiles of both entities in patients diagnosed with NHL. The concentrations quantified with this method coupled to other information has allowed the development of a population PK model for the parent drug and its main metabolite with a successful internal evaluation. The sparse data of our analysed population did not permit us to establish significant PK/PD relationships, taking into account the haematological toxicity. Nevertheless, the model proposed can be useful for DOX dosage adjustments in TDM purpose, according to Bayesian algorithms. Finally, additional studies are required to confirm previously reported results.

6.- CONCLUSIONS

- An analytical method of ultra high liquid chromatography (UHPLC) coupled to fluorescence detector quantifying doxorubicin and its main active metabolite, doxorubicinol, in human plasma has been successfully developed and validated. Its simplicity, speed, low cost and volumes required, as well as its appropriate lower limit of quantification provide this method as an adequate tool for therapeutic drug monitoring in clinical routine of both active entities pharmacologically.

- A population pharmacokinetic model of doxorubicin and doxorubicinol in patients diagnosed of non-Hodgkin´s lymphoma has been developed. The structural model includes three compartments for the parent drug and two additional ones for the metabolite with first order distribution and elimination.

- None of the covariates evaluated (weight, height, body surface area, lean body weight, creatinine clearance, bilirubin, sex and aspartate aminotransferase) showed a statistically significant influence on the parent drug clearance.

- The pharmacokinetic parameters estimated with the final pharmacokinetic model proposed were similar to those previously published in different populations.

- The internal evaluation techniques based on Monte Carlo simulations (Visual Predictive Check and Normalized Prediction Distribution Error) and bootstrapping were successful. Thus, the model developed could be used for doxorubicin dosage adjustment in this population using Bayesian algorithms.

- No statistically significant relationship between drug exposure (area under the curve and maximum concentration of doxorubicin and doxorubicinol and total area under the curve for both entities) and haematological toxicity parameters (neutrophils and leukocytes count, neutropenia and leukopenia grade) has been shown.

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