UNIVERSIDADE FEEVALE

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DESENVOLVIMENTO E VALIDAÇÃO DE ESTRATÉGIAS BIOANALITICAS PARA AVALIAÇÃO DA EXPOSIÇÃO AO PACLITAXEL EM PACIENTES ONCOLÓGICOS

Novo Hamburgo

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Dissertação apresentada ao Curso de Mestrado em Toxicologia e Análises Toxicológicas como requisito para obtenção do título de Mestra em Toxicologia e Análises Toxicológicas.

Professor orientador: Prof. Dr. Rafael Linden

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RESUMO

O câncer é uma doença crônica considerada uma das principais causas de morte no mundo. O paclitaxel (PCT) é um fármaco antineoplásico amplamente utilizado para o tratamento de tumores sólidos, sendo sua dose normalmente calculada a partir da área de superfície corporal do paciente. Apesar da sua eficácia clínica, a administração de PCT geralmente está associada a efeitos adversos severos e o tratamento apresenta ampla variabilidade interindividual na tolerabilidade a esses efeitos, causada pelas diferenças interindividuais na farmacocinética, especialmente na depuração, o que leva a grandes diferenças na exposição a este fármaco. Nesta perspectiva, o presente estudo teve como objetivo desenvolver e validar estratégias bioanalíticas para aplicação do monitoramento terapêutico de PCT, particularmente empregando amostras de plasma e de sangue seco em papel. PCT foi extraído do plasma com uma mistura de solventes orgânicos e determinado em concentrações clinicamente relevantes por cromatografia líquida de alta eficiência com detector de arranjo de diodos através de um ensaio completamente validado. Adicionalmente, um método para determinação de PCT em sangue seco em papel (*dried blood spots*, DBS) empregando cromatografia líquida associada a espectrometria de massas sequencial também foi desenvolvido e validado extensivamente. Amostras de 34 voluntários em tratamento com PCT foram dosadas pelos métodos desenvolvidos. Os resultados das concentrações de PCT foram utilizados para calcular o tempo em que a concentração de PCT esteve acima de 0,05 μM, parâmetro relacionado à resposta a exposição sistêmica ao PCT. Cerca de dois terços (65%) dos pacientes apresentaram exposições ao PCT fora da faixa terapêutica, evidenciando a necessidade de implementação do monitoramento terapêutico para otimização das doses. Neste trabalho é apresentado o primeiro relato de um método para determinação de PCT em DBS aplicado clinicamente em pacientes em tratamento quimioterápico.

Palavras-chave: paclitaxel, câncer, monitoramento terapêutico de fármacos, individualização de dose, sangue seco em papel.

ABSTRACT

Cancer is a chronic disease considered a major cause of death in the world. Paclitaxel (PCT) is an antineoplastic drug widely used for the treatment of solid tumors, and dosing is based on body surface area of the patient. Despite its clinical efficacy, PCT administration is usually associated with severe adverse effects, and the treatment has wide interindividual variability in the tolerability of this effects, caused by the individual differences in pharmacokinetics parameters, especially in clearance, which lead to large differences in exposure to this drug. In this context, the present study aimed to develop and validate bioanalytical strategies for application in therapeutic drug monitoring of paclitaxel, particularly using samples of plasma and dried blood spots. PCT was extracted from plasma with a mixture of organic solvents and determined at clinically relevant concentrations by high performance liquid chromatography with diode array detector through a fully validated assay. Additionally, a method for determination of PCT in dry blood spots (DBS) using liquid chromatography associated with tandem mass spectrometry was also developed and extensively validated. Samples of 34 volunteers undergoing PCT treatment were measured using the developed assays. The results of PCT concentrations were used to calculate the time that the systemic concentration remains above a threshold of 0.05 μM, the parameter related to the systemic exposure to PCT. Approximately two-thirds (65%) of the patients presented PCT exposures outside of the therapeutic range, evidencing a need to implement therapeutic monitoring for dose optimization. This work brings the first report of a method for the determination of PCT in DBS applied clinically in patients undergoing chemotherapy.

Keywords: paclitaxel, cancer, therapeutic drug monitoring, dose individualization, dried blood spots.

SUMÁRIO

1. INTRODUÇÃO GERAL

O câncer é uma doença crônica relacionada, entre as cinco principais causas de morte no mundo, sendo considerado um dos principais problemas de saúde pública (NIH, 2015). Um dos fundamentos no tratamento do câncer é a quimioterapia (VERBRUGGHE et al., 2013). Dentre os quimioterápicos mais amplamente utilizados está o paclitaxel (PCT), um alcalóide natural isolado da casca de uma árvore, *Taxus brevifolia*. PCT é utilizado para o tratamento de vários tipos de tumores, incluindo câncer de mama, de ovário, cabeça e pescoço, pulmonar de células não pequenas e de esôfago. Sua atividade antitumoral foi descoberta em meados de 1970 e foi aprovada pelo *Food and Drug Administration* (FDA) em 1992 (RODRÍGUEZ-ANTONA, 2010). A dose de PCT é determinada com área de superfície corporal do paciente, geralmente são indicadas doses de 45 a 260 mg/m^2, de acordo com cada tipo de tumor, e levando em consideração a função hepática dos pacientes.

Apesar da eficácia clínica no tratamento de diferentes tumores (ETTINGER et al., 1995; FORASTIERE et al., 1998; SCHILLER et al., 2002) o PCT possui uma janela terapêutica estreita e diversos efeitos adversos (KUMAR et al., 2010). Pacientes tratados com PCT apresentam ampla variabilidade interindividual na tolerabilidade aos efeitos adversos, onde toxicidade neurológica e hematológica são as mais proeminentes (KRENS et al., 2013). Esta tolerabilidade está relacionada às diferenças farmacocinéticas interindividuais, relacionadas à depuração (STEED & SWAYER, 2007), variantes genéticas nas enzimas envolvidas na sua metabolização e no transporte (RODRÍGUEZ-ANTONA, 2010; DE GRAAN et al., 2013; PACI et al., 2014), variáveis demográficas, fisiológicas, patológicas, assim como interações medicamentosas (KRAFF et al., 2015A).

A morte resultante de toxicidade gerada por fármacos quimioterápicos é rara, mas a toxicidade excessiva é comum (BARDIN et al., 2014). Além disto, subdosagens podem ocorrer em função da imprecisão dos cálculos de dose e frequentemente não serem notadas. O monitoramento terapêutico de fármacos (MTF) envolve a quantificação do fármaco em fluidos biológicos para individualização de doses com o intuito de maximizar os efeitos terapêuticos e minimizar a toxicidade (GAO et al., 2012; BARDIN et al., 2014). Geralmente, é considerado para fármacos que apresentam ampla variabilidade farmacocinética, janela terapêutica estreita e uma relação bem definida entre exposição sistêmica e toxicidade ou resposta. A parâmetro farmacocinético que apresenta a relação mais forte com a farmacodinâmica do PCT é o tempo que a concentração plasmática de PCT permanece acima

de 0,05 µM (Tc>0,05) após o final da infusão (KRENS et al., 2013). Este parâmetro está relacionado com uma variedade de efeitos adversos, como neutropenia e neuropatia (OHTSU et al., 1995; JOERGER et al., 2007; ZHANG et al., 2016).

A coleta de amostras para o cálculo do Tc>0,05 dos pacientes deve ser feita de 18 a 30 horas após o início da infusão, a cada ciclo de tratamento (JOERGER et al., 2007). A realização desta coleta pode apresentar dificuldades práticas, pois requer que o paciente retorne a um centro especializado para a coleta de sangue e posterior separação do plasma. Para que o parâmetro Tc>0,05 seja calculado apropriadamente é necessária uma abordagem matemática sofisticada. Por isso, Kraff e col. (2015a) desenvolveram uma ferramenta baseada no Microsoft Excel® para determinação do Tc>0,05 empregando apenas uma amostra de sangue, com precisão e exatidão similar ao software de referência NOMEM. Além do desenvolvimento do software, os mesmos desenvolveram um algoritmo para ajuste de doses de PCT quando utilizado em um esquema de administração semanal, baseados em dados retrospectivos, com alvo de Tc>0,05 de 10 a 14 horas (KRAFF et al., 2015b). Para o regime de PCT com infusão a cada 21 dias, foi proposto um alvo terapêutico de 26 a 31 horas (JOERGER et al., 2012). Apesar de sua importância clínica, uma fração significativa dos pacientes apresenta exposições subterapêuticas ou excessivas, o que compromete a segurança e a eficácia dos seus tratamentos. Em um estudo realizado com 96 pacientes em uso de PCT, 35% dos pacientes estavam subdosados e 11% acima da faixa terapêutica (ZHANG et al., 2016). Já no estudo de Joerger et al. (2016a), conduzido com 175 pacientes, 29% estavam subdosados e 38% apresentaram exposições excessivas ao PCT.

Considerando que o PCT exerce atividade citotóxica em concentrações muito baixas (LIEBMANN et al., 1993), métodos bioanalíticos de elevada sensibilidade são necessários para sua determinação e quantificação em fluídos biológicos. A cromatografia líquida de alta eficiência com detector de arranjo de diodos (CLAE-DAD) e com detector de massas (CL-EM/EM) são os métodos mais utilizados devido a sua reprodutibilidade, especificidade, eficiência e sensibilidade (KIM et al., 2005). Para a recuperação do PCT de fluídos biológicos, podem ser realizados vários tipos de técnicas de extração, como extração líquido-líquido (RAZAZADEH et al., 2015), extração em fase sólida (WANG et al., 2003), ou extração líquido-líquido seguida de extração em fase sólida (SPARREBOOM et al., 1995). Uma abordagem alternativa para coleta de amostras biológicas são as manchas de sangue seco em papel (*dried blood spots*, DBS), que permite a realização de esquemas de amostragem mais complexos, eventualmente realizados na residência do próprio paciente, sendo menos

invasiva que a flebotomia convencional e pode estar correlacionada com as concentrações plasmáticas do fármaco (KROMDIJK et al., 2013; ANTUNES et al., 2015).

O PCT é um fármaco candidato para o MTF, sendo necessários métodos analíticos confiáveis e convenientes para sua determinação para posterior estimativa da exposição individuais e eventuais decisões clínicas. Neste contexto, neste dissertação uma ampla revisão sobre as características farmacológicas, as fontes de variabilidade interindividual na exposição ao fármaco e a experiência clínica de individualização de doses dos taxanos foi realizado e é descrita no capítulo 1. Adicionalmente, um ensaio para determinação de PCT em plasma empregando CLAE-DAD foi desenvolvido e validado, tal como apresentado no capítulo 2. Por fim, para permitir uma aplicação facilitada do MTF de PCT em ambientes clínicos, uma ensaio baseado em CL-EM/EM para determinação de PCT em DBS foi desenvolvido e validado, sendo aplicado a um grupo de pacientes em tratamento quimioterápico.

2. CAPÍTULO 1

PHARMACOGENETIC AND PHARMACOKINETIC DOSE INDIVIDUALIZATION OF THE TAXANE CHEMOTHERAPEUTIC DRUGS PACLITAXEL AND DOCETAXEL

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ABSTRACT

The taxane drugs paclitaxel and docetaxel, widely used on cancer chemotherapy, are currently dosed mainly based on body-surface area. This approach is associated with wide interindividual variability in drug exposure, leading to suboptimal dosing for many patients. We reviewed the available evidence supporting dose individualization strategies for paclitaxel and docetaxel, focusing mainly on the application of therapeutic drug monitoring by *a priori* pharmacogenetic data or *a posteriori* drug measurement in biological fluids. The PubMed database was searched, in the period of 1987-2017, using the keywords pharmacogenetics, metabolic genotyping, dose individualization, therapeutic drug monitoring, personalized medicine, taxanes paclitaxel and docetaxel, either alone or in combination. The current knowledge of pharmacology of the taxane drugs paclitaxel and docetaxel, mainly its pharmacokinetics and the proteins responsible for their biotransformation and transport, along with the genetic polymorphism responsible for variations in the activities of these proteins, opens new opportunities for dose selection for individual patients. Considering the relation between systemic exposure to these drug and clinical responses, *a posteriori* TDM, with measurement of drug concentrations in plasma of treated patients, is currently the most straightforward approaches for dose individualization of paclitaxel and docetaxel.

Keywords: Pharmacogenetics, metabolic genotyping, dose individualization, therapeutic drug monitoring, personalized medicine, taxanes paclitaxel, docetaxel.

1. INTRODUCTION

Taxanes, mainly paclitaxel and docetaxel, are commonly prescribed chemotherapeutic drugs used in the treatment of various solid tumors, such as prostate, breast and non-small-cell lung cancer [1]. Despite their clinical utility, taxanes, as many other anticancer drugs, present a narrow therapeutic window and its use is associated with potentially severe hematopoietic and neurologic toxicities [2].

The natural source of taxanes are the bark, needles and roots of several Taxus species, such as *T. brevifolia, T. baccata, T. chinensis, T. canadensis, T. cuspidata, T. globosa, T. floridana* and *T. wallichiana* [3, 4]. Besides natural sources, paclitaxel could be also produced by total chemical synthesis, which is usually not cost-effective [5-7], or by semi-synthesis, which uses intermediates such as 10-deacetylbaccatin III or baccatin III, found in needles of *Taxus*. Docetaxel, a semi-synthetic analog of paclitaxel, is also synthesized from 10-deacetylbaccatin III [10]. Alternatively, paclitaxel can be also obtained for plant cell cultures of *Taxus* spp. [4, 5, 8-13].

Currently, taxane dosing is based mostly on body surface area (BSA). This dose selection approach is associated to a wide interindividual variation in drug expose and, consequently, on drugs effects [2]. However, with the increased knowledge of the pharmacogenetics pharmacokinetics and pharmacodynamics of taxanes, dose individualization of these toxic drugs may become a reality.

In the context of personalized medicine, paclitaxel and docetaxel are potential candidates for therapeutic drug monitoring (TDM) strategies, including *a priori* pharmacogenetic pre-emptive testing, as well as *a posteriori* evaluation of drug exposure, fitting into a broader definition of TDM, which is considered as a drug dose individualization approach that can be based on the knowledge of the pharmacogenetic, demographic and clinical characteristics of the patients, prior to the treatment, or on the measurement of pharmacokinetic of pharmacodynamics markers after initiation of therapy [14].

The aim of this manuscript is to review relevant information on the pharmacology of the taxane drugs paclitaxel and docetaxel, with special focus on data relevant to dose individualization. For this purpose, the PubMed database was searched using the keywords pharmacogenetics, metabolic genotyping, dose individualization, therapeutic drug monitoring, personalized medicine, taxanes paclitaxel and docetaxel, either alone or in combination.

2. PHARMACODYNAMICS

Taxane drugs present a unique mechanism of action on polymerization of tubulin, rather than a direct action on the DNA, being usually used in combination with other antimitotic agents and exhibit a selective anti-vascular reaction at therapeutic doses [29]. To date, there are only three taxane drugs approved for cancer therapy (cabazitaxel, docetaxel and paclitaxel). Paclitaxel and docetaxel are considered first-generation taxanes. Paclitaxel is used in Kaposi's sarcoma, non- small-cell lung, breast and ovarian cancer [15, 16]. Docetaxel is currently used in the therapy of head and neck, stomach, prostate, breast and non-small-cell lung cancers [15]. Cabazitaxel, a second-generation taxane, is indicated in metastatic hormone-refractory prostate cancer [15, 17]. Taxanes drugs are antimitotic agents [18].

Paclitaxel is a polyoxygenated diterpenoid, obtained from *Taxus brevifolia*, a Pacific yew. The chemical structure of paclitaxel consists of a 15-membered tricyclic taxane ring system (tricyclo [9.3.1.0] pentade- cane) linked to an oxetane ring (D). At C-13 hydroxyl position, it has a *N*-benzoyl-b-phenylisoserine group attached through an ester bond [19, 20]. Cytotoxic activity is conferred by the oxetane ring of taxanes, as well as C1- hydroxyl, C2-benzoyloxy and C4-acetate are crucial moieties for keeping this effect [21]. The oxetane ring plays a crucial role in microtubulin binding through hydrogen bonding, also allowing to orient the C4-acetyl moiety interaction within its hydrophobic binding pocket [22]. Knowing the importance of some functional groups of the paclitaxel molecule has permitted more active and water-soluble chemical analogues, such as docetaxel [23]. Docetaxel exhibits potent anticancer effect and differs in substitution pattern at C10 and C13 (Fig. 1). The presence of the free C10-hydroxyl (secondary alcohol) and the C13-*t*-butoxycarboxamide confers higher water solubility to docetaxel when compared to paclitaxel, leading to more favored pharmacokinetic properties [20].

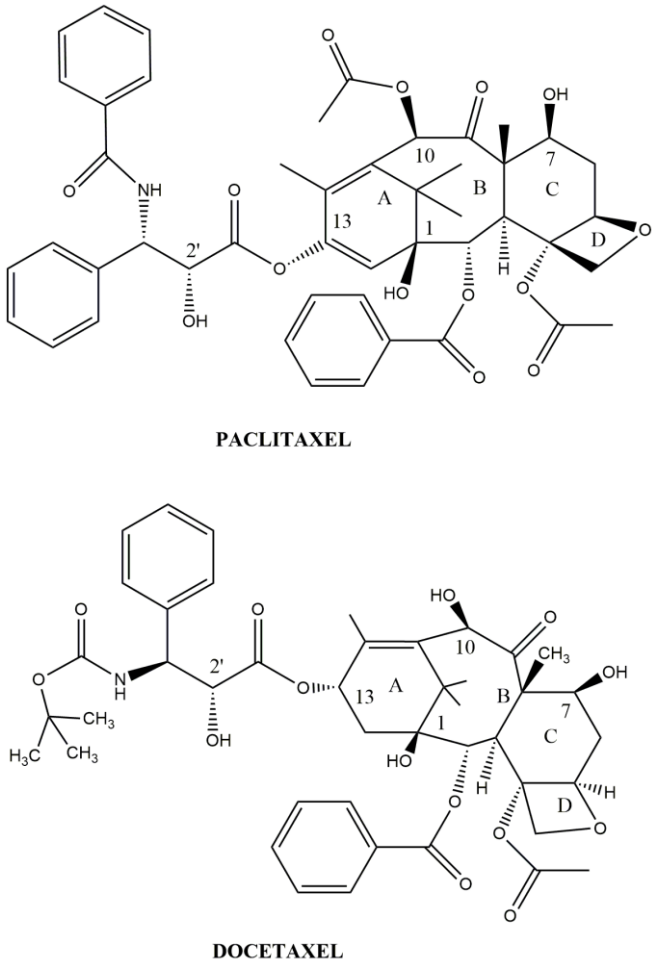

Figure 1. Chemical structures of paclitaxel and docetaxel

Taxanes act on the tubulin present in microtubules [15], increasing polymerization of this cellular structure [24]. Microtubules are structures that form part of the cytoskeleton, participating in the processes of mechanics, transport and cell division. In addition, they play important role in the carcinogenic process, promoting cell proliferation and, consequently, metastasis [25]. They resemble cylinders formed from heterodimers of α-β-tubulin and act by separating chromosomes during mitotic stage of the cell cycle [16]. This structure presents a dynamic instability, which is responsible for the alternation of polymerization and depolymerization states of α-β-tubulin heterodimers. This process is triggered by the hydrolysis of a GTP that is bound to tubulin[25,26]. Taxanes target β-tubulin in order to alter the microtubules stability, altering the separation of chromosomes and cell division [27–29]. Taxanes connect to a taxane binding site on β-tubulin, which is found in the microtubule inner surface [24]. The most important mechanisms of actions of taxanes are cyclin B-1 stabilization, activation of cell division control 2 (cdc-2) kinase, induction of apoptosis

through B-cell lymphoma 2 (bcl-2) phosphorylation, spindle assembly checkpoint activation and cell proliferation inhibition [30].

Although the structural resemblance among the taxanes molecules, they have distinct conformations to bind the β-tubulin. Churchil *et al.* [15] demonstrated that docetaxel and paclitaxel form hydrogen bonds with the β-tubulin, on β:Arg369 and β:Asp26, respectively.

Docetaxel has twice the efficacy of paclitaxel because acts on the mitotic spindle centrosome. Despite of this, paclitaxel and docetaxel have comparable therapeutic efficacy in the treatment of cancer [1]. On the other hand, taxanes also have varying effects on the interactions of the tubulin M-loop with its side chains. Therefore, the contact between the side chains and affinity of the bonds is not related to the potency and also to the IC_{50} of these drugs [1].

Drug resistance imposes limitations to the efficacy of paclitaxel and docetaxel [31]. Resistance to these drugs is linked to increased expression of the gene encoding the P-glycoprotein (PGP), i.e., the multidrug resistance 1 (MDR). PGP is an efflux pump that reduces taxane concentration in the intracellular environment [18,31]. Expression of PGP by cancer cells can be responsible for both constitutive and acquired resistance to taxanes. Overexpression of class III β-tubulin was also identified as the cause of taxane resistance [18]. Paclitaxel increases the expression of the MDR1 gene by altering cellular mechanisms, such as tubulin mutations and changes in β-tubulin binding regions, reduction of function pf Bcl-2 and p53, that are proteins that promote cellular apoptosis, increases in expression of ABC transporters and cytocine expression alterations, (IL 6 for example) [16]. Resistance to docetaxel was associated with expression of different isoforms of β-tubulin, activation of drug efflux pumps, phosphatase and tensin homolog (PTEN) gene loss, activation of survival pathways (PI3K/AKT and mTOR) and NOTCH2/ Hedgehog signaling pathways [32]. The mechanisms of taxanes resistance also include alterations in the tubulin, the target component of taxanes. The main mechanisms reported are increase expression of tubulin, especially class III β-tubulin isotype, alterations in the expression profile of microtubule associated proteins (MAPs) and alteration of the expression pattern of α- and β-tubulin in various cell lines [33].

The principal adverse effects related to the use of paclitaxel are related to the suppression of bone marrow activity, including anemia, thrombopenia and neutropenia. These effects are accompanied by toxicity on central nervous system, including myalgia, arthralgia, paresthesia in peripheral limbs and hypoesthesia, hypotension and potentially severe

hypersensitivity reactions. Regarding docetaxel, main toxicities are diarrhea, loss of hair, mucositis, toxicity in nails and neutropenia febrile [1].

3. PHARMACOKINETICS

The pharmacokinetics profiles of paclitaxel and docetaxel have been characterized by population pharmacokinetic studies, demonstrating fast distribution to tissues and considerably large volumes of distribution. In a Phase I trial, Markman *et al*. [34] have demonstrated that paclitaxel is not effectively absorbed following oral administration. However, other authors attempted to administrate paclitaxel and docetaxel orally together with PGP inhibitors, based on the knowledge that overexpression of multidrug transporter PGP by intestinal enterocytes limits the oral absorption of taxanes [35–38]. Peak plasma levels are usually reached at the end of the infusion, following a rapid decrease of plasma concentration levels due to distribution [39].

Several dose regimens for taxanes have been used in patients with solid tumors. For paclitaxel, infusion lengths varied from 1 to 24 h, and administered doses between 60 and 175 mg/m^2, either in combination therapy or as a single chemotherapeutic agent. Weekly scheduling of paclitaxel may increase antitumor activity by reducing tumor regrowth between cycles and maximizing the drug's antiangiogenic effects [40]. Moreover, a meta-analysis including 10 randomized studies showed a more favorable toxicity profile for weekly compared to the 3-weekly paclitaxel regimens [41]. Weekly doses of paclitaxel usually are in the range of 60-80 mg/m^2, combined with variable exposure levels of carboplatin [42,43]. Docetaxel doses usually varied from 25 to 100 mg/m^2, with 1 to 2 h intravenous infusion times [44–49].

The pharmacokinetics of paclitaxel is nonlinear due to saturable distribution, metabolism and elimination [50], and by interaction with CrEL, a micelle-forming agent usually present on its pharmaceutical formulations [51–53]. CrEL is a pharmaceutical vehicle used to dissolve paclitaxel for intravenous administration, and has been suggested to reduce plasma clearance, affecting the disposition of the drug and altering the distribution of paclitaxel by entrapment in micelles [52,54,55]. Therefore, the free fraction of paclitaxel decreases with increasing concentrations of CrEL [55]. About 90% of administrated paclitaxel is bound to plasma proteins, most to serum albumin and a minor contribution of α1-acid glycoprotein [2].

Paclitaxel pharmacokinetics is described either by two or three-compartment models, with a terminal half-life estimated to be between 8 and 12 h [56–59]. Currently, taxane dosing is based only on body surface area (BSA), and for patients without hepatic impairment, paclitaxel prescribed doses are between 135 and 175 mg/m^2 [60]. Huizing *et al*. [48] studied the pharmacokinetics parameters of paclitaxel in ovarian cancer patients following 3 and 24 h infusions of paclitaxel at doses levels of 135 and 175 mg/m^2. The terminal half-life ranged from 13.1 to 52.7 h and the total body clearance from 12.2 to 23.8 L/h/m^2. Average of maximal plasma concentration (C_{max}) values were 0.23 ± 0.03 μmol/L at 135 mg/m^2 and 0.43 ± 0.14 μmol/L at 175 mg/m^2 for the 24 h infusion, with 2.54 ± 0.52 μmol/L at 135 mg/m^2 and 4.27 ± 1.26 μmol/L at 175 mg/m^2 for the 3 h infusion. Volumes of distribution at steady state, when administered as 24 h infusion, were in the range of 227 to 688 L/m^2. Alternatively, when paclitaxel is administered as shorter infusions, with 3 or 6 h, volumes of distribution were in the range of 50-100 L/m^2 [57]. The large volumes of distribution are mainly due by the binding to tissue proteins [61].

The pharmacokinetics of docetaxel is characterized as a three-compartment model [62,63]. Unlike paclitaxel, docetaxel has a linear pharmacokinetics, and peak levels of docetaxel vary according to the dosing and schedule of administration [1,64]. AUC increased with dose from 0.96 μg/mL/h at 20 mg/m^2 to 5.2 μg/mL/h at 115 mg/m^2, and increased with hours of infusion from 4.6 μg/mL/h at 1-2 h infusion to 6.8 at 6 h infusion [65]. In accordance, Garland *et al*. [66] found an increase in AUC with higher dose of docetaxel, from 2.4 μg/mL/h at 60 mg/m^2 to 3.6 μg/mL/h at 75 mg/m^2. The C_{max} was also increased at higher doses. Total plasma clearance was verified to be independent of dose, with an average of 21.1 ± 5.3 L/h/m^2, with distribution volume of 72 ± 40 L/m^2 [65,67]. In contrast, plasma clearance was higher with more prolonged exposure of docetaxel [68]. With high doses in the 1 or 2 h infusions, the pharmacokinetics fit a triexponential curve. The α, β, and γ half-lives, after a 115 mg/m^2 1 h infusion, were 4 min, 36 min, and 11.1 h, respectively, and total body drug clearance was 21.1 ± 5.3 L/h/m^2 [65]. The C_{max} has an average of 2.9 μg/mL at the dose range of 60-100 mg/m^2 [69,70]. Clinically recommended doses of docetaxel are 60-100 mg/m^2 administered as a 1 h intravenous infusion at 3 weeks interval, including the injection of 3-5 g of polysorbate 80 and 0.5-0.8 mL of ethanol to the patient [60]. As in paclitaxel formulations, the presence of CrEL also affects the disposition of the drug and may cause hypersensitivity reactions [39,71].

The biotransformation of taxanes is mediated by hepatic metabolism and biliary excretion (Fig. 2). According to the Figure 2, the protein responsible for the hepatocellular uptake of taxanes is the organic anion-transporting polypeptide 1B3 (OAT1B3), also known as solute carrier organic anion transporter family member 1B3 (SLCO1B3), encoded by the gene *SLCO1B3*. Taxanes also enter the hepatocytes through passive diffusion, due their lipophilic characteristics [72–74]. Once inside the hepatocytes, taxanes are metabolized mainly by enzymes from cytochrome P450 system [75].

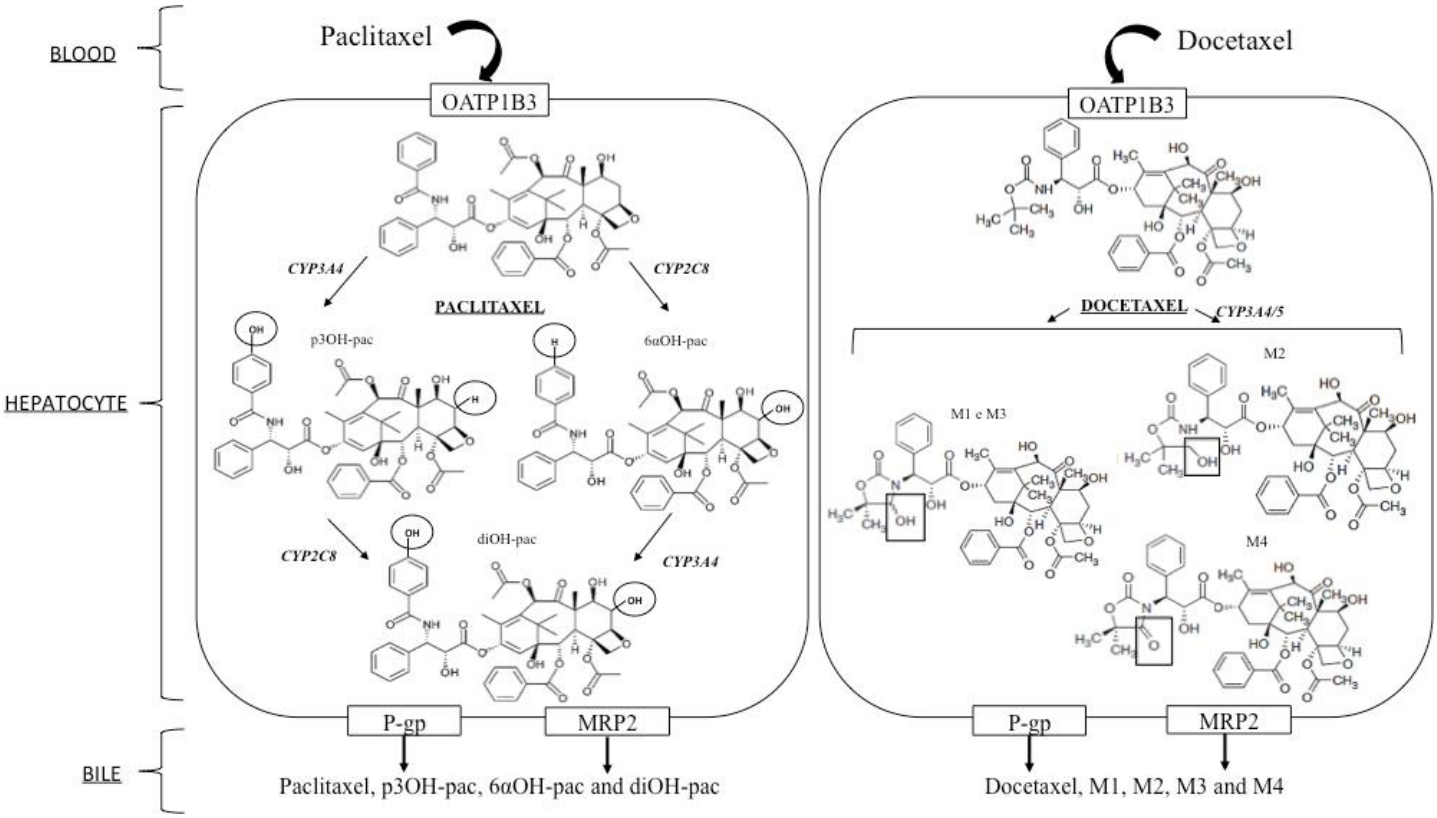

Figure 2. Biotransformation of paclitaxel and docetaxel

The enzyme CYP2A metabolizes both docetaxel and paclitaxel. However, the major metabolite of paclitaxel, 6α-hydroxypaclitaxel, is formed by CYP2C8 by modification C6 position of the taxane ring [76]. The metabolite 3'-p-hydroxypaclitaxel is formed mainly by CYP3A4 as a product of hydroxylation of the phenyl moiety at the C3' position on the compound's side chain. The minor paclitaxel metabolite 6α,3'-*p*-dihidroxypaclitaxel is formed by the action of both CYP2C8 and CYP3A4 [76–78]. Inside the hepatocytes, docetaxel is oxidized in the side chain of the *tert*-butylpropionate to four inactive metabolites

by the action of the CYP3A4 and CYP3A5 enzymes [79]. The main metabolite is the hydroxydocetaxel (M2), which may also be oxidized by an unstable carboxylic acid, the oxazolidinedione [80,81]. CYP3A4/5 also form other docetaxel metabolites, which also can be formed by spontaneous cyclization of intermediate metabolites, forming the diastereoisomers M1 and M3, and a ketone metabolite (M4) [82]. The metabolites of both paclitaxel and docetaxel have no significant cytotoxic activity. For instance, 6α-hydroxypaclitaxel presents 30 times less cytotoxic activity than the parent drug [77].

After hepatic metabolization, taxanes and their metabolites are secreted to bile by the efflux proteins PGP and multi-drug resistance protein 2 (MRP2). The excretion trough the bile, and subsequently by feces, represents 70-80% of the body clearance, and only 5-10% are eliminated by renal system as the parent drugs [39,80,83–85].

4. RELATION BETWEEN EXPOSURE AND RESPONSE TO PACLITAXEL AND DOCETAXEL

Taxanes have a narrow therapeutic window and a broad profile of adverse events [2]. Treatment with these drugs has wide interindividual variability in the tolerability of adverse effects, caused by the individual differences in pharmacokinetics parameters, especially in clearance, which lead to large differences in exposure to the drug. These differences in exposure are evaluated as the AUC [86–88]. For paclitaxel, it can also be evaluated by the time that the systemic concentration remains above a threshold of 0.05 or 0.1 μM (Tc > 0.05; Tc > 0.1) [48,88,89]. Other contributive factors to the pharmacokinetics (PK) interindividual variability are the individual germline genome, environmental factors that influence the activity of the enzymes involved in the biotransformation of taxanes, as drug-drug interactions and demographic, pathological and physiological variables [2].

The most notable adverse events of paclitaxel are hematologic and neurologic toxicities [1,2,59,90]. Several studies have reported the relationship between PK and toxicity or efficacy [48,86–88,91–94]. Hensing *et al*. [95] studied a group of 230 patients with advanced non-small cell lung cancer (NSCLC) which were treated with paclitaxel (200 mg/m^2 every 3 weeks), combined to carboplatin, to verify the influence of age on toxicity and response. They found no significant differences in any of the most common toxicities comparing the age of the patients, but the overall incidence of neutropenia and neuropathy were high, ranged between 35-38% and 16-13%, respectively.

In a study conducted in 105 patients with ovarian cancer treated with paclitaxel (175 mg/m^2 for 3 h every 3 weeks) in combination with carboplatin, was found a correlation between Tc > 0.05 μM and an increase of severe neutropenia (*P*=0.01) but also with tumor complete response. Tc > 0.05 μM was significantly higher in those patients which presents a complete response when compared with patients with progressive disease (*P*=0.02) [91]. Similarly, Huizing *et al*. [96] found a positive relation between survival and Tc ≥ 0.1 μM in NSCLC patients treated with 10-250 mg/m^2 as a 3 h paclitaxel infusion, combined with carboplatin. A recent study with 96 ovarian cancer patients from China, receiving paclitaxel 175 mg/m^2 combined with carboplatin, showed a statistically significant difference in paclitaxel Tc > 0.05 μM between complete remission (CR) + partial remission (PR) and stable disease (SD) + progressive disease (*P* = 0.00185). Paclitaxel Tc > 0.05 μM in most patients with CR and PR was in the range of 26–30 h, which is concordant with the target previously proposed [59,97]. Paclitaxel Tc > 0.05 μM significantly correlated with the occurrence of leukopenia (*P* = 0.0002) and leukopenic fever (*P* = 0.0211), and higher paclitaxel Tc > 0.05 correlated with increased incidence of severe leukopenia, leukopenic fever and peripheral neuropathy [98].

Based in these studies, paclitaxel Tc > 0.05 μM seems to be the best parameter to predict toxicities and response in the patients with solid tumors, and it was used as the target PK marker in a recent dose adjustment trial [99].

The toxicity profile of docetaxel is mainly hematological, and less common toxicities include neuropathy and fluid retention, of which several studies have reported its association with PK parameters, particularly clearance or AUC [100–105]. Bruno *et al.* [106] studied 582 in the first course of docetaxel based treatment, where 64 % experienced neutropenia, and reported a strong association between docetaxel clearance and neutropenia (*p*>0.0001), where a 50 % reduction in docetaxel clearance was associated with 4.3 and 3.0 fold increases of the odds of grade 4 neutropenia and febrile neutropenia, respectively. They also observed that patients with elevated levels of hepatic enzymes presented a 27 % reduction in docetaxel clearance, consequently having an increased risk of toxicity. Ozawa *et al.* [104], reported the AUC as a predictor of febrile neutropenia (*p*=0.001) in 200 Japanese patients with solid tumors in treatment with docetaxel 60 mg/m^2, 1 h, 3-weekly. Yamamoto *et al.* [107] had shown minimal interindividual variation in docetaxel clearance along several chemotherapy cycles, proposing a target AUC of 4.9 mg/L.h for 100 mg/m^2 doses. Due to the linear

pharmacokinetics of docetaxel, Engels *et al.* [108] proposed clinical AUC targets of 2.5; 3.7 or 4.9 mg/L.h to doses of 50, 75 and 100 mg/m^2, respectively.

5. PHARMACOGENETICS

Variations on genes encoding proteins responsible for taxanes transport and metabolism contribute to interindividual differences on treatment outcomes [79,109–111]. These include the efflux transport adenosine triphosphate-binding cassette (ABC) genes (*ABCC1, ABCC2* and *ABCB1*), enrolled on hepatobiliary and intestinal secretion, the solute carrier organic anion transporting polypeptide (OATP1B3 or SLCO1B3), as well as cytochrome P450 metabolism enzymes (CYP2C8, CYP1B1 and CYP3A4) [2,80].

Several studies relating pharmacogenetics and pharmacokinetics of taxanes were reported, [79–81,112–137] (Table 1). The *ABCB1* gene (also known as MDR1, encoding the P-glycoprotein) have been associated to taxane efficacy and toxicity. An overexpression of MDR1 is the strongest predictive biomarker of taxane resistance in general. To date, several *ABCB1* polymorphisms are known, but the *2677G> T/A* (rs2032582), *3435C>T* (rs1045642) and *1236C>T* (rs1128503), stating for the *ABCB1*2* haplotype, are the most relevant in taxane pharmacogenetic, even with conflicted data in the literature [138]. The presence of these three polymorphisms have been previously related to a greater clinical response and survival of paclitaxel-treated patients [126] and decreased risk of toxicity, including neutropenia and neurotoxicity [114,123]. In contrast, a few studies indicated a significantly increased risk of toxicity, as hematologic [121,128], neutropenia [122,124]; diarrhea [126] and neuropathy [112]. More recently, other studies, including larger cohorts, failed to found significant association between *ABCB1*2* polymorphism and hematological and neurological toxicity or treatment response, assessed as overall survival (OS), clinical/radiological response, progression free survival (PFS), during taxane treatment [113,115,116,125,127,136].

Table 1. Overview of paclitaxel and docetaxel of pharmacogenetic studies

Gene	Population (N)	Chemotherapy	Type of Cancer	Polymorphisms	Main findings		Ref
					Association with taxane exposure	Association with outcomes (Toxicity and/or response)	
ABCB1	European 1,303	Paclitaxel containing	Breast	-129T>C 2677G>T/A	-	-129T>C decreased risk of sensory neuropathy (grade ≥2) OR = 0.47; 95% CI: 0.28–0.79 (P=0.004) 2677G>T/A increased risk of sensory neuropathy (grade ≥2) OR=1.22; 95% CI: 1.03–1.45 (P=0.02)	[112]
	Caucasian (n=79) Afro-American (n=27) Other (n=5)	Paclitaxel containing	Breast	1236C>T 2677G>T/A 3435C>T	-	Not significantly associated with paclitaxel complete response or toxicity	[113]
	Italian 152	Docetaxel and paclitaxel based	Breast	1236C>T 3435C>T	-	C1236T not associated to grade 3-4 toxicity C3435T is a predictive factor for toxicity not influenced by other genotypic characteristics (P=0.0264). Grade 3-4 toxicity TT *vs*. CC/CT OR= 0.48 (P=0.05).	[114]
	Caucasian 92	Docetaxel containing	Breast, Prostate, Lung, Head/neck	1236C>T 2677G>T/A 3435C>T	Not significantly associated with docetaxel CL	-	[80]
	Scandinavian 119	Paclitaxel + carboplatin	Ovarian	1236C>T 2677G>T/A 3435C>T	-	Not significantly associated with neutropenia, sensory neuropathy or overall survival.	[115]
ABCB1	Spanish	Paclitaxel	Breast	1236C>T 2677G>T/A	-	Not significantly associated with risk of	[116]

	118	containing		3435C>T		neurotoxicity	
	Scandinavian Caucasian 93	Paclitaxel + carboplatin	Ovarian, Fallopian tube, Peritoneal	1236C>T 2677G>T/A 3435C>T	Not significantly associated with paclitaxel CL	-	[117]
	Caucasian 38	Paclitaxel + carboplatin	Ovarian	1236C>T 2677G>T/A 3435C>T	Carriers of the variant 2677 GA had higher paclitaxel clearance compared with wild-type GG and TT patients (26.0 l/h *vs* 18.9 l/h and 17.4 l/h, P=0.036 and P=0.048 respectively).	Not significantly associated with toxicity	[118]
	Scandinavian Caucasian 92	Paclitaxel + carboplatin	Ovarian	1236C>T 2677G>T/A 3435C>T	-	Carriers of C3435T variant alleles had progressively more pronounced neutrophil decrease (63%, 72% and 80% for 3435CC, CT and TT, respectively; P= 0.03). Similarly, to G2677T/A (68%, 76% and 82%; P=0.02)	[119]
	European 914	Paclitaxel or Docetaxel + carboplatin	Ovarian	1236C>T 2677G>T/A 3435C>T	-	Not significantly associated with toxicity or response (progression-free survival, CA-125 response, clinical/radiological response)	[136]
ABCB1	European 322	Paclitaxel + carboplatin	Ovarian	1236C>T 3435C>T 1199G>A	-	1236C>T increased risk of anemia (OR = 1.71, 95% CI = 1.07-2.71, P=0.023)	[121]
	Korean 92	Docetaxel containing	Lung, Stomach, Head and neck, Esophagus	1236C>T 2677G>T/A 3435C>T	-	2677 GT was significantly associated with leukopenia/neutropenia (P=0.025) and with higher risk of chemo-resistance (OR= 6.48; 95% CI 1.92 - 21.94; P=0.003). No	[122]

						association with tumor response.	
	Taiwanese 59	Docetaxel + epirubicin + cyclophosphamide	Breast	−41A>G -145C>G 1236C>T 2677G>T/A 3435C>T	-	Febrile neutropenia occurred more frequently in *ABCB1* 2677 GG variant (RR 4.10; 95% CI 1.14–14.73; P<0.05). *ABCB1* 3435 CC tended to suffer leucopenia (p=0.057).	[123]
	Korean 218	Docetaxel+ adriamycin+ cyclophosphamide	Breast	1236C>T 2677G>T/A 3435C>T	-	*ABCB1* 3435 TT increased risk of neutropenia (RR=1.689; 95% CI 1.183–2.416; P=0.015). Other genotypes not significantly associated with toxicity.	[124]
	European 58	Docetaxel	Breast, Prostate, Lung, other	2677G>T/A 3435C>T	Not significantly associated with with docetaxel AUC or CL	Not significantly associated with neutropenia	[139]
	US (n=184) Japanese (n=145 + n=197)	Paclitaxel + carboplatin	Lung (Non–Small-Cell)	3435C>T	-	Not significantly associated with response (PFS and OS) or toxicity.	[125]
	Korean 216	Docetaxel + doxorubicin	Breast	1236C>T 2677G>T/A 3435C>T	*ABCB1* 3435TT genotype had a higher AUC than CC⁄CT for docetaxel	*ABCB1* 3435TT genotype had a longer OS than CC⁄CT (HR=0.22; 95% CI 0.05-0.9; P=0.024 for risk of death compared to CC/CT). C3435TT was associated with increased toxicities (neutropenia RR=4.6; 95% CI 1.3-11.6; P=0.037 and diarrhea RR=3.3; 95% CI 1.3-15.1; P=0.017, compared to CC/CT).	[126]
ABCB1	Danish 150	Docetaxel containing	Breast	1236C>T 2677G>T/A 3435C>T	-	Not significantly associated with peripheral neuropathy.	[127]
	European 92	Docetaxel containing	Breast, Lung, Prostate, Other	1236C>T 2677G>T/A 3435C>T	1236 TT genotype was significantly associated with a decreased docetaxel CL (-25%; P=0.0039)	-	[79]

	Asian 54	Docetaxel	Nasopharyngeal	1236C>T 2677G>T/A 3435C>T	Not significantly associated with docetaxel exposure (AUC, CL, C_{max})	The heterozygous genotypes 2677 GA/GT/TA had the highest percentage decrease in nadir haemoglobin from cycle 1 baseline compared to GG/TT genotypes (P=0.006). Similar trend for 3435 CT compared to those with CC/TT genotypes (P=0.066).	[128]
	European non-Hispanic white women 4616	Paclitaxel containing	Epithelial ovarian (Ovarian Cancer Association Consortium-OCAC and Cancer Genome Atlas-TCGA)	1236C>T 2677G>T/A 3435C>T	-	No significant association between any of the three SNPs and either PFS or OS or any other subset for residual disease or treatment groups. Only a marginal inverse association between 1236C>T and OS in patients with nil residual disease treated with any chemotherapy (HR=0.88, 95% CI 0.77-1.01; P = 0.07)	[129]
ABCC2	European 1,303	Paclitaxel containing	Breast	4544G>A	-	Decreased risk of sensory neuropathy (grade≥2) OR = 0.63; 95% CI, 0.42–0.93 (P=0.02)	[112]
ABCC2	White patients 92	Docetaxel containing	Breast, Prostate, Lung, Head/neck	–1019A>G –24C>T 1249G>A –34T>C 3972C>T 4544G>A	Not significantly associated with docetaxel CL	-	[80]
	Scandinavian Caucasian 93	Paclitaxel + carboplatin	Ovarian, Fallopian tube, Peritoneal	1249G>A 3563T>A 4544G>A	Not significantly associated with paclitaxel CL	-	[117]

	European 914	Paclitaxel or Docetaxel + carboplatin	Ovarian	1249G>A –24C>T 148A>G	-	Not significantly associated with toxicity or response (PFS, CA-125 response, clinical/radiological response)	[136]
	Korean 92	Docetaxel containing	Lung, Stomach, Head and neck, Esophagus	rs12762549	-	Significantly associated with leukopenia/neutropenia (P=0.028) No association with tumor response.	[122]
	Japanese 253	Docetaxel containing	n.a.	rs12762549	-	Increased risk of neutropenia grade 3/4 (OR 4.83; 95% CI 1.44– 16.26; P=0.012) Combined analysis indicate association of rs12762549 in *ABCC2* (P=0.00022) and 2677G>T/A in *SLCO1B3* (P=0.00017) with neutropenia.	[81]
ABCC2	Korean 218	Docetaxel+ adriamycin+ cyclophosphamide	Breast	–24C>T 1249G>A 3972C>T	-	Not significantly associated with toxicity	[124]
SLCO1B3	White patients 92	Docetaxel containing	Breast, Prostate, Lung, Head/neck	334T>G 439A>G 699G>A 767G>C 1559A>C 1679T>C	Not significantly associated with docetaxel CL.	-	[80]
	Spanish 118	Paclitaxel containing	Breast	334T>G 699G>A rs4149056	-	Not significantly associated with risk of neurotoxicity	[116]
	European 322	Paclitaxel + carboplatin	Ovarian	334T>G IVS12-5676A>G	-	Not significantly associated with toxicity	[121]

	Korean 218	Docetaxel+ adriamycin+ cyclophosphamide	Breast	334T>G 699G>A	-	Not significantly associated with toxicity	[124]
	Japanese 253	Docetaxel containing	n.a.	IVS12-5676A>G	-	Increased risk of neutropenia grade 3/4 (OR 5.44; 95% CI 2.22– 13.34; P=0.013) Combined analysis indicate association of rs12762549 in *ABCC2* (P=0.00022) and 2677G>T/A in *SLCO1B3* (P=0.00017) with neutropenia.	[81]
	Asian 54	Docetaxel	Nasopharyngeal	334T>G 439A>G 699G>A 767G>C 1559A>C 1564G>T 1679T>C IVS12-5676A>G	The homozygous IVS12-5676 variant GG was associated with increase of docetaxel exposure (higher AUC P=0.026 and lower CL P=0.036).	Not significantly associated with toxicity	[128]
SLCO1B3	Korean 92	Docetaxel containing	Lung, Stomach, Head and neck, Esophagus	IVS12-5676A>G	-	Increased risk of toxicity (OR: 9.44; 95% CI 1.39-64.01; P=0.022).	[122]
CYP2C8	Spanish 113	Paclitaxel containing Docetaxel containing	Breast	HapC *35707G>A* **4* **3* (rs10509681) **3(*rs11572080)	-	HapC, rs11572080 and rs1934951 significantly associated with paclitaxel toxicity: anemia grade ≥2 (P=0.003; P=0.016; P= 0.008, respectively) in paclitaxel treated patients.	[130]
	European 1,303	Paclitaxel containing	Breast	**3 (*rs11572080) **4*	- -	**3 not* associated with sensory neuropathy **4* increase of taxane-related sensory neuropathy (grade ≥2) OR=1.48; 95% CI:	[112]

						1.02–2.15 (P = 0.04)	
	Caucasian (n=79) Afro-American (n=27) Other (n= 5)	Paclitaxel containing	Breast	**3* (rs11572080) **3* (rs10509681)	-	*CYP2C8*3* was significantly associated with clinical complete response (OR=3.92; 95 % CI 1.46–10.48; P=0.046).	[113]
	European-American (n=209) Afro-American (n=107) Mixed-race (n=411)	Paclitaxel containing	Breast	**3* (rs10509681)	-	*3 was not associated with risk of neuropathy in European-American (HR per allele = 1.93; 95% CI: 1.05–3.55 P=0.006), African-American (HR per allele= 3.30; 95% CI: 1.04–10.45, P=0.043) or Mixed-race (HR per allele= 1.98; 95% CI: 1.25–3.13, P= 0.004)	[131]
CYP2C8	Spanish 118	Paclitaxel containing	Breast	**3* (rs11572080) HapC **4* **1B*	-	*CYP2C8*3* was significantly associated with increased risk of neurotoxicity (HR per allele= 1.72; 95% CI: 1.05–2.82; and P=0.032) Haplotype C was significantly associated with reduced risk of neurotoxicity (HR per allele= 0.55; 95% CI: 0.34-0.89; and P=0.014) *CYP2C8*4* and *CYP2C8*1B* not associated with neurotoxicity	[116]
	Scandinavian Caucasian 93	Paclitaxel + carboplatin	Ovarian, Fallopian tube, Peritoneal	**3* (rs10509681) **4*	**3* was associated with 11% lower CL of unbound paclitaxel (P= 0.03) *4 was associated with an 18% lower clearance of unbound paclitaxel (P= 0.04)	-	[117]
	Caucasian 38	Paclitaxel + carboplatin	Ovarian	**3* (rs10509681) **1B* **1C*	**3* was associated with decrease of paclitaxel clearance within *ABCB1* 2677G/T patients (median **1/*3*:	*CYP2C8*1/*3* had a higher risk of motor neuropathy (P=0.034)	[118]

				**4*	14.7 l/hr *vs 1*/*1*: 22.8 l/hr, P=0.032) **1B, *1C* and **4* showed no association with paclitaxel clearance	**3* allele also was associated with hematological toxicity, especially leucopenia (P= 0.067) and thrombocytopenia (P = 0.02)	
CYP2C8	Caucasian 97	Paclitaxel or Paclitaxel + carboplatin	Breast, Bladder, Esophagus, Lung, Ovarian	**2* **3* **4*	Not associated with paclitaxel CL	-	[132]
	Scandinavian 119	Paclitaxel + carboplatin	Ovarian	*3 (rs10509681)	-	*CYP2C8*3* was not associated with neutropenia, sensory neuropathy or response (OS).	[115]
CYP3A4/5	Spanish 118	Paclitaxel containing	Breast	*CYP3A5*3* *CYP3A4*1B*	-	*CYP3A5*3* was associated with reduced risk of neurotoxicity (HR per allele= 0.51; 95% CI 0.30-0.86; and P=0.012) *CYP3A4*1B* was not associated with neurotoxicity	[116]
	European Validation cohort n=239 Exploratory cohort n=261	Paclitaxel containing	Ovarian, Breast, Esophageal, Others	*CYP3A4*22* (rs35599367)	*CYP3A4**22 was not associated with paclitaxel pharmacokinetic parameters (CL, AUC, C_{max}, or T>0.05)	*22 was associated with neurotoxicity in female carriers in the exploratory and validation cohort (P = 0.043 and P=0.036) *22 increased risk of developing grade 3 neurotoxicity in female and male carriers in validation cohort (OR = 19.1; 95% CI 3.3–110; P = 0.001)	[133]
	American 93	Paclitaxel + carboplatin + 5FU	Esophageal	*CYP3A4*1B*	-	*CYP3A4*1B* was not associated with pathologic complete response, time to progression/recurrence, OS, or toxicity.	[134]

	Caucasian (n=79) Afro-American (n=27) Other (n= 5)	Paclitaxel containing	Breast	*CYP3A4*1B* *CYP3A5*3C*	-	*CYP3A4*1B* and *CYP3A5*3C* were not associated with paclitaxel response or toxicity.	[113]
CYP3A4/5	Korean 216	Docetaxel + doxorubicin	Breast	*CYP3A5*3*	*CYP3A5*1/*1* and **1/*3* carriers had higher docetaxel AUC than **3/*3* carriers (P= 0.024)	*CYP3A5*3* was not associated with survival or toxicity.	[126]
	European 322	Paclitaxel + carboplatin	Ovarian	*CYP3A4*3*	-	**3* was associated with increased risk of thrombocytopenia (OR = 4.99, 95% CI = 1.22-20.31, P=0.025)	[121]
	Korean 92	Docetaxel containing	Lung, Stomach, Head and neck, Esophagus	*CYP3A4*1B* *CYP3A4*18* *CYP3A4*3* *CYP3A5*2* *CYP3A5*3*	-	Not significantly associated with toxicity or response	[122]
	Taiwanese 59	Docetaxel + epirubicin + cyclophosphamide	Breast	*CYP3A4*4* *CYP3A4*5* *CYP3A4*18* *CYP3A5*3*	-	CYP3A5 **1/*3* was related with increased risk of neutropenia (RR= 3.29, 95% CI 1.03-10.50; P<0.05) and febrile neutropenia (RR = 7.17; 95% CI 1.10-53.55; P<0.05)	[123]
	Korean 218	Docetaxel+ adriamycin+ cyclophosphamide	Breast	*CYP3A5*3*	-	Not significantly associated with toxicity	[124]
	European 58	Docetaxel	Breast, Prostate, Lung, other	*CYP3A4*1B* *CYP3A5*3*	*In CYP3A4*1A/*1B* docetaxel CL was significantly higher and AUC was lower than in **1A/*1A* (55.2 ± 13.5 L/h *vs* 37.3 ± 11.7 L/h [P=0.01] and 31.4 ± 6.2 [µg · h/L] *vs* 52.7 ± 18.2 [µg · h/L] [P=0.005],	Not significantly associated with neutropenia	[139]

					respectively) No significantly association of *CYP3A5*3* with docetaxel exposure.		
CYP3A4/5	Korean 92	Docetaxel containing	Lung, Stomach, Head and neck, Esophagus	*CYP3A4*1B*, *CYP3A4*18*, *CYP3A4*3* *CYP3A5*3* *CYP3A5*2*	-	Not significantly associated with hematological toxicity or tumor response (complete response and progression of disease)	[122]
	US (n=184) Japanese (n=145 + n=197)	Paclitaxel + carboplatin	Lung (Non–Small-Cell)	*CYP3A4*1B* *CYP3A5*3C*	-	CYP3A4*1B was associated with reduced PFS (HR=0.36; 95% CI, 0.14-0.94; P=0.04), but not with OS or with toxicity. CYP3A5*3C was not associated with response (PFS and OS) or toxicity.	[125]
	Italian 152	Docetaxel and paclitaxel based	Breast	*CYP3A4*1B* *CYP3A5*3*	-	Not significantly associated with toxicity	[114]
	Caucasian 92	Docetaxel containing	Breast, Prostate, Lung, Head/neck	*CYP3A4*1B* *CYP3A5*3C*	The simultaneous presence of the *CYP3A4*1B* and *CYP3A5*1A* was associated with a 64% increase in docetaxel CL (P = 0.0015)	-	[80]
	Asian 54	Docetaxel	Nasopharyngeal	*CYP3A4*1B* *CYP3A5*3* *CYP3A5*6*	Not significantly associated with docetaxel exposure (AUC, CL, C_{max})	Not significantly associated with hematological toxicity	[128]
CYP1B1	Italian 95	Paclitaxel or Docetaxel	Breast	**3*	-	*3 associated with lower occurrence of hypersensitivity reactions (P<0.0001). Allele not associated to hematologic or neurologic toxicity.	[135]
CYP1B1	Caucasian (89%) Asian (8%) African-	Paclitaxel + doxorubicin + cyclophosphamide	Breast	**3*	-	Significantly associated with response, *3/*3 carriers had longer PFS than patients *1/*1 or *1/*3 (P=0.037)	[120]

	American (3%) 93						
	European 914	Paclitaxel or Docetaxel + carboplatin	Ovarian	**3*	Not significantly associated with paclitaxel CL	Not significantly associated with toxicity or response (progression-free survival, CA-125 response, clinical/radiological response)	[136]
	52	Docetaxel	Prostate	*3	Not significantly associated with docetaxel CL	Significantly associated with response, *3/*3 carriers had a poor prognosis compared with *1/*1 or *1/*3 (12.8 *vs* 30.6 months; P=0.0004)	[137]
TUBB2A	European 1,303	Paclitaxel containing	Breast	rs9501929	-	Increased risk of sensory neuropathy (grade ≥2) OR=1.80; 95% CI: 1.20–2.72 (P=0.005)	[112]
	Danish 150	Docetaxel containing	Breast	rs909964 rs909965 rs9501929 rs3734492 rs13219681	-	Not significantly associated with peripheral neuropathy.	[127]

OR: Odds ratio; HR: Hazard ratio; RR: Relative risk; n.a. not available; CL: clearance; AUC: Area under the concentration–time curve; C_{max}: Maximum concentration; T time above 0.05 mmol/L; CA-125: cancer antigen 125; PFS: Progression free survival; OS: Overall survival

The impact of *ABCB1* variant alleles on docetaxel pharmacokinetic have been studied as well [79,80,115,118,126,128,139]. *ABCB1* polymorphisms and systemic exposure to taxanes, evaluated as Area under the curve (AUC), clearance (CL) or Maximum concentration (C_{max}), have been associated. Bosch *et al*. [79] demonstrated a 25% decrease on docetaxel CL in 1236TT patients (P=0.0038). Furthermore, the authors recommended a 25% reduction on docetaxel dose in such patients. Similarly, Kim *et al*. [126] found higher docetaxel AUC in 3435TT patients (P=0.031) and in accordance to the higher drug exposure, there was also an improvement on response (OS) and increase of toxicity (neutropenia). In addition, Green *et al*. [118] found higher paclitaxel CL for 2677GA in comparison to wild type GG (P=0.036) and variant TT (P=0.048). Other four studies found no association between docetaxel or paclitaxel exposure and ABCB1 polymorphisms [79,80,115,128,139]. In contrast, Chew *et al*. [128] found an increase on hematological toxicity within 2677G>TA variant carries (P=0.006), as a similar trend with 3435C>T (P=0.066), but with no significant association with docetaxel exposure (AUC, CL or C_{max}). Thus, suggesting that these *ABCB1* polymorphisms may influence the susceptibility to toxicity independent of an effect on clearance.

In addition to *ABCB1, ABCC2* has also been associated to taxane therapy. Kiyotani *et al*. [81] found a significant association of the *ABCC2* rs12762549 and *SLCO1B3* rs11045585 mutations with leukopenia/neutropenia induced by docetaxel in Japanese (P=0.00022 and P=0.00017, respectively). The authors proposed a classification system based on patient's genotypes for these two single nucleotide polymorphisms, patients with a score 0 were at minor risk of leukopenia/neutropenia induced by docetaxel as compared to those with a score 1 or 2 (P=0.0000057). By means of the proposed prediction approach, the authors accurately classified 75.7% of non-leukopenia/neutropenia and 62.9% of severe neutropenia/leukopenia into the respective category, indicating that the polymorphisms in *SLCO1B3* and *ABCC2* genes may successfully predict the probability of docetaxel-induced neutropenia/leukopenia [81]. Differently, Abraham *et al*. [112] found in a European population with breast cancer (N=1,303) a positive effect of ABCC2 4544G>A on paclitaxel therapy, reducing the risk to develop sensory neuropathy (P=0.02). In addition, other studies failed to find a significant association between *ABCC2* polymorphisms and toxicity [124,136], response [122,136] or docetaxel/paclitaxel exposure [80,115].

Polymorphisms in genes encoding enzymes from the cytochrome P450 system, such as CYP3A4/5, CYP2C8, and CYP1B, have also been associated to taxanes efficacy and

toxicity. There are several alleles described for the *CYP3A4* gene, among which are the alleles **1B, * 2, *3, *4, *5, *6, *7, *8, *10, *11, *12, *14, *15A/B, *16, *17, *18, *19, *22* [140,141]. According to Tran *et al*. [139], the *1B allele located in the regulatory region (-392A-G) of the *CYP3A4* gene has been associated with increased enzyme expression, but with frequency of <1%, a factor that limits its clinical relevance. Recently, the allele **22* present in 5-7% of Caucasians has been shown to be the main responsible for the decrease in the expression and enzymatic activity of CYP3A4 [141,142]. This allele results from a C>T substitution at position 15389 in intron 6 (rs35599367) of CYP3A4 [142]. De Graan *et al*. [133] related the presence of the *CYP3A4*22* allele with the occurrence of neurotoxicity grade 3 in patients treated with paclitaxel (OR=19.1; 95% CI 3.3–110; P=0.001), but the variant allele was not significantly associated with paclitaxel exposure thus indicating that the variant allele **22*, may not play a significant role on paclitaxel pharmacokinetic. To date there are no reports of the association between the present of allele *22 and docetaxel pharmacokinetics and treatment outcomes, which support the development of additional studies to evidence this relation. The CYP3A5 gene has more than 20 allelic variations, among which are **3, *6, *7* and **9* alleles. The most studied is the **3* allele, that leads to a marked decrease in CYP3A5 enzyme activity and, consequently, to higher concentrations of drugs that are preferentially metabolized by this enzyme. The frequency of this allele in the Caucasian population is 85 to 95% [140,143]. Gandara *et al*. [125] observed lower survival rates in patients carrying *CYP3A5*3* allele treated with paclitaxel. This mutation has also been related to adverse events, in a study conducted by Gréen *et al*. [144], patients with *CYP3A5 *1/*3* genotype had marginal significantly lower (P=0.07) nadir values of white cell counts than homozygous carriers of the allele **3*. The association of *CYP3A5*3* polymorphism with hematological toxicity in patients treated with docetaxel has also been was reported [124,123]. In the study conducted by Tsai *et al*. [123] patients with the *CYP3A5 *1/*3* genotype had higher frequency of side effects, including pleural effusion, fever and febrile neutropenia, in comparison to carriers of the homozygous genotype. In addition to these reports, similarly to transporters genes, several studies found no significant association between CYP3A4/5 polymorphisms and toxicity, response [114,122,124,126,128,134,145] or taxane exposure [128,136,146].

The *CYP2C8* gene, expressed mainly in the liver, is located at chromosome 10q24 [147]. The influence of its polymorphisms on paclitaxel metabolism was studied, with controversial results [132,148,118,113,116,149]. Besides the wild-type **1*, two different

allelic variants were reported, the *CYP2C8*2* and *CYP2C8*3* [148,118]. The *CYP2C8*3* variant, present in 2% of Afro- Americans and 13% of Caucasians, was associated with a significant reduction of paclitaxel metabolism, leading to 15% of the *CYP2C8*1* activity [148]. Differently, Henningsson *et al.* [132] found no correlation between the *CYP2C8*3* variant and paclitaxel clearance in 97 patients. Hertz *et al.* [113] reported that breast cancer patients with the *CYP2C8*3* variant were more probable to achieve clinical complete response to paclitaxel treatment, than patients carrying the wild-type allele. However, the authors observed a tendency of increased risk of severe neuropathy in *CYP2C8*3* carriers (22 % versus 8 %; OR= 3.13, P=0.075) The association between the *CYP2C8*3* allele and paclitaxel-induced severe neurotoxicity was reported by others as well [116,149]. Additionally, Yasar *et al.* [150] indicated that the *CYP2C8*3* allele variant seems to be associated to the *CYP2C9*2*, since 96% of the *CYP2C8*3* carriers had the CYP2C9*2 variant as well. The *CYP2C8*2* variant is present in 18% of the African-Americans and has been related to a decrease in the paclitaxel clearance in comparison to the wild-type allele *CYP2C8*1* [148].

In a pilot study Rizzo *et al*. [135] demonstrated a reduced rate of hypersensitivity reactions to taxanes in the presence of the *CYP1B1*3* (4326 C>G) polymorphism. Similarly, Marsh *et al.* [120] reported positive outcomes in the presence of the *CYP1B1*3* variant during paclitaxel treatment, with a significantly longer progression-free survival in homozygous patients. In contrast, Sissung *et al.* [146], showed that prostate cancer patients homozygous for *CYP1B1*3* had a poor prognosis after docetaxel (P=0.0004) treatment in comparison to carriers of the wild-type *CYP1B1*1* allele. In this study, the docetaxel clearance was not influenced (P=0.39) by the *CYP1B1* genotype, demonstrating that the relation between *CYP1B1*3* polymorphism and clinical response is unlikely to be associated to drug metabolism.

In addition to the polymorphisms associated to taxanes pharmacokinetic, there has also been reports on mutations modifying these drugs pharmacodynamics (Table 1). In a study conducted by Leandro-Garcia *et al*. [151], the 101T>C, 112A>G and 157A>G polymorphisms of the TUBB2A gene were evaluated to verify whether polymorphisms could affect β-tubulin mRNA expression levels and influence the response to treatment with the drug. The results obtained showed that the patients carrying variants (101T>C, 112A>G) may have a risk of developing paclitaxel-induced neurotoxicity. Finally, DNA damage repair genes (*ERCC1-2*) may contribute to the cellular activity and toxicity of taxanes [130].

A significant association was found between mucositis (P≤0.01) and neuropathy (P≤0.01) and *ERCC1* Gln504Lys (rs3212986) polymorphism within 70 patients treated with docetaxel and 43 with paclitaxel. [130]. Similarly, Kim *et al.* [152] found a significant association between the *ERCC1* Gln504Lys polymorphism and the incidence of grade3/4 neurological toxicity in ovarian cancer patients after taxane and platinum chemotherapy (25.0% GT/TT versus 8.5% GG; P = 0.019).

Although the link between taxane pharmacogenetics and treatment outcomes have already been recognized, to date there still is a lack of consistent data for the introduction of genotyping tests in routine clinical setting. The impact of polymorphisms on a number of genes encoding transport and metabolism proteins on taxane therapy outcomes remain unclear, being sample size a limiting factor in many of the previously reported studies. Many studies have had inadequate statistical power to make a meaningful comment on the potential effect of the variants under investigation. Thus, it is not surprising that genotypes with a low frequency are unlikely to yield a statistically significant association with clinical outcomes. To our knowledge, there is no report of prospective studies of taxane dose-adjustment guided by genotypes of transporters or metabolism enzymes. Moreover, it is important to emphasize that patient's comorbidities in addition to drug interactions, as the concomitant use of P450 enzymes inducers or inhibitors, would increase or decrease drug exposure and may also affect chemotherapy outcomes. In this aspect, genotyping evaluation itself cannot fully predict exposure to taxanes. However, the use of *in vivo* probe drugs as midazolam for CYP3A [153] have been successfully used in means to provide a phenotypic evaluation of the protein.

6. DOSE INDIVIDUALIZATION STUDIES

Based on the association of prolonged survival and plasma concentrations of paclitaxel of 0.1 μM for more than 15 h presented for Huizing *et al*. [96], de Jonge *et al*. [53] evaluated the feasibility of dose individualization, used Baysean pharmacometric approaches, to reach the same paclitaxel plasmatic concentrations in advanced NSCLC patients. Patients were treated with paclitaxel, administered as 3 h intravenous infusions, together with carboplatin, for up to six courses, every 3 weeks. Paclitaxel was administered in a fixed dose of 175 mg/m^2 in the first course. In subsequent courses, paclitaxel doses were individualized to in order to obtain the desired plasma concentrations (0.1 μM for more than 15 h after the beginning of the infusion). Authors also evaluated the relationship between the occurrence of

hematological toxicities with the time in which the plasma paclitaxel concentration was higher than 0.1 µM. The study included 25 patients and for those with an increased individualized dose did not presented an increased frequency of toxic events when compared with the patient group receiving unchanged doses.

In 2012, Joerger *et al*. [59] developed a dosing algorithm with the purpose of reducing severe neutropenia in patients receiving paclitaxel, targeting an individual paclitaxel Tc > 0.05 µM between 26 and 31 hours, based on previous data [88,91,93]. They compared the conventional dose of paclitaxel 200 mg/m^2 every 3 weeks (concentration-time data of patients obtained from two previous clinical trials developed by the same group [58,91]) with the personalized dose, and observed a reduction of 15 % to 7% in grade 4 neutropenia in the first cycle, and a further reduction to 4 % in the second cycle of chemotherapy. Data simulations using the PK/PD model developed by the authors and the proposed dosing algorithm resulted in first-cycle doses in the range of 150 to 185 mg/m^2 for women and from 165 to 200 mg/m^2 for men. Dose individualization for the subsequent cycles ranged from 40% reductions to 30% increases, with an overall median paclitaxel dose of 167 mg/m^2, with a widely-dispersed range of 76 to 311 mg/m^2. Kraff *et al*. [97] developed a dosing algorithm for weekly schemes of paclitaxel, based on retrospective data, targeting a Tc > 0.05 µM of 10 to 14 h. Using the dosing algorithm proposed by the authors, 70% of the simulated patients reached the Tc > 0.05 µM target range in the second cycle and 97% of the patients in the fourth treatment cycle. Bayesian simulations using the proposed dosing algorithm showed a reduction in average paclitaxel-related chemotherapy induced peripheral neuropathy grade 2 from 9.6% with conventional dosing to 4.4% with pharmacokinetically based dosing. The dosing adjustment algorithms proposed by these authors are summarized in table 2.

Table 2. Proposed pharmacokinetically-based dose adjustment algorithms for paclitaxel.

Weekly scheme [97]					
$T_{C>0,05\ \mu M}$	Dose Adjustment				
≥18	-25%				
14 to <18	-15%				
10 to <14	0%				
6 to <10	+15%				
<6	+25%				
Three-weekly scheme [59,142]					
Neutropenia 0-2 in previous cycle		Neutropenia 3 in previous cycle		Neutropenia 4 in previous cycle	
$T_{C>0,05\ \mu M}$	Dose Adjustment	$T_{C>0,05\ \mu M}$	Dose Adjustment	$T_{C>0,05\ \mu M}$	Dose Adjustment
>50	-30%	>50	-30%	>50	-40%
41-50	-25%	41-50	-25%	41-50	-30%
31-41	-20%	31-41	-20%	31-41	-25%
26-31	0%	<31	0%	<31	-20%
20-26	+10%				
10-20	+20%				
<10	+30%				

More recently, Joerger *et al.* [154] studied paclitaxel dose individualization in a group of patients with NSCLC, aiming to achieve drug exposures within the previously targets. In this study, 365 patients scheduled to receive paclitaxel, combined with carboplatin AUC 6 or cisplatin 80 mg/m^2, as first line treatment for advanced NSCLC were randomized to receive standard paclitaxel doses at 200 mg/m^2 (arm A) or pharmacokinetically-adjusted doses (arm B). In the dose individualization arm, the initial dose of paclitaxel was selected according to age, sex and body surface area, and the subsequent doses determined by previous-cycle paclitaxel exposure, calculated as the time plasma concentrations were higher than 0.05 µm. The occurrence of severe neutropenia (grade 4) was similar in both arms (19% versus 16%; P = 0.10). However, the occurrence of neuropathy grade ≥2 (38% versus 23%, P < 0.001) and grade ≥3 (9% versus 2%, P < 0.001) was significantly reduced in the dose individualization arm. The median paclitaxel dose was significantly higher in the dose individualization arm (199 versus 150 mg/m^2, P < 0.001). Response rate was similar in both arms of the study (31% versus 27%, P=0.405), as well the adjusted median progression-free survival (5.5 versus 4.9 months) and overall survival (10.1 versus 9.5 months. According to authors, the employed strategy of paclitaxel dose individualization did not result in reduced occurrence of severe neutropenia, but was related to a lower incidence of paclitaxel-associated neuropathy, potentially being useful to reduce the toxic risk of paclitaxel chemotherapy in advanced

NSCLC patients. These studies support the development of further studies in dose individualization for paclitaxel and, eventually, its clinical application.

The proper calculation of Tc > 0.05 μM for paclitaxel dose individualization could be challenging, once requires specialized software. To overcome this difficulty, Kraff *et al.* developed a user-friendly Excel® tool for the estimation of Tc > 0.05 μM, with accuracy and precision similar to the reference software NOMEM [97]. This tool is available from the authors under request.

According to an early report of the Therapeutic Drug Monitoring Group of the French Society of Pharmacology and Therapeutics, published before the larger studies from 2010 onwards, considered the levels of evidence for paclitaxel TDM between recommended and potentially useful, the latter being exceed due to the strong PK interindividual variability and the strong correlation between exposure, toxicity and PK parameters [155]. Considering the current available evidence on the relation between exposure to paclitaxel and occurrence of toxicity, the existence of a well-established PK exposure parameter and the availability of easy to use computational tools of its estimation, as well as readily available laboratory assays for measuring drug levels, pharmacokinetically dose individualization of paclitaxel can be considered as a valid and routine-feasible approach to increase safety of cancer patients. PK-guided dosing of paclitaxel may be of particular value in clinical situations dealing with patient considered of higher risk of severe toxicity, such as frail individuals, extremes of body weight, liver dysfunction and increased risk of paclitaxel-induced neuropathy. However, clinical studies to validate the PK target, simulated by Kraff *et al.* [97], with the more recent weekly administration schedules are needed.

In the case of docetaxel, the clinical experience with dose individualization is more limited. Engels *et al.* [108] performed a small dose individualization study with docetaxel, where 15 patients were treated for at least 2 chemotherapy cycles with standard docetaxel doses, based on body surface area, and 15 with at least 1 cycle of pharmacokinetically individualized doses. Authors used a limited sampling strategy in combination with a validated population pharmacokinetic model and Bayesian analysis in order to estimate docetaxel AUCs (target AUCs of 2.5; 3.7 or 4.9 mg/L.h for doses of 50, 75 and 100 mg/m^2, respectively). The individualization strategy allowed a reduction in AUC interindividual variability (SD of ln AUC) by 35% after one pharmacokinetically guided chemotherapy cycle and of 39% (P=0.055) when all cycles were evaluated. Individualized dose selection also reduced the interindividual variability of the decrease in total leukocyte and neutrophil counts

by 50%. However, there were no significant differences in the incidence of toxicity between both doses selection groups. Ma *et al*. [156] performed a randomized clinical trial of docetaxel dose individualization including 99 Asian advanced NSCLC patients. Patients were randomly assigned to receive single agent 3-weekly docetaxel chemotherapy for up to 6 cycles, either at a fixed standard dose of 75 mg/m^2 or a PK-guided dose, with the same starting dose and further adjustments to obtain an AUC of 2.5 to 3.7 mg/L.h. There was a 25.4% lower incidence of grade ≥3 neutropenia incidence in PK-guided dose arm (47.5% vs. 72.9%, p = 0.002). Overall response rate showed no differences between standard and PK-adjusted doses (20.0% vs. 17.5%, p = 0.769), so is progression free survival (4.6 vs. 3.4 months, p = 0.567).

Considering the current knowledge, the level of evidence of TDM for docetaxel still needs to be assessed in larger, randomized, dose adjustment studies and evidence is necessary to clarify the potential benefits of dose individualization in docetaxel chemotherapy. Also, differently from paclitaxel, a consensual target for the main PK exposure-related parameter for docetaxel is still lacking.

7. CONCLUSION

The current knowledge of the pharmacology of the taxane drugs paclitaxel and docetaxel, mainly its pharmacokinetics, along with the characterization of the genetic polymorphism responsible for variations in activities of biotransformation and transport proteins, opens new opportunities for dose selection for individual patients. Although strong evidence of the predictive capability of pharmacogenetic testing to predict individual response to paclitaxel and docetaxel is still lacking, consistent scientific evidences support pharmacokinetically guided dose individualization, particularly for paclitaxel. The main pharmacokinetic marker for dose adjustment of paclitaxel is the time in which the plasma concentration is above a threshold of 0,05 µM, and dose adjustment algorithms are already available. Clinical studies with paclitaxel had demonstrated significant reduction of toxicities in pharmacokinetically dose adjustment arms, without reduction in treatment efficacy. In the case of docetaxel, further randomized dose individualization studies are needed to clarify the relation between exposure to the drug and toxicity and clinical outcomes.

Considering the relation between systemic exposure to these drug and clinical responses, *a posteriori* TDM, with measurement of drug concentrations in plasma of treated

patients, is currently the most straightforward approach for dose individualization of paclitaxel and docetaxel. Besides the strong rationale beyond the use of pharmacokinetic dose individualization for taxanes, which express both genotypic and phenotypic variations among patients, additional and larger studies are needed to increase evidence and awareness on this potentially useful tool to obtain maximum clinical benefit of paclitaxel and docetaxel in cancer treatment.

CONFLICT OF INTEREST

There is no conflict of interest to report.

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3. CAPÍTULO 2

AN OPTIMIZED HIGH-PERFORMANCE HPLC-PDA METHOD FOR THE CLINICAL APPLICATION OF PACLITAXEL THERAPEUTIC DRUG MONITORING

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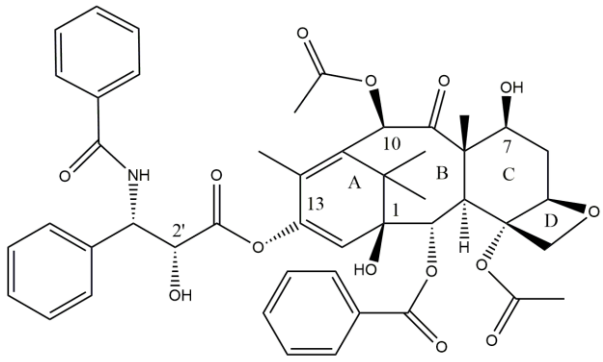
ABSTRACT

Objectives: The aim of this study was to develop a method for the determination of paclitaxel in human plasma by high-performance liquid chromatographic with photodiode array detector, suitable for the clinical implementation of therapeutic drug monitoring of this chemotherapeutic drug. **Methods:** Liquid-liquid extraction was performed to extract paclitaxel from 500 µL of plasma samples with a mixture of acetonitrile and 1-chlorobutane. Separation was performed in a Hypersil Gold C18 column (150 x 4.6 mm, 5 µm), eluted with a mixture of triethylammonium phosphate buffer 0.5 mM pH 3,4 and acetonitrile (52:48, v/v). The wavelength monitored was 227 nm. **Results:** Retention time was 6.99 min for paclitaxel and 6.5 min for the internal standard (docetaxel 2 µg mL^{-1}), with total run time of 8 min. The method was linear from 10 to 500 ng mL^{-1}. Accuracy was 97.06-110.18%, intra-assay precision was 1.29 to 5.59%, and inter-assay precision was 3.34 to 9.27%. Processed samples are stable up to 12 h in the autosampler and for three freeze and thaw cycles. Paclitaxel concentrations obtained from 18 cancer patients were all within the linear of the assay. **Conclusions:** The method for determination of paclitaxel using high-performance liquid chromatography was developed, and presented suitable characteristics for the use in therapeutic monitoring of this antineoplastic drug.

Keywords: paclitaxel, antineoplastic drug, high-performance liquid chromatography, therapeutic drug monitoring.

1. INTRODUCTION

Paclitaxel (PTX, figure 1) is an antineoplastic drug widely used for the treatment of solid tumors, and belongs to the taxane group of cytotoxic drugs (1). The mechanism of action is based on selective tubulin polymerization that leads to apoptosis (2). PTX pharmacokinetics is nonlinear, and could be affected by interaction with the solvent Cremophor EL (CrEL), which affects the distribution of PTX by entrapment in micelles, leading to a reduction of its plasma clearance (3,4). Additionally, PTX interaction with CrEL also affects the reproducibility of PTX analytical measurements by chromatographic methods (5). Metabolic clearance of PTX is also affected by genetic polymorphisms, mainly in the *CYP2C8* gene, which also contributes to the interindividual variability in its pharmacokinetics (6).



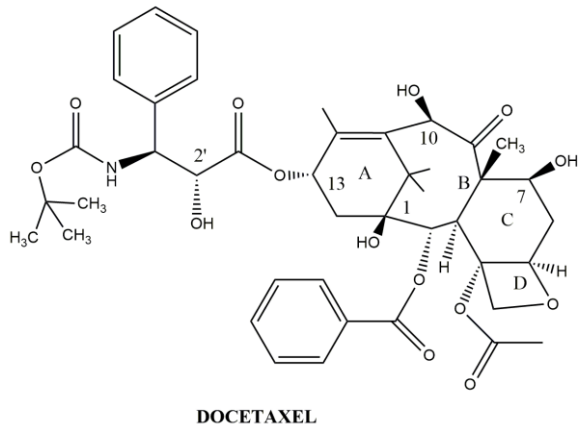


Figure 1. Chemical structures of paclitaxel and docetaxel

The clinical use of PTX can be associated with potentially severe hematopoietic and neurologic toxicities, even at low doses (1,7), and the variable tolerability to these effects was related to interindividual pharmacokinetics differences, especially in clearance (8). The

clinical response to PCTX is strongly associated with the pharmacokinetic marker denominated as time with plasma concentration above the threshold of 0.05 μM (Tc > 0.05) (7,9,10). The current proposed therapeutic targets for Tc > 0.05 are 26-31 hours for three-weekly chemotherapy regimens (9) and 10-14 hours for weekly regimens (10). Based on these observations, algorithms for dose individualization had been proposed based on the individual exposure to the drug (11). Therefore, therapeutic drug monitoring (TDM) could be a useful tool to optimize PTX chemotherapy, leading to increased efficacy and safety of this important drug.

The use of high-performance liquid chromatography (HPLC) coupled to spectrophotometric detection is an affordable alternative for the implementation of paclitaxel TDM. Several HPLC methods were already described for the determination of PTX in biological samples. However, most of the described methods involved laborious sample extractions (12,13,14), large specimen amounts (13,15), or long chromatographic runs (16).

In this manuscript, we describe a simple, fast, selective, sensitive and cost-effective HPLC–PDA method for the quantification of PTX, suitable for clinical use on TDM.

2. MATERIALS AND METHODS

2.1 Reagents and standards

PTX and docetaxel (internal standard) (Figure 1) were purchased from Toronto Research Chemical (Toronto, Canada). Acetonitrile, trietilamonium phosphate buffer 0.5 M, ammonium acetate and Cremophor EL were obtain from Sigma-Aldrich (St. Louis, USA). The solvent 1-chlorobutane was obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained through an Elga Purelab Ultra® apparatus from Elga Labwater (High Wycombe, UK).

2.2 Preparation of solutions and solvents

Stock (1000 μg mL^{-1}), intermediate (100 μg mL^{-1}) and working (0.20, 0.50, 1.00, 2.00, 5.00, 10.00 μg mL^{-1}) solutions of PTX were prepared in methanol. The internal standard working solution was prepared by dilution of an intermediate solution of docetaxel (100 μg mL^{-1}) with methanol in order to obtain the concentration of 2 μg mL^{-1}. Ammonium acetate

buffer pH 5.0 was prepared by weighting 0.1925 g of the ammonium acetate buffer and diluted in 0.25 mL of ultra- purified water. The drug extraction solvent was a mixture of 1-chlorobutane and acetonitrile (4:1, v/v). The chromatographic mobile phase was a mixture of trietilamonium phosphate buffer 0.5 M pH 3.4 and acetonitrile (52:48, v/v).

2.3 Sample preparation

Liquid-liquid extraction was performed by adding 500 µL of plasma, 50 µL of the internal standard solution, 500 µL of ammonium acetate buffer pH 5.0 and 3.5 mL of extraction solvent to 5 mL polypropylene tubes. The mixture was homogenized for 10 minutes at 15 rpm, followed by 10 min of centrifugation at 3,000 rpm. An aliquot of 3.0 mL of the supernatant was transferred to clean 5 mL polypropylene tubes and dried at 45 °C with a vacuum centrifuge (Concentrator Plus, Eppendorf). The dried extract was recovered with 100 µL of the chromatographic mobile phase and centrifuged for 10 min at 3,000 rpm. The resulting supernatant was transferred to a vial, and 50 µL was injected into the HPLC-PDA system.

2.4 Equipment and chromatographic conditions

The high-performance liquid chromatographic system was an Acquity®, with a photodiode array detector (PDA) (Waters, Milford, USA). Separation was performed in a Hypersil Gold® C18 (150 x 4.6 mm, 5 µm), from Thermo Scientific (San Jose, USA). The column temperature during all analyses was set at 30 °C. Elution was performed at isocratic mode, with a mobile phase flow rate of 1 mL min^{-1}. Total run time was 8 min. Chromatograms were acquired at the wavelength of 227 nm.

2.5 Selectivity

For testing the selectivity of the method, 6 different human sources of blank plasma samples were extracted as described above to check the possible interfering peaks in the retention times of PTX and the internal standard.

2.6 Linearity

The calibration curve was prepared at 6 levels, with quintuplicate analysis in each level. Calibrators were prepared by adding 25 µL of the PCT working solutions to 475 µL of blank plasma to obtain calibration samples at the concentrations of 10, 25, 50, 100, 250 and 500 ng mL^{-1}, and were processed as described above. Calibration curves were constructed by calculating the ratios of the peak area of the analyte to the peak area of the internal standard and relating these ratios with nominal concentrations of the calibration samples. Homoscedasticity of calibration data was evaluated with *F*-test at the confidence level of 95%. Curves were fitted by least-squares linear regression using several weighting factors, and calibration models were evaluated by their correlation coefficients (*r*) and cumulative percentage relative error (∑%RE) (17).

2.7 Precision and accuracy

Quality control (QC) samples were prepared by adding 25 µL of the PTX working solutions to 475 µL of blank plasma to obtain PTX concentrations of 20 (quality control low, QCL), 75 (quality control medium, QCM), and 300 ng mL^{-1} (quality control high, QCH). Quality controls were processed as described above in triplicate for 5 days. Within-assay precision and between-day precision were calculated by one-way ANOVA with the grouping variable "day" and were expressed as CV%. Accuracy was defined as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criteria for accuracy were mean values within ±15% of the theoretical value, and a maximum CV of 15% was accepted for precision (18).

2.8 Lower limit of quantification

The lower limit of quantification (LLQ) were performed by including a quality control sample at the lowest point of the calibration curve (10 ng mL^{-1}) in the precision and accuracy experiments and tested in triplicate in 5 different days. The acceptance criteria established for the LLQ was a maximum CV of 20% and accuracy within 100±20% of the nominal value (19).

2.9 Stability

The extract stability at the chromatograph's autosampler was tested at the concentration levels of QCL and QCH (n=8 each). Pooled extracted were injected into the HPLC system at time intervals of 1 h, during 12 h under the conditions of a regular analytical run. Stability of analytes was tested by regression analysis plotting absolute peak areas corresponding to each compound at each level vs. injection time. Using the obtained linear regression, the concentration after 12 h was calculated. A decrease or increase of up to 10% in the measured peak areas was considered as acceptable. The freeze-thaw stability was evaluated by preparing quality control samples containing PTX the concentration levels of QCL, QCM, and QCH. The quality controls samples were analyzed in triplicate on the same day that was prepared and after 3 freeze-thaw cycles, on days 2, 5 and 7. The samples were frozen at -20 °C for 48 h, thawed, and kept at ambient temperature for 1 h before extraction, for each cycle. The concentrations of control and stability samples were calculated from daily calibration curves and compared with the concentration at the beginning of the series. Variations of up to 15% were considered as acceptable.

2.10 Extraction efficiency

The extraction efficiency was determined by comparing the peak areas of PTX obtained at the QC samples of the precision and accuracy experiments to those obtained with solutions with concentrations corresponding to complete recovery, measured in triplicate in three different days. Extraction efficiency was expressed as percentage of the concentration of the reference samples.

2.11 Influence of Cremophor EL

The influence of CrEL on the quantification of PTX was tested by adding 0,1% of CrEL in 7 mL of blood, which was consistent with the amount of this pharmaceutic vehicle in blood up to 48 hours after the start of the infusion (20), followed by obtaining plasma by centrifugation. The resulting plasma was used to prepare quality control samples of 20 (quality control low, QCL), and 300 ng mL^{-1} (quality control high, QCH), in triplicate. Quality control samples without CrEL, at the same concentration levels and prepared as

described before, were also extracted to compare the peak areas with those where CrEL was added. Differences of up to 15% were considered as acceptable.

2.12 Method application

The method was applied to the measurement of PTX in plasma samples of 19 patients from the Oncology Service of the Hospital de Clínicas de Porto Alegre. The study was cleared by the Ethics Review Board of the hospital and all participants provided informed consent. Samples were collected once for each patient between 18 and 30 h after the beginning of the infusion, according to a limited sampling strategy previously described (9). The Tc > 0.05 μM was calculated using a Microsoft Excel tool developed by Kraff *et al.*, 2015 (11).

3. RESULTS AND DISCUSSION

3.1 Chromatography and sample preparation

Previously described HPLC-UV assays for PCT measurements had run times in the range of 15 to 30 min (12,14,15), using single wavelength UV detection. Huizing *et al.*, 1995 (13) and Kim *et al.*, 2005 (21), as in our method, used photodiode-array detectors, also allowing the use UV spectrum for the identification of PCT and IS. The optimized chromatographic condition of our assay allowed a fast turnaround time, with retention times of 6.99 min for PTX and 6.5 min for docetaxel (IS), resulting in a total run time of 8 min (Figure 2). The chromatographic conditions employing a reversed phase column and gradient elution with PDA detection proved capable of selectively separating analytes and endogenous compounds. No interfering peaks were detected in the tested blank samples both at the retention times of PTX and the IS, which was confirmed by spectrum purity evaluation with the PDA detector. The employed internal standard, docetaxel, is structurally similar to PTX, which can contribute to the assay's reproducibility.

Previous studies reported the use solid phase extraction (SPE), mainly using cyano cartridges (12,13) and liquid-liquid extraction followed by SPE (22,23) to measure PTX plasma levels of PTX by HPLC-UV. Reported sample volumes were in the range of 0.5 to 1 mL (12,13,22,23). As these approaches are laborious and time-consuming, we evaluated several solvents (methyl-tert-butyl ether, ethyl ether, 1-chlorobutane, and acetonitrile) and

solvent mixtures to obtain an optimized liquid-liquid extraction procedure. The best results were obtained with a mixture of 1-chlorobutane and acetonitrile (4:1, v/v), which provided extracts with a high degree of purity, with no interferences even at the low measured levels, presenting extraction yields between 94.6 and 97.1%. The optimized extraction procedure allowed sufficient sensibility to measured PTX concentrations up to 30 h after the infusions.

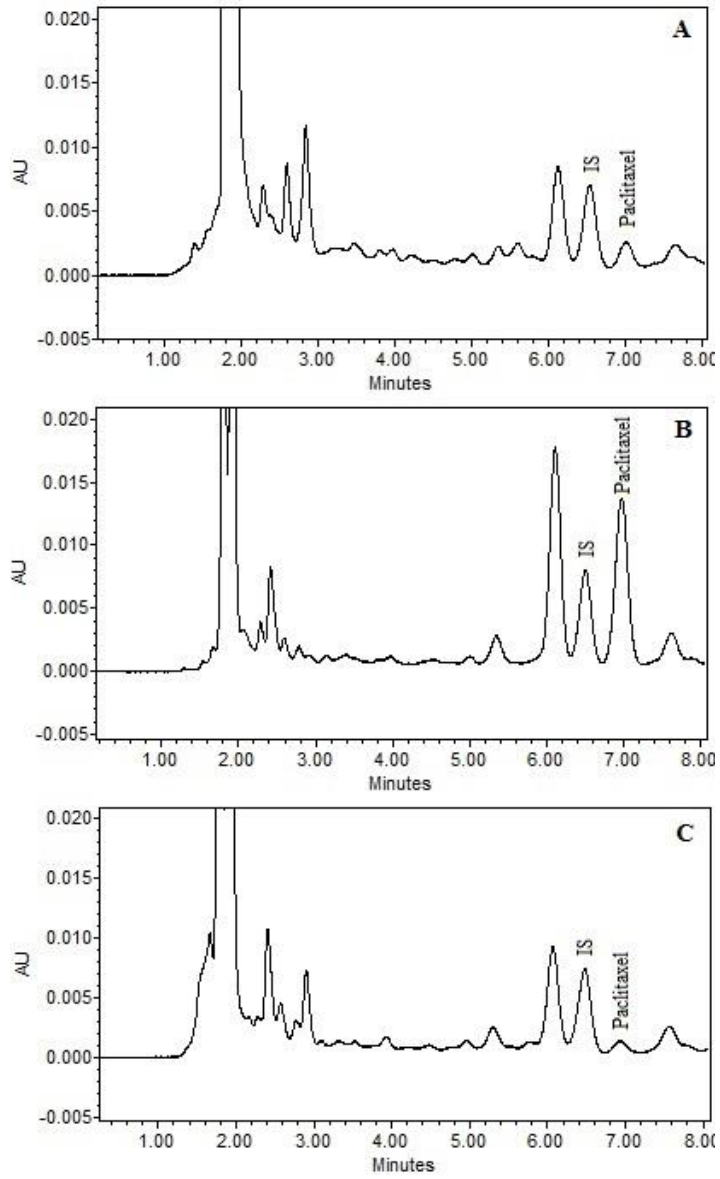


Figure 2. Chromatograms obtained with the assay (227 nm). A: Patient sample with paclitaxel at 41,87 ng mL^{-1}. B: QCH with paclitaxel at 300 ng mL^{-1}. C: QCL with Paclitaxel at 20 ng mL^{-1}.

3.2 Method validation

Linearity was demonstrated in the range of 10 to 500 ng mL^{-1}, covering plasma concentrations that are to be expected for patients receiving usual infusional regimens of PTX. With this calibration range, it was possible to quantify with a single calibration curve samples from patients corresponding to collection times between 18 and 30 hours after the start of the infusion, allowing the calculating of the pharmacokinetic marker Tc > 0.05 µM. Calibration data had significant heteroscedasticity (F = 59.984, $F_{crit\ (5,5;\ 0,95)}$ = 5.05) and the best weighting factor for PTX was $1/x^2$, with ∑%RE of 11 x 10^{-13}, which was used for the further validation studies and the routine application of the method. Coefficients of correlation of calibration curves were above 0.999, exhibiting acceptable linearity.

The method's accuracy was within the range of 97.06-110.18%, with intra-assay precision in the range of 1.29-5.59%, and inter-assay precision in the range of 3.34-9.27% (Table 1). At the lowest limit of quantification (10 ng mL^{-1}), accuracy was 99.3 %, intra-assay precision was 2.06%, and inter-assay precision was 8.38%.

Table 1. Method validation parameters: precision, accuracy, effect of CrEL on accuracy and extraction yield.

QC sample	Concentration (ng/mL^{-1})	Precision (CV%)		Accuracy (%)	Effect of CrEL on accuracy (%)	Extraction yield (%)
		Intra-assay	Inter-assay			
QCLOQ	10	2.06	8.38	99.30	-	-
QCL	20	1.29	3.34	97.06	109.72	97.17
QCM	75	1.97	4.93	103.21	-	98.79
CQH	300	5.59	9.27	110.18	108.50	94.66

QCLOQ: quality control at the limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control high, CrEL: Cremophor EL, (precision and accuracy n=45, extraction yield n=27).

There was no indication of instability of the analytes in any of the tested conditions, demonstrating the feasibility of its processing in usual laboratory conditions. The extracts maintained in the autosampler were stable with a maximum variation in peak area ratios after 12 h of 1.6% from the values at the beginning of the series. Additionally, there was no indication of instability after three freeze-thaw cycles (Table 2). The maximum concentration change after the third cycle was 3.7%, indicating that even after freezing and thawing the

sample, for at least 3 times, and up to 7 days, it was possible to find the same concentrations that in fresh samples. The concentrations of PCT were not influenced by the presence of CrEL in any of the evaluated quality control levels, showing a maximum variation of 9.72% (Table 2).

Table 2. Processed sample stability at autosampler and freeze-thaw cycles.

QC sample	Concentration (ng mL^{-1})	Benchtop stability	Freezer and thaw stability
		Concentration change after 12 h (%)	Concentration change after third cycle
QCL	20	-1.60	-3.7
CQH	300	-0.30	0.5

QCL: quality control low, QCH: quality control high, PLL: patient low level, PHL: patient high level, (processed sample stability n=24, freeze-thaw cycles n= 24).

3.3 Method application

Plasma concentrations of PTX were measured in plasma samples obtained from 18 patients, which received doses from 50 to 200 mg per square meter of body surface are, with blood collection times between 18 and 30 hours after the starting of the infusion. Measured concentrations were in the range of 11.52 to 125.1 ng mL^{-1}, all within the linear range of the assay. PTX Tc > 0.05 µM was in the range of 9.5 to 34.4 h. A large proportion of the patients (78.9%) presented Tc > 0.05 µM outside the recommended therapeutic range, being 52.6% below the minimum recommended exposure. These preliminary results support the routine application of TDM for optimizing PTX doses in cancer chemotherapy once a significant part of the patients treated with PTX presented drug exposures outside recommended ranges. This is the first report of an HPLC-DAD assay applied to human dose optimization of PTX using the Tc > 0.05 µM approach.

4. CONCLUSIONS

This work provides a fully validated and easily implementable method for the determination of paclitaxel in human plasma samples using a standard HPLC-PDA system, with adequate sensibility and analytical performance to the clinical application in therapeutic

drug monitoring in patients receiving common infusional chemotherapeutic regimens, also allowing the use of currently available dose individualization algorithms.

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4. CAPÍTULO 3

ANALYTICAL AND CLINICAL VALIDATION OF A DRIED BLOOD SPOT ASSAY FOR THE DETERMINATION OF PACLITAXEL USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Background: Paclitaxel (PCT) is a chemotherapeutic drug widely used for the treatment of several types of tumors and its use is frequently associated with severe adverse events, mainly neurologic and hematopoietic toxicities. The relation between exposure and response to PCT was previously described and this drug is a potential candidate for therapeutic drug monitoring (TDM). The use of dried blood spot (DBS) sampling could allow the use of complex sampling schedules, required for TDM of PCT. The aim of this study was to develop and clinically validate a LC-MS/MS assay for the quantification of PCT in DBS. **Methods:** PCT was extracted from one 8 mm DBS punch with a mixture of methanol and acetonitrile, followed by chromatographic separation in a Kinetex C18 (50 x 4.6 mm, 2.6 μm) column. Detection was performed in a 5500-QTRAP® mass spectrometer, with a run time of 2.3 minutes. **Results:** The assay was linear in the range of 2.5 to 400 ng mL^{-1}. Precision (CV%) and accuracy at the concentration levels of 7.5, 40 and 150 ng mL^{-1} were 1.69-4.9% and 106.25 to 109.92 %, respectively. PCT was stable for 21 days at 25 and 45 °C. The method was applied to DBS samples obtained from 34 patients under PCT chemotherapy. The use of a simple correction factor, derived from the correlation between PCT concentrations in plasma and DBS from the same patients, allowed unbiased estimation of PCT plasma concentrations from DBS measurements, leading to similar clinical decisions using either plasma or DBS measurements.

Conclusions: DBS testing of PCT concentrations represents a promising alternative for the dissemination of PCT dose individualization.

Keywords: paclitaxel; dried blood spots; therapeutic drug monitoring; liquid chromatography; mass spectrometry.

1. INTRODUCTION

Paclitaxel (PCT) is a chemotherapeutic drug used for the treatment of many tumors, including breast [1], head and neck [2], ovarian [3] and non-small cell lung cancer [4]. PCT was isolated from a Pacific Yew tree in 1971 [5] and was approved for clinical use by the US FDA in 1992. PCT mechanism of action is based on selective tubulin polymerization, preventing the cellular microtubule depolymerization, which in turn inhibits cell division [6].

PCT administration is usually associated with several adverse events, mainly neurologic and hematopoietic toxicities [7]. The interindividual tolerability to these effects is partially related to pharmacokinetics and pharmacogenetics differences among patients, particularly in PCT clearance [8]. PCT pharmacokinetics is nonlinear due to saturable elimination and distribution of the drug [9]. PCT metabolic clearance can be affected by genetic polymorphisms [10], demographic, physiologic and pathologic factors and by drug interactions [11].

The pharmacokinetic parameter that better represents the relation between exposure to PCT and clinical response is the time that plasma concentration remains above a threshold of 0.05 μM (Tc > 0.05 μM) [11–13]. Considering this target, algorithms had been proposed to individualize treatment with PCT based on the individual exposure to the drug [14]. The proposed therapeutic range for Tc > 0.05 μM in three weekly chemotherapy regimens is 26-31 h, and for weekly regimens is 10-14 h [12,13]. The determination of Tc > 0.05 μM can be performed with only one plasma concentration, obtained 24 h after the beginning of the drug infusion, using a proper pharmacokinetic model [12]. In this context, a simple Excel®-based tool to calculate Tc > 0.05 μM was recently described, based on a single PCT concentration [13]. Recent studies showed that PCT dose adjustment based on Tc > 0.05 μM lead to a significant reduction in the occurrence of adverse effects, without a reduction in clinical efficacy [4].

Therapeutic drug monitoring (TDM) of PCT is a promising tool to optimize chemotherapy with this drug, but is highly dependent on the availability of reliable and clinically implementable assays. The current sampling strategy to estimate pharmacokinetics parameters of PCT requires plasma separation from venous blood, after phlebotomy. This conventional sampling approach requires specialized professionals and infrastructure [12,13,15] Alternatively, the use of dried blood spots (DBS) samples could allow remote or

self-sampling, also being an alternative for sample transportation from distant sites to reference laboratories due to higher analyte stability, increased biosafety, and simplified logistics, usually not requiring refrigeration [15–17]. These DBS advantages are particularly relevant for TDM of PCT in limited-resources settings, where outpatients have significant difficulties to return to a specialized center to collect blood samples at the required time of 24 h post-infusion.

The clinical application of TDM using DBS as the sampling strategy demands analytical methods with high sensitivity and specificity, such as liquid chromatography-mass spectrometry (LC-MS/MS), particularly considering the small amount of sample available for testing [15,18]. Additionally, before clinical application, DBS drug measurement assays require extensive validation, including tests to evaluate the impact of blood hematocrit in its accuracy [15–17]. Moreover, a clinical application study is mandatory to adequately translate DBS concentrations to plasma levels [15]. Despite the previous description of PCT assays in DBS [19,20], there is no report of a comprehensive, DBS-specific, method validation, as well as no data on the clinical performance of the methods.

Considering its potential clinical relevance and the lack of a comprehensively validated bioanalytical method for the determination of PCT in DBS, in the context of TDM, this study aimed to develop and validate a clinically applicable LC-MS/MS method for the quantification of PCT.

2. EXPERIMENTAL

2.1 Standards, solvents and materials

PCT and deuterated PCT (PCT-D5) were acquired from Toronto Research Chemicals (NorthYork, Canada). Acetonitrile and methanol were purchased from Merck (Darmstadt, Germany) and ammonium formate, ammonium acetate, formic acid and 1-chlorobutane were acquired from Sigma Aldrich (Saint Louis, USA). Whatman 903® paper was obtained from GE Healthcare (Westborough, USA). Ultra-pure deionized water was supplied by a Milli-Q RG unit from Millipore (Billerica, MA, USA).

2.2 Solutions

Stock (1000 μg mL^{-1}), intermediate (100 μg mL^{-1}) and working (0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 8.00 μg mL^{-1}) solutions of PCT were prepared by dissolution in methanol. The internal standard (IS) working solution, at 1 μg mL^{-1}, was prepared by dilution of intermediate solution of PCT-D5 with methanol. Working solutions of PCT were prepared at concentrations 20 times higher than calibration and control levels by dilution with methanol. DBS extraction solution was a mixture of methanol and acetonitrile (90:10, v/v), containing PCT-D5 at 4 ng mL^{-1}. The IS solution for plasma analysis was PCT-D5 at 1 μg mL^{-1}, also in methanol.

2.3 Chromatographic and mass spectrometric conditions

DBS and plasma samples were analyzed using a 1260 Infinity liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA), coupled to a 5500-QTRAP® hybrid triple quadrupole mass spectrometer (ABSciex, Concord, Canada). The chromatographic separation was performed with a Kinetex C18 (50 x 4.6 mm, 2.6 μm) column, from Phenomenex (Torrance, CA, USA), maintained at 30 °C. The mobile phase consisted of ultra-pure water (A, 25%) and methanol (B, 75%), both containing formic acid (0.1%, v/v) and 2 mmol $L^{-1}$of ammonium formate, eluted at isocratic mode. The mobile phase flow rate was 0.5 mL min^{-1}. Chromatographic run time was 2.3 min. The 5500-QTRAP® mass spectrometer was equipped with a TurboIonSpray™ interface using electrospray ionization in the positive ionization mode. Nitrogen was used as curtain, collision and nebulizer gas. The source parameters were: ion source temperature, 650 °C; ion spray voltage, 4.5 kV; entrance potential (EP), 10 V; nebulizer gas (GS1) pressure, 60 psi; auxiliary gas (GS2) pressure, 60 psi; and curtain gas pressure, 15 psi. The analyses were performed in multiple reaction monitoring (MRM) mode. For each compound, two MRM transitions were chosen for quantification and confirmation, and optimized by constant infusion of working solutions of each analyte (20 ng/mL in water/methanol, 1:1 v/v). Table 1 shows the optimized conditions of declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and the retention time for PTC and PTC-D5. Analyst 1.6.2 software was used for data collection and MultiQuant 3.0.1 for data processing.

Table 1. Optimized parameters for analysis of paclitaxel and paclitaxel-D5 by liquid chromatography-tandem mass spectrometry.

Analyte	MRM transitions (*m/z*)[a]	DP (V)[b]	CE (V)[c]	CXP (V)[d]	Retention time (min)
Paclitaxel	854.3→509.2	186	17	11	2.0
	854.3→569.2	186	13	27	
	854.3→551.2	186	15	13	
Paclitaxel-D5	859.4→569.2	181	13	13	2.0
	859.4→509.2	181	19	25	

[a] Quantifier transitions were underlined, *MRM* multiple reaction monitoring [b] *DP* declustering potential [c] *CE* collision energy [d] *CXP* collision cell exit potential

2.4 Preparation of DBS

Calibration and quality control DBS samples were prepared by pipetting 50 µL of blood on Whatman 903® paper, followed for a minimum drying time of 3 hours before extraction. Calibrators and quality control samples were prepared by diluting working solution of PCT with venous blood in the proportion 1:20 (v/v). Blood used for the preparation of validation DBS samples had a Hct% of 40 unless otherwise stated. DBS samples from finger-pricks were obtained by application of one drop of blood to the paper, directly from the patient finger, without touching the surface of the collection area. DBS samples obtained from patients were allowed to dry at room temperature for 3 h after collection and then stored in plastic bags with desiccants at room temperature.

2.5 DBS sample preparation

One DBS disk (fixed punch diameter of 8 mm) was cut in 4 pieces and transferred to a 2 mL polypropylene micro tube, followed by addition of 500 µL of the extraction solution. The tube was agitated at 500 RPM for 60 min in a ThermoMixer® (Eppendorf), at 25 ºC. An aliquot of 450 µL from the supernatant was evaporated in a vacuum centrifuge at 45 °C and recovered with 150 µL of mobile phase, followed by 10 min of centrifugation at 10.000 g. An aliquot of 20 µL of the resulting supernatant was injected into the LC-MS/MS system.

2.6 Linearity

Calibration samples had concentrations of 2.5, 5, 10, 25, 50, 100, 200 and 400 ng mL^{-1}, processed in quintuplicate. Calibration curves were obtained relating the area ratios from PCT to PCT-D5 peaks. Homoscedasticity of calibration data was evaluated with *F*-test at the confidence level of 95%. Weighted least-squares linear regression was used to generate calibration models, which were evaluated through their coefficients of correlation (*r*) and cumulative percentage relative error (Σ%RE) [21].

2.7 Precision and accuracy

QC samples were prepared at the concentration levels of 7.5 (quality control at low concentration, QCL), 40 (quality control at medium concentration, QCM) and 150 ng mL^{-1} (quality control at high concentration, QCH). QC samples were processed and analyzed in triplicate, in each of 5 days. Within-assay precision and between-day precision were calculated by one-way analysis of variance and expressed as CV%. Accuracy was evaluated as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criteria for accuracy were mean values within ±15% of the theoretical value, and for precision, a maximum CV of 15% was accepted [22].

2.8 Effect of hematocrit on accuracy

Aliquots of blood presenting Hct% of 25 and 50 were prepared by centrifuging EDTA whole blood and then combined with appropriate volumes of cells and plasma [23]. PCT was added to these aliquots of blood to achieve the concentrations of QCL and QCH, previously described. The DBS obtained were analyzed in triplicate for each concentration level and Hct% value. The influence of the Hct% on PCT measurements was determined as the percentages of nominal concentrations that were measured in the DBS. Acceptance criteria were values in the range of 85-115%.

2.9 Selectivity

Blank DBS samples obtained from six different human sources were prepared as described above to check for the presence of chromatographic peaks that might interfere with detection of PCT or IS.

2.10 Lowest limit of quantification

Precision and accuracy were evaluated at the concentration level of the lowest calibrator, 2.5 ng mL^{-1} (QCLOQ), which was tested in triplicate on five different days. The acceptance criteria were accuracy within 100±20% of the nominal concentration and a maximum CV% of 20 [22].

2.11 Extract stability at the autosampler

For estimation of stability of processed samples at the chromatograph's autosampler, DBS QC samples at low (QCL) and high (QCH) concentrations were extracted as described above, in duplicate. The extracts obtained at each concentration were pooled. Aliquots of these pooled extracts were injected under the conditions of a normal analytical run at time intervals of 1 h, during 10 h. Peak area ratios between beginning and end of the series were compared. A decrease or an increase of up to 15% in the measured peak areas was considered as acceptable [22].

2.12 Stability at DBS maintained at different temperatures

For evaluation of thermal stability of PCT in DBS samples, QC DBS samples at QCL and QCH levels were kept at 25 and 45 °C and analyzed in triplicate on days 1, 7, 14 and 21 after spotting on the paper. Stability was considered acceptable if all results were within the range of 85-115% of the concentrations measured at the beginning of the series.

2.13 Matrix effect and extraction yield

Matrix effect (ME) was evaluated by a standard experimental design, using a post extraction spike method [23]. Three series (A, B and C) of the QC samples QCL, QCM and QCH, previously described, were prepared and analyzed in order to assess extraction yield (EY) and ME on ionization as follows: (A) solutions of PCT and IS in mobile phase, at concentration equivalent to complete recovery, (B) DBS extracts samples from five different sources (mixed before application on paper, whole spot containing 18 µL of blood), spiked post extraction with PCT in mobile phase containing IS and (C) DBS extracts samples from five different sources (mixed before application on paper, whole spot containing 18 µL of blood), enriched with PCT before extraction. Each QC sample was analyzed in quintuplicate. ME on ionization was estimated as the percentages of reduction or increase of PCT and IS areas on post extraction spiked (B), comparing to the solutions (A), calculated as ME=[100-(B/A%)]. Extraction yield was calculated comparing the analyte/IS area ratio before extraction (C) and after extraction (B), using the formula EY = C/B%.

2.14 Impact of hematocrit in extraction yield

Aliquots of 18 µl of blood (Hct% 25 and 50) containing PCT at the concentrations of QCL and QCH and non-spiked blood were added to Whatman 903® paper to obtain DBS. Whole spots were cut and extracted. Non-spiked extracts were also added with PCT to obtain concentrations equivalent to 100% extraction yield. Extraction yield was calculated comparing the area ratio of PCT to the IS in control and non-spiked samples.

2.15 Impact of spotted blood volume on accuracy

Blood with Hct% of 40 was prepared as described above and PCT was added to achieve the concentrations QCL and QCH and was then pipetted onto Whatman 903® paper at the volumes of 30, 40 and 55 µL, consistent with finger prick blood drops. After drying, the obtained DBS were analyzed as described above and PCT was quantified with a calibration curve prepared after pipetting 50 µL of blood to paper. In all extractions, an 8-mm disk was used for testing. The influence of spotted volume on PCT measurements was

determined as the percentages of nominal concentrations that were measured in the DBS. The acceptance criterion was a maximum deviation of ± 15%.

2.16 Determination of PCT in plasma

Plasma samples were prepared using liquid-liquid extraction. Briefly, 250 µL plasma samples were transferred to polypropylene tubes and added with 25 µL of IS solution (PCT-D5, 1 µg mL^{-1}), 500 µL ammonium acetate buffer pH 5 and 3,500 µL of the 1-chlorobutane:acetonitrile (80:20, v/v). After 10 minutes of homogenization, followed by 10 min centrifugation at 2,000 g, an aliquot of 3,200 µL supernatant was evaporated at 45 °C in a vacuum centrifuge. The resulting dried extract was recovered with 100 µL of initial mobile phase, and 20 µL was injected into the LC-MS/MS system. Calibration ranges, as well as chromatographic and mass spectrometric conditions, were the same as applied to DBS samples. The assay has inter and intra-assay CV%, tested at the concentration levels of 7.5, 40 and 150 ng mL^{-1}, of 3.4-8.9 and 3.0-6.3%, respectively, and accuracy in the range 95.6-97.6%.

2.17. Assay application

DBS from fingerpicks and from venous blood samples (50 µL pipetted on the paper), from which plasma was separated by centrifugation, were obtained simultaneously (within 10 min) from 34 volunteer outpatients prescribed with PCT, and concentrations were measured with the developed assay. Hct was measured from an aliquot of venous blood by standard hematology procedures. The study was approved by the institutional review board of Hospital de Clínicas de Porto Alegre and performed according to the Helsinki Declaration principles. Informed consent was obtained from the volunteers.

2.18 Comparison between concentrations measured in DBS and plasma samples

Estimated plasma concentrations (EPC) were estimated by three different approaches. Firstly, EPC were calculated considering each patient's Hct% values using the equation: $EPC_{Hct} = (DBS_{conc}/[1-(Hct\%/100)]) \times f_p$, where DBS_{conc} is the concentration measured in DBS, Hct% is the individual hematocrit of patient and f_p is the fraction of the

drug in plasma, according to Antunes *et al.* [15]. The f_p value was adjusted to obtain a mean ratio between the measured PCT plasma concentrations and EPC of 1, using the above equation. In a second approach, EPC was also calculated using a correction factor based on the mean ratio of PCT plasma to DBS concentrations, in each chemotherapy scheme (weekly or three weekly), without considering the individual Hct% nor f_p ($EPC_{coorrection\ factor}$). A third approach for the calculation of estimated plasma concentrations from DBS measurements ($EPC_{equation}$) was to apply the regression equation obtained after correlating plasma (*x*) to DBS concentrations (*y*).

Tc > 0.05 μM for paclitaxel was calculated using a simple Excel®-based tool described by Kraff *et al*. [13]. Agreement between measured plasma and estimated plasma concentrations was evaluated using Passing-Bablok regression and Bland-Altman relative difference plots. Aditionally, the concordance between dose adjustment decisions using either plasma or EPC concentrations was also evaluated. Statistical analyses were done with Medcalc version 12.3 (Ostend, Belgium) and Microsoft Excel® (Microsoft Corporation, USA).

3. RESULTS AND DISCUSSION

3.1. Chromatography and sample preparation

The chromatography conditions described in this manuscript allowed a retention time of 2.0 min for both PCT and PCT-D5, and a total run time of 2.3 min (Figure 1), with no interfering peaks in the tested blank samples. The determination of PCT in DBS was already described in two previous reports. However, these reports did not perform any human clinical evaluation of the performance of the assays [19,20]. Additionally, neither of both publications performed specific DBS validation tests, even considering the critical impact of varying Hct% on the ability of DBS assays to predict plasma concentrations [15]. Rao *et al.* [19] quantified paclitaxel by LC-MS/MS using docetaxel as the IS, with retention times of 4.3 min for IS and 4.9 min for PCT [19]. Xie *et al.* [20] also used LC-MS/MS for measuring PCT concentration in DBS obtained from mice, with retention time of 1.69 min.

The sample preparation employed in this study was simple and based on a single-step extraction with a mixture of organic solvents containing the IS, which allowed accurate

quantification of paclitaxel from the small amount of sample present in DBS (about 17 µL)[16].

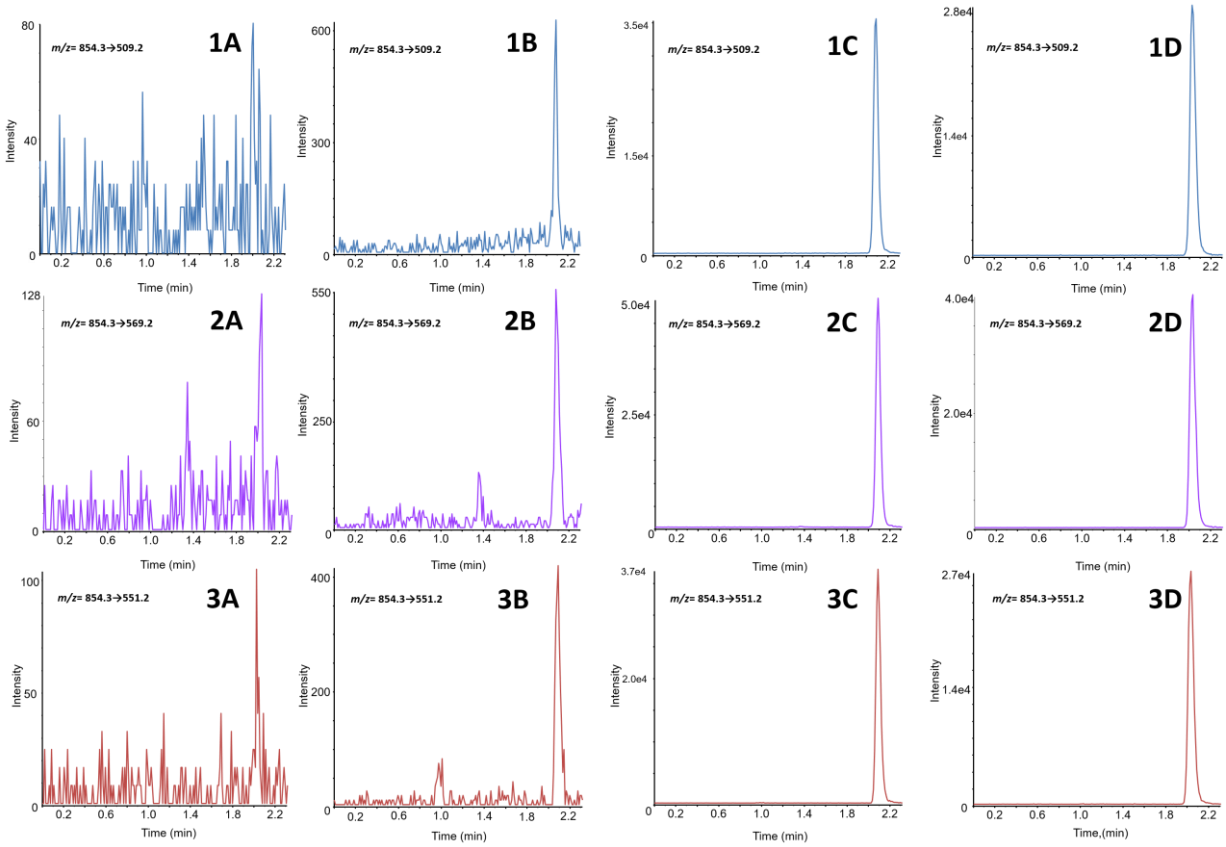

Figure 1. Chromatograms obtained with DBS analyses. **A**: Blank DBS. **B:** Quality control at the limit of quantification (QCLOQ). **C**: Quality control high (QCH). **D:** Patient sample, with PCT concentrations of 157.1 ng mL^{-1}. **1:** *m/z* =854.3→509.2**. 2:** *m/z* =854.3→569.2. **3:** *m/z* =854.3→551.2.

3.2. General method validation

Precision, accuracy, linearity, stability and matrix effect tests were performed using blood with Hct% of 40 (Table 2). Calibration data presented significant heteroscedasticity (F=99.12; $F_{crit(4,4,0.95)}$=6.39) and several weighting factors were tested. Among the evaluated weighting factors, $1/x^2$ presented the lowest Σ%RE, of -7.11×10^{-15} and was used for all quantitative measurements. General method validation data is presented in Table 2. Processed sample stability for 10 h at the autosampler was acceptable, with peak area variations for QCL and QCH in the range of +8.1 to -4.4, at the end of the series. Intra-assay precision ranged between 1.69 and 4.42 %, and inter-assay between 3.54 and 4.90 %.

Accuracy was in the range of 106.25 to 109.92 %. The lowest limit of quantification was 2.5 ng mL^{-1}, a concentration below the expected clinical levels of PCT at about 24 h after infusion in the current chemotherapy regimens. QCLOQ presented intra-assay and inter-assay precision of 6.86 and 8.74 %, and accuracy of 101.16 %. The method had a minimum matrix effect, with a maximum deviation of -7.55 % for PCT and 0.85 % for PCT-D5. An additional indication of minimal matrix effects was the relatively low CV% of the peak area of the internal standard among all patient samples (n=34), of 9.1%, with no outliers.

Table 2. General method validation parameters for paclitaxel determination in DBS: precision, accuracy, extraction yield, matrix effect and processed sample stability at autosampler (AS).

QC sample	Nominal concentration (ng mL^{-1})	Precision (CV %)		Accuracy (%)	Extraction yield (%)	Matrix effect (%)	Processed sample concentration change after 10 h in AS (%)
		Intra-assay	Inter-assay				
QCLOQ	2.5	6.86	8.74	101.16	-	-	-
QCL	7.5	4.42	4.90	107.09	66.8	+1.43	+8.1
QCM	40	1.69	4.36	106.25	-	-7.55	-
QCH	150	3.15	3.54	109.92	68.1	-0.98	-4.4
PCT-D5						0.85	

QCLLOQ: quality control at the lower limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control (linearity n=40, precision and accuracy n=45, extraction yield n= 30, processed sample stability n=24).

3.3. Specific DBS method validation

The stability of PCT in DBS was tested to simulate the time and conditions of usual handling and transport of samples by the mail service. Therefore two temperatures were tested, room (25 °C) and high (45 °C) temperatures. PCT was stable for up 21 days at both 25 and 45 °C, with maximum variation from the nominal concentrations of -11 %, after three weeks (Table 3). Considering the time the regular mail service takes to deliver the orders, PCT stability is acceptable for clinical use. These results were similar to a previous study, where PCT was stable for up to 45 days at high (45 °C) and refrigerated temperatures (4 °C), with maximum variation of 5.02 % of the nominal concentrations [19]. PCT stability in DBS was also demonstrated for up 20 days at room temperature [20].

Table 3. Three-week stability of paclitaxel in DBS maintained at different temperatures (percentage of nominal concentration).

QC sample	Nominal concentration (ng mL^{-1})	Temperature (°C)	Day 7	Day 14	Day 21
QCL	7.5	25	106.2	106.7	108.4
		45	106.2	106.6	98.8
QCH	150	25	101.3	97.2	104.6
		45	106.7	109.0	89.0

QCL: quality control low, QCH: quality control (n=3 for each concentration at each day and temperature).

The amount of blood in a spot with a fixed diameter, and the extraction yield of the analyses, can be affected by the blood's Hct. Therefore, we evaluated the impact of Hct on measurement accuracy and extraction yield at the Hct% of 25 and 50, at two control levels (QCL and QCH) (Table 4). Accuracy ranged between 97.7 to 114.8 % for Hct% of 25, and 108.6 to 112.5 % for the Hct% of 50. The accuracy was considered acceptable by the 15% variation criterion. Extraction yield was minimally affected by Hct, and presented variations according to PCT concentrations, decreasing at high concentrations, with minor effects on the quantitative performance of the assay. Extraction yield was in the range of 71.5 to 73.1 % for Hct% 25 and 64.9 to 69.5 % for Hct% 50 (Table 4).

Table 4. Evaluation of the influence of Hct on accuracy and extraction yield in paclitaxel DBS measurements.

Hct%	QC sample	Nominal concentration (ng mL^{-1})	Accuracy (%)	Extraction yield (%)
25	QCL	7.5	114.8	73.1
	QCH	150	97.7	71.5
50	QCL	7.5	112.5	69.5
	QCH	150	108.6	64.9

QCL: quality control low. QCM: quality control medium. QCH: quality control high (n =3 for each control sample, at each Hct% value).

The evaluation of the influence of spotted volume on the accuracy of PCT was performed using two QC levels prepared by spotting different volumes of whole blood (30, 40, 55 µL) added with PCT on Whatman 903® paper (Table 5). No significant impact on the accuracy of PCT measurements was observed, with measured PCT concentrations in the range of 98.5 to 112.7 % of nominal levels.

Table 5. Evaluation of the influence of spotted volume on the accuracy of paclitaxel measurements in DBS.

Volume (µL)	Nominal concentration (ng mL^{-1})	Accuracy (%)
30	7.5	98.5
	150	112.7
40	7.5	102.1
	150	106.1
55	7.5	111.9
	150	107.8

n =3 for each control sample, at each volume.

3.4. Method application

The method was applied to DBS and plasma samples of 34 patients in treatment with PCT. Patients received PCT as a single chemotherapy agent or in association with other chemotherapy drugs (carboplatin, gemcitabine, cisplatin and trastuzumab). Nineteen patients received weekly PCT scheme (45-80 mg m^{-2}, 1 h infusion) and 15 patients received three weekly PCT scheme (100-200 mg m^{-2}, 3 h infusion). The samples were collected between 18 and 30 hours after the start of the infusion of PCT [12]. The Hct% of the patients was in the range of 25 to 46.3.

Considering the presence of Cremophor EL® (CrEL, a pharmaceutical vehicle used to dissolve PCT for intravenous administration) in the administered formulation of PCT used in this group of patients and its clinical implication on the measured concentrations, we evaluated the correlation between measured and estimated plasma concentrations in two groups, separated by the dosing scheme. With increasing concentrations of CrEL, the disposition of drug is affected by entrapping it in micelles and reducing its free fraction [9,24,25].

Plasma concentrations of PCT for patients receiving weekly treatment were in the range of 10.9 to 49.6 ng mL^{-1}, and DBS capillary concentrations ranged between 11.7 and 83.1 ng mL^{-1}. For patients receiving the three weekly PCT scheme, plasma concentrations were in the range of 51.6 to 220.9 ng mL^{-1} and DBS concentrations in the range of 60.9 to 249.2 ng mL^{-1}. PCT concentrations measured in capillary DBS, obtained from finger pricks, were highly correlated with DBS obtained from venous blood, which were obtained by usual phlebotomy (r=0.986), with a difference of 3.7% between the average of measured concentrations in capillary and venous DBS and a mean ratio between capillary and venous

DBS of 1.04 (supplementary data, table s1). Plasma and capillary DBS concentrations presented a higher correlation for the patients receiving three weekly scheme when compared with weekly scheme, with *r*-values of 0.930 and 0.896, respectively.

Table 6. Comparison of measured plasma and DBS paclitaxel concentrations and estimated paclitaxel concentrations (n=34).

PCT Scheme	Patient	Plasma (ng mL^{-1})	DBS (ng mL^{-1})	EPC (% of plasma levels)		
				Equation	Multiplying factor	Hct correction formula
Weekly	1	10.9	11.7	96.2	75.9	86.3
	2	17.4	16.6	76.6	67.4	74.4
	3	17.4	27.9	114.2	113.3	121.7
	4	19.2	26.7	99.9	98.3	93.7
	5	20.4	27	94.9	93.5	88.2
	6	21	33.4	109.8	112.4	120.3
	7	21.2	33.4	108.8	111.3	108.7
	8	23.4	28.5	86.4	86.1	103.9
	9	26.1	37.4	97.2	101.3	110.4
	10	26.2	43.9	111.2	118.4	117.3
	11	26.6	42.4	106.3	112.6	131.3
	12	27.5	42.3	102.6	108.7	102.3
	13	28.6	47.9	110.0	118.4	148.5
	14	28.7	36.2	86.0	89.1	91.0
	15	29.5	49.8	110.4	119.3	123.8
	16	31.6	53.2	109.3	119.0	127.3
	17	33.7	46.5	91.0	97.5	113.1
	18	37.3	47	83.0	89.0	98.8
	19	49.6	83.1	104.6	118.4	115.9
Three weekly	20	51.6	60.9	100.9	106.7	90.9
	21	33.7	50.9	125.5	136.5	122.6
	22	46.3	38	64.2	74.2	59.1
	23	56.5	72.9	112.9	116.6	97.2
	24	62.1	74.2	104.8	108.0	100.6
	25	63.6	82.2	114.6	116.8	104.1
	26	69.5	78.3	99.4	101.8	85.6
	27	78.1	79.3	89.7	91.8	94.5
	28	83	94.4	102.1	102.8	85.5
	29	89	97.4	98.6	98.9	96.8
	30	89.1	115.9	118.7	117.5	99.1
	31	100.7	83.7	73.8	75.1	66.5
	32	130.3	157.1	112.1	109.0	92.0
	33	171.9	158	85.4	83.1	68.2
	34	220.9	249.2	106.8	101.9	84.1
	Range			64.2-118.7	67.4-119.3	59.1-148.5
	Average			100.2	102.7	100.7

Plasma concentrations were estimated from DBS obtained from fingerpicks using three different approaches ($EPC_{equation}$, EPC_{Hct} and $EPC_{correction\ factor}$). Comparison of measured and estimated paclitaxel concentrations, using these three approaches, are presented in Table 6. The estimation of PCT plasma concentrations from capillary DBS using the equation EPC_{Hct} approach for the weekly treated patients, using a f_p value of 0.5, allowed to estimate 15 of 19 samples within ±25% of the measured plasma concentrations. For the group of three weekly scheme, the f_p value was 0.56, and this estimation approach presented 13 of 15 samples within ± 25% of the measured plasma concentrations. The other two approaches, $EPC_{equation}$ and $EPC_{correction\ factor}$, had better performance to predict plasma concentrations from DBS, and $EPC_{correction\ factor}$ was selected due to its simpler calculation. The regression equation for weekly group was *DBS concentration=(1.725*plasma concentration)-6.379*, with $EPC_{equation}$ presenting all samples values within ± 25 % of the measured plasma concentrations. For the three weekly group, the regression equation was *DBS concentration=(1.024*plasma concentration)+7.575*, with 12 of 15 samples values within ± 25 % of the measured plasma concentrations. The correction factor was based on the mean ratio of PCT plasma to DBS concentrations, without considering the individual Hct nor f_p. The correction factor for the weekly group was 0.707, with $EPC_{correction\ factor}$ presenting 18 of 19 samples within ±25% of the measured plasma concentrations, and for the three weekly group the factor was 0.904, which allowed 13 of 15 samples to be within ± 25 % of the measured plasma concentrations.

$EPC_{correction\ factor}$ was used to evaluate if clinical decisions for PCT dose adjustments would be the same using DBS or plasma measurements. This estimation approach was selected due to its better predictive performance and simpler application than the other two approaches. Tc>0.05 μM was calculated using both plasma and $EPC_{correction\ factor}$ concentrations (Table 6), using a simple Excel®-based tool described by Kraff *et al*. [13]. Tc>0.05 μM for plasma concentrations were in the range of 6.6 to 20 h for the weekly PCT scheme and 20.2 to 40.1 h for the three weekly scheme. Tc>0.05 μM calculated with $EPC_{correction\ factor}$ were in a range of 6.4 to 21.6 h for the weekly scheme and 20 to 38.3 h for the three weekly scheme. Fifteen (44.1 %) of the 34 patients reached the Tc>0.05 μM target range, six (17.7%) were below, and 13 (38.2 %) were above the therapeutic range proposed by Joerger *et al.* [12] and Kraff *et al.* [14]. Previous studies also reported a significant number of patients with sub-optimal exposure to PCT. In a study conducted in 96 females with ovarian cancer treated with PCT in the first cycle of the treatment, 52% of the patients

reached the Tc>0.05 μM target range of 26-30 h, 35% were below and 11 % were above the target [26]. In another study with 175 patients, Joerger *et al.* [4] found that only 34% of treated patients had a Tc>0.05 μM in the target range, 29% were below and 38% of the patients were below the therapeutic range.

Table 7. Comparison of calculated PCT Tc>0.05 μM calculated using plasma and estimated plasma concentrations, with correspondent proposed dose adjustments based on published algorithms.

PCT Scheme	Patient	Tc>0.05 μM (h) plasma	Dose adjustment (%)	Tc>0.05 μM (h) EPC	Dose adjustment (%)
Weekly	1	6.6	+15	6.4	+15
	2	9.5	+15	8.1	+15
	3	10	0	10.8	0
	4	9.2	+15	9.3	+15
	5	12.5	0	12.1	0
	6	10	0	11.8	0
	7	10	0	10.8	0
	8	9.7	+15	9.2	+15
	9	11.8	0	12.5	0
	10	13.3	0	16	-15
	11	14.4	-15	16.7	-15
	12	15	-15	15.7	-15
	13	11.4	0	13	0
	14	13.1	0	12.1	0
	15	14.2	-15	16.5	-15
	16	15.5	-15	17.8	-15
	17	16.1	-15	15.1	-15
	18	17.2	-15	15.5	-15
	19	20	-25	21.6	-25
Three weekly	20	21.5	-25	22.1	-25
	21	20.2	+10	29.6	0
	22	27	0	20	+10
	23	27.2	0	29.1	0
	24	29.5	0	29.7	0
	25	36	-20	38.4	-20
	26	29.3	0	29.3	0
	27	24.9	+10	24.1	+10
	28	33.5	-20	34	-20
	29	30.5	0	30.5	0
	30	31.1	-20	32.4	-20
	31	40.1	-20	38.3	-20
	32	30.8	0	31.2	-20
	33	30.3	0	29.7	0
	34	36.3	-20	36.5	-20

Once the Tc>0.05 μM was calculated, it was possible to evaluate the concordance between proposed dose adjustments based on the algorithms suggested by Kraff *et al*. [14] for the weekly scheme and by Joerger *et al*. [12] for three weekly scheme. The Tc>0.05 μM calculated by plasma and by $EPC_{correction\ factor}$ concentrations resulted, in 88.2 % of the cases (30 out of 34), in the same decision of dose adjustment (Table 7).

Passing-Bablok regression and Bland-Altman plot comparison of measured plasma and $EPC_{correction\ factor}$ are presented in Figure 2. The Passing-Bablok regression equation, comparing plasma measurements of PCT with $EPC_{correction\ factor}$, presented a 95% confidence interval for slope of 0.9805 to 1.1717 and -4.6371 to 2.8749 for intercept, with no significant deviation from linearity (P=0.42). Based on Passing-Bablok regression data, no systematic or proportional differences between plasma concentration and $EPC_{correction\ factor}$ were identified. The mean relative difference between actual and estimated plasma concentrations was 1.4, with one (2.94 %) measurement outside of the ± 1.96 standard deviation range for PCT (-30.6 to +33.4 % difference range) (Figure 2). While this wide dispersion could be attributed to samples with higher differences between predicted and measured plasma concentrations, in general the clinical evaluation was concordant, was previously discussed. Differences between EPC and actual measured concentrations were distributed randomly around the mean, indicating the absence of systematic errors. Besides our promising results, future studies, with a higher number of patients, are necessary to confirm the clinical utility of the correction factor, as applied here.

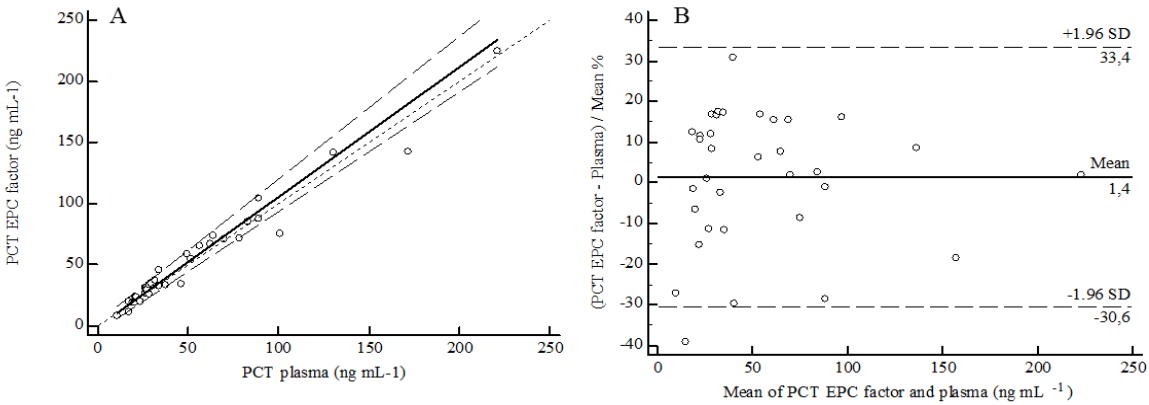

Figure 2. Passing-Bablok (PB) and Bland-Altman (BA) comparison of measured and estimated plasma concentrations from DBS measurements for paclitaxel (PCT). **A:** PB regression PCT plasma vs. $EPC_{correction\ factor}$. **B:** BA plot PCT plasma vs. $EPC_{correction\ factor}$.

4. CONCLUSIONS

An assay for the determination of paclitaxel in DBS using LC-MS/MS was developed and validated. PCT was stable up to three weeks at 45 °C and 25 °C, indicating the possibility of transportation by normal postal conditions. After applying a correction factor, plasma concentrations of PCT were estimated from DBS measurements without systematic or proportional bias. In 88.2 % of the cases, the clinical decision of PCT dose adjustment based on plasma or DBS measurements is the same. PCT can be accurately measured in DBS and represents an interesting alternative for the dissemination of dose individualization of PCT.

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Table S1. Comparison of paclitaxel measured concentration in venous and capillary.

Patient	Venous DBS (ng mL^{-1})	Capillary DBS (ng mL^{-1})	Ratio Capillary/venous
1	12.5	11.7	0.94
2	17.5	16.6	0.95
3	25.4	27.9	1.10
4	26.7	26.7	1.00
5	25.9	27.0	1.04
6	30.5	33.4	1.10
7	33.4	33.4	1.00
8	27.5	28.5	1.04
9	38.7	37.4	0.97
10	37.9	43.9	1.16
11	45.4	42.4	0.93
12	38.7	42.3	1.09
13	44.7	47.9	1.07
14	34.6	36.2	1.05
15	44.7	49.8	1.11
16	48.1	53.2	1.11
17	53.0	46.5	0.96
18	43.5	47.0	1.07
19	42.3	83.1	1.11
20	35.4	60.9	1.07
21	71.4	50.9	1.16
22	57.1	38.0	1.07
23	74.1	72.9	0.98
24	80.8	74.2	0.92
25	77.4	82.2	1.06
26	79.6	78.3	0.98
27	82.4	79.3	0.96
28	99.7	94.4	0.95
29	104.3	97.4	0.93
30	94.4	115.9	1.23
31	79.0	83.7	1.06
32	144.3	157.1	1.09
33	145.9	158.0	1.08
34	247.6	249.2	1.01
Range	12.5-247.6	11.7-249.2	0.92-1.23
Average	63.07	65.5	1.04

DBS (n=34).

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5. CONSIDERAÇÕES FINAIS

O PCT, formulado com Cremophor EL, é um medicamento quimioterápico muito utilizado no tratamento de tumores sólidos, principalmente em países subdesenvolvido e em desenvolvimento, em vista de seu baixo custo em relação a outras formulações mais novas, como o Abraxane (JOERGER et al., 2016B). Embora eficiente em muitas condições, o PCT apresenta efeitos adversos potencialmente severos, mesmo em doses baixas (LIEBMANN et al., 1993). Esses efeitos indesejados se devem em grande parte a diferenças interindividuais na forma com que o organismo metaboliza e excreta o fármaco. A atividade de enzimas envolvidas no metabolismo (ANDRIGUETTI et al., 2017), interações medicamentosas, e características demográficas e patofisiológicas impactam a exposição individual ao PCT (KRENS et al., 2013). Entretanto, a seleção de doses de PCT ainda é realizada através do cálculo baseado na área de superfície corporal do paciente. Evidências crescentes indicam o MTF de PCT é uma alternativa útil, visto sua ampla variabilidade farmacocinética, janela terapêutica estreita e uma relação bem definida entre exposição sistêmica e toxicidade ou resposta (KUMAR et al., 2010; KRENS et al., 2013).

Com a revisão realizada neste estudo (capítulo 1) foi possível descrever como o conhecimento da farmacogenética e farmacocinética pode ajudar na individualização das doses de PCT, além de demonstrar evidências da aplicabilidade do MTF para este fármaco. O método bioanalítico desenvolvido para quantificar as amostras de plasma dos pacientes em terapia com o PCT por CLAE-DAD (capítulo 2), representando uma alternativa facilmente implantável em laboratórios com recursos limitados. O uso do DBS como alternativa de amostragem, apresentado no capítulo 3, apresenta diversas vantagens potenciais para aplicação clínica, tal como a praticidade da coleta, que é minimamente invasiva, associada com a elevada estabilidade do PCT pela secagem da amostra, que possibilita o transporte por correio. Além disto, permite que, com devido treinamento, o próprio paciente possa realizar a coleta em sua residência e enviar ao laboratório por correio. Além disto, a avaliação clínica dos resultados usando tanto a amostra convencional de plasma como DBS apresentou resultados comparáveis, demonstrando o potencial clínico desta abordagem alternativa de amostragem.

Os resultados das concentrações de PCT dos 34 pacientes avaliados foram utilizados para determinar a exposição sistêmica ao quimioterápico através do cálculo do Tc>0,05 µM.

Dezenove (55,9%) pacientes apresentaram Tc>0,05 fora das faixas terapêuticas propostas por Joerger et al. (2012) e Kraff et al. (2015a), evidenciando que existe uma grande necessidade de estratégias de seleção de doses mais efetivas para o PCT, particularmente através do emprego do MTF através de metodologias bioanalíticas tais como as desenvolvidas nesta dissertação.

Os métodos de quantificação de PCT desenvolvidos neste estudo poderão ser usados em estudos futuros de avaliação da relação entre variáveis farmacocinéticas e farmacogenéticas e respostas clínicas ao fármaco, assim como em estudos de individualização de doses deste importante quimioterápico.

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ANEXO I

Comprovante de publicação do artigo intitulado "Pharmacogenetic and pharmacokinetic dose individualization of the taxane chemotherapeutic drugs paclitaxel and docetaxel" em Current Medicinal Chemistry.

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REVIEW ARTICLE

Pharmacogenetic and Pharmacokinetic Dose Individualization of the Taxane Chemotherapeutic Drugs Paclitaxel and Docetaxel

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Abstract: The taxane drugs paclitaxel and docetaxel, widely used on cancer chemotherapy, are currently dosed mainly based on body-surface area. This approach is associated with wide interindividual variability in drug exposure, leading to suboptimal dosing for many patients. We reviewed the available evidence supporting dose individualization strategies for paclitaxel and docetaxel, focusing mainly on the application of therapeutic drug monitoring by *a priori* pharmacogenetic data or *a posteriori* drug measurements in biological fluids. The PubMed database was searched, in the period of 1987-2017, using the keywords pharmacogenetics, metabolic genotyping, dose individualization, therapeutic drug monitoring, personalized medicine, taxanes paclitaxel and docetaxel, either alone or in combination. The current knowledge of pharmacology of the taxane drugs paclitaxel and docetaxel, mainly its pharmacokinetics and the proteins responsible for their biotransformation and transport, along with the genetic polymorphism responsible for variations in the activities of these proteins, opens new opportunities for dose selection for individual patients. Considering the relation between systemic exposure to these drug and clinical responses, *a posteriori* TDM, with measurement of drug concentrations in plasma of treated patients, is currently the most straightforward approaches for dose individualization of paclitaxel and docetaxel.

Keywords: Pharmacogenetics, metabolic genotyping, dose individualization, therapeutic drug monitoring, personalized medicine, taxanes paclitaxel, docetaxel.

1. INTRODUCTION

Taxanes, mainly paclitaxel and docetaxel, are commonly prescribed chemotherapeutic drugs used in the treatment of various solid tumors, such as prostate, breast and non-small-cell lung cancer [1]. Despite their clinical utility, taxanes, as many other anticancer drugs, present a narrow therapeutic window and its use is associated with potentially severe hematopoietic and neurologic toxicities [2].

The natural source of taxanes are the bark, needles and roots of several Taxus species, such as *T. brevifolia, T. baccata, T. chinensis, T. canadensis, T. cuspidata, T. globosa, T. floridana and T. wallichiana* [3, 4]. Besides natural sources, paclitaxel could be also produced by total chemical synthesis, which is usually not cost-effective [5-7], or by semi-synthesis, which uses intermediates such as10-deacetylbaccatin III or baccatin III, found in needles of *Taxus*. Docetaxel, a semi-synthetic analog of paclitaxel, is also synthesized from 10-deacetylbaccatin III [10]. Alternatively, paclitaxel can be also obtained for plant cell cultures of *Taxus* spp. [4, 5, 8-13].

Currently, taxane dosing is based mostly on body surface area (BSA). This dose selection approach is associated to a wide interindividual variation in drug expose and, consequently, on drugs effects [2]. However, with the increased knowledge of the pharmacoge-

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ANEXO II

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An Optimized High-Performance HPLC-PDA Method for the Clinical Application of Paclitaxel Therapeutic Drug Monitoring

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SUMMARY. **The aim of this study was to develop a method for the determination of paclitaxel in human plasma by high-performance liquid chromatographic with photodiode array detector, suitable for the clinical implementation of therapeutic drug monitoring of this chemotherapeutic drug. Liquid-liquid extraction was performed to extracted paclitaxel from 500 µL of plasma samples with a mixture of acetonitrile and 1-chlorobutane. Separation was performed in a Hypersil Gold C18 column (150 x 4.6 mm, 5 µm), eluted with a mixture of triethylammonium phosphate buffer 0.5 mM pH 3,4 and acetonitrile (52:48, v/v). The wavelength monitored was 227 nm. Retention time was 6.99 min for paclitaxel and 6.5 min for the internal standard (docetaxel 2 µg/mL), with total run time of 8 min. The method was linear from 10 to 500 ng/mL. Accuracy was 97.06-110.18%, intra-assay precision was 1.29 to 5.59%, and inter-assay precision was 3.34 to 9.27%. Processed samples are stable up to 12 h in the autosampler and for three freeze and thaw cycles. Paclitaxel concentrations obtained from 18 cancer patients were all within the linear of the assay. The method for determination of paclitaxel using high-performance liquid chromatography was developed, and presented suitable characteristics for the use in therapeutic monitoring of this antineoplastic drug.**

RESUMEN. El objetivo de este estudio fue desarrollar un método para la determinación de paclitaxel en plasma humano mediante cromatografía líquida de alta resolución con detector de matriz de fotodiodos, adecuado para la implementación clínica de la monitorización de fármacos terapéuticos de este fármaco quimioterapéutico. La extracción líquido-líquido se realizó para extraer paclitaxel de 500 µL de muestras de plasma con una mezcla de acetonitrilo y 1-clorobutano. La separación se realizó en una columna Hypersil Gold C18 (150 × 4,6 mm, 5 µm), se eluyó con una mezcla de tampón de fosfato de trietilamonio 0,5 mM de pH 3,4 y acetonitrilo (52:48, v/v). La longitud de onda monitoreada fue de 227 nm. El tiempo de retención fue de 6,99 min para paclitaxel y de 6,5 min para el estándar interno (docetaxel 2 µg/mL), con un tiempo de ejecución total de 8 min. El método fue lineal de 10 a 500 ng/mL. La precisión fue de 97.06-110.18%, la precisión dentro del ensayo fue de 1.29 a 5.59%, y la precisión entre ensayos fue de 3.34 a 9.27%. Las muestras procesadas son estables hasta 12 h en el automuestreador y durante tres ciclos de congelación y descongelación. Las concentraciones de paclitaxel obtenidas de 18 pacientes con cáncer estaban todas dentro de la linealidad del ensayo. El método desarrollado para la determinación de paclitaxel usando cromatografía líquida de alta resolución presentó características adecuadas para el uso en la monitorización terapéutica de este fármaco antineoplásico.

INTRODUCTION

Paclitaxel (PTX, **Fig. 1**) is an antineoplastic drug widely used for the treatment of solid tumors, and belongs to the taxane group of cytotoxic drugs [1]. The mechanism of action is based on selective tubulin polymerization that leads to apoptosis [2]. PTX pharmacokinetics is nonlinear, and could be affected by interaction with the solvent Cremophor EL (CrEL), which affects the distribution of PTX by entrapment in micelles, leading to a reduction of its plasma clearance [3,4]. Additionally, PTX interaction with CrEL also affects the reproducibility of PTX analytical measurements by chromatographic methods [5]. Metabolic clearance of PTX is also affected by genetic polymorphisms, mainly in the *CYP2C8* gene, which

KEY WORDS: antineoplastic drug, high-performance liquid chromatography, paclitaxel, therapeutic drug monitoring.
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ANEXO III

Comprovante de aceite do artigo intitulado "Analytical and clinical validation of a dried blood spot assay for the determination of paclitaxel using high-performance liquid chromatography-tandem mass spectrometry" à Clinical Biochemistry.

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