

Review Article

ANALYTICAL METHODS FOR TAXANES QUANTIFICATION IN DILUTED FORMULATIONS AND BIOLOGICAL SAMPLES AND THEIR APPLICATIONS IN CLINICAL PRACTICE

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ABSTRACT

Improvement of the rational use of classic chemotherapeutic drugs is currently a concern for health professionals, as these still constitute an important strategy for cancer treatment. Taxanes are a group of chemotherapeutic agents routinely used in the treatment of a variety of cancers. Their administration to patients is parenteral, which implies dilutions from commercial stock solutions to obtain the final individualized quantity of drug prescribed—that is, however, not usually confirmed. The absence of this post-dilution quality control can contribute to the variability of clinical response. Analysis of these diluted solutions is extremely important to assure an accurate content and, as a result, a safe and effective chemotherapy. On the other hand, therapeutic drug monitoring is essential to optimize individual therapy of drugs with a narrow therapeutic window such as taxanes. Drug quantification in plasma samples is important to establish pharmacokinetic parameters that allow dose adjustment and assess inter- and intra-individual metabolism variability, improving the effectiveness of therapy while minimizing adverse effects. Methods to quantify plasmatic concentrations of taxanes are, therefore, important in clinical practice, contributing to the individualization of treatments. This work made a bibliographic review and comparison of currently available analytical methods for these objectives. High performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and micellar electro-kinetic chromatography (MEKC) analytical methods were considered and compared for quantification of taxanes in hospital laboratories and it was concluded that, although LC-MS/MS is an extremely powerful technique, HPLC is versatile and cost-effective for both purposes.

Keywords: Analytical methods, Compounding, HPLC, LC-MS/MS, MEKC, Quality control, Taxanes, Therapeutic drug monitoring.

INTRODUCTION

Despite the development of more selective therapies for cancer, treatment schemes with classic chemotherapeutic drugs still continue to be an important strategy for cancer treatment [1] and the improvement of their rational use is currently a concern for health professionals.

Taxanes are a group of very effective chemotherapeutic agents routinely used in the treatment of a broad range of cancers, including ovarian, gastric, prostate, breast and non-small cell lung cancer [2]. These drugs are microtubule stabilizing agents (MSAs), also known as microtubule inhibitors (MIs) and their mechanism of action, as their designations suggest, involves stabilization of microtubules by binding to polymeric tubulin, preventing its disassembly, inhibiting the metaphase-anaphase transition, blocking mitosis and ultimately inducing apoptosis [3, 4]. The most commonly used taxanes in oncology are paclitaxel and docetaxel. Paclitaxel allows microtubule attachment but alters the tension across the kinetochore in mitosis [5, 6]. Docetaxel causes the disruption of the centrosome organization, affecting the late S, G₂ and M phases, resulting in incomplete mitosis, accumulation of cells in the G₂-M phase and cell death [7].

Paclitaxel was firstly extracted from the bark of Pacific yew tree *Taxusbrevifolia* in 1971 [7, 8] and it has been on the market since 1993 [9]. It is a highly hydrophobic molecule and is, therefore, administered in a solution together with ethanol and purified Cremophor® EL (CrEL), a formulation vehicle consisting in polyoxyethylated castor oil [10]. Alopecia, myelosuppression, gastrointestinal symptoms and febrile neutropenia are some of the common side effects of treatment with paclitaxel and peripheral neuropathy is especially related to this drug, increasing with cumulative dosage [7]. Moreover, CrEL can also cause severe hypersensitivity reactions, which may require premedication [10].

Docetaxel is semi-synthetically derived by from 10-deacetylbaccatin III, the inactive taxane precursor [11] isolated from the needles of

the European yew tree *Taxusbaccata* [7] and has been marketed since 1996 [9]. Docetaxel differs from paclitaxel at two positions in its chemical structure, originating a less hydrophobic molecule and is administered dissolved in polysorbate 80 (Tween 80) and ethanol, which causes considerably less hypersensitivity reactions comparatively to CrEL. Docetaxel presents similar side effects to those of paclitaxel and, additionally, causes edema and fluid accumulation, which can be dose-limiting in clinical practice. Increased neutropenia, skin and nail disorders and fluid retention are associated with the three-weekly regimen [7].

Adverse reactions and acquired and/or intrinsic resistance to paclitaxel and docetaxel present important concerns in clinical practice and, therefore, a number of new-generation taxanes, some with new delivery systems, such as albumin-bound macromolecules and antibody-drug conjugate technology [12] have been developed and are currently being assessed in Phase II/III clinical trials, with relevant clinical evidence [13-25]. Namely, cabazitaxel, a semi-synthetic derivative of 10-deacetylbaccatin III with low affinity to P-glycoprotein [26-28] has been approved recently by the Food and Drug Administration (FDA) [29] and European Medicines Agency (EMA) [30] for the treatment of castration-resistant metastatic prostate cancer in patients previously treated with docetaxel [31] and is currently being evaluated in metastatic breast cancer persistent after taxane or anthracycline treatments [28, 32].

Administration and quality control of taxanes diluted formulations in clinical practice

Paclitaxel and docetaxel are parenterally administered to patients. This implies dilutions from commercial stock solutions in sodium chloride (NaCl 0.9%) or glucose (5%) intravenous fluids to obtain the final prescribed individualized quantity of drug, in an appropriate concentration. Even though these diluted formulations are normally performed in controlled environment at the hospital pharmacy by specialized treatment, the process is not error-free [33, 34]. Careful supervision and double-checking of calculations, measurements and compounding and visual inspection for

preparation integrity are usually performed in order to reduce the risk of medication errors [35]. However, it does not constitute an accurate and sufficient post-dilution quality control in terms of compounding accuracy and can contribute to the variability of clinical response to taxanes treatment. Therefore, the analysis of the intravenous infusions that are administered to the patients is extremely important to assure their accurate drug content and, as a result, a safer and more effective chemotherapy [36]. Besides this ethical point of view, this quality assurance step is also necessary for health institutions accreditation. After implementation of a cost- and time-saving acceptance sampling plan, that would allow to reduce the number of samples assayed to provide accurate quality level estimates, the rate of non-conformity should be improved by identifying risk factors and taking correcting actions [37, 38].

Parenteral administration of taxanes is performed with infusion times ranging from 1 to 24 hours. It has been suggested that the efficacy is similar when comparing shorter versus longer infusion times and, therefore, in most clinical protocols taxanes are infused over 1 to 3 hours [39]. Taxanes are administered at fixed doses: paclitaxel is given at doses in the range of 100 to 200 mg/m² every 21 days, and docetaxel at doses ranging from 50 to 100 mg/m². Weekly treatment protocols for paclitaxel and docetaxel have also been evaluated at doses of 80–100 mg/m² and 30–40 mg/m², respectively [40, 41].

Paclitaxel and docetaxel pharmacokinetics

After intravenous administration, paclitaxel binds extensively to plasma proteins (89–98%) [42] and a triphasic elimination has been reported, with half-lives of 0.19, 1.9 and 20.7 hours. Paclitaxel has a large volume of distribution, between 50 and 650 L/m² [43], which indicates extensive tissue binding. Nevertheless, especially after a 3 hour infusion, the values of C_{max} and area under the concentration curves (AUC) increase non-proportionally with dose, suggesting nonlinear kinetics for these infusions with a saturable elimination process [44–48]. Hepatic metabolism and excretion into the bile is the primary elimination route for paclitaxel. Its transport is mediated by membrane-bound transporter proteins and CYP450 enzymes are involved in its metabolism [45, 47, 49], resulting in several pharmacologically inactive oxidation products. Some of these enzymes, namely CYP3A4, CYP3A5 and CYP2C8 [50], can present phenotypic variability and influence pharmacokinetic (PK) and pharmacodynamic (PD) parameters and are also responsible for paclitaxel's propensity to several drug-drug interactions.

Docetaxel is distributed in deep tissue, exhibiting a volume of distribution between 82 and 149 L/m² [51–54] and a terminal elimination half-life between 60.7 and 120 hours, with weekly

administrations (35 mg/m²) or administration every 3 weeks (60–100 mg/m²) [55]. As paclitaxel, it is strongly bound to plasma proteins, mainly metabolized by CYP3A4 and CYP3A5, influenced by P-glycoprotein and its elimination is mostly fecal. Its kinetic seems to be linear over the dosages between 55 and 100 mg/m² [53, 55].

The need for therapeutic drug monitoring

It is well accepted that the reported PK characteristics of taxanes result in inter- and intra-individual variability, which can alter the way the patient is exposed to drugs [47, 51]. Nevertheless, taxane dosing is currently based on body surface area (fixed doses), leading to significant inter-individual differences in plasmatic concentrations and the risk of severe, treatment-limiting adverse events. Diminished liver function is considered in some cases [42, 54].

Therapeutic drug monitoring (TDM) is an approach used to individualize the therapy employing systematic drug-level monitoring to adjust future dosages. It has been considered as essential to optimize individual therapies for drugs with extensive PK variability, narrow therapeutic windows and a well-defined relationship between systemic exposure (PK) and toxicity or response (PD) [56, 57], where the possibilities of over or under dosage exist, originating excessive toxicity or a suboptimal treatment, respectively [58].

Regardless of the TDM theoretical advantages, it is not required for the majority of cytotoxic drugs [58]. Besides, there are serious challenges to its widespread implementation: it requires the development of an assay for the drug of interest that quickly returns accurate results, introducing added healthcare costs (collection and analysis of patient samples, highly trained staff to interpret the results and recommend appropriate dose adjustments). It could also represent an increased burden for the patient [59].

Despite those challenges, drug quantification in blood or plasma samples is important to establish PK parameters that allow dose adjustment and assess inter- and intra-individual metabolism variability [60, 61], thus improving the effectiveness of therapeutics while minimizing side effects. Immunoassay methods have in some cases been used for TDM, although it is recognized that they can suffer from non-specific interference from related compounds, metabolite interference or matrix effects [58].

Fast, easy to perform, well developed and successfully implemented methods to quantify plasmatic concentrations of taxanes are, therefore, urgently needed in clinical practice, contributing to the individualization of treatments.

Table 1: Current analytical methods for the quantification of paclitaxel and docetaxel

Method	Sample	Sample volume (µL)	Sample pretreatment	Run time (min)	LLOQ	Refs.
HPLC	Human plasma	100	LLE	25	10 ng/mL	[62]
HPLC	Human plasma	1000-2000	SPE	15	0.8 ng/mL; 0.9 ng/mL	[63]
HPLC	Human plasma	500	SPE	30	3 ng/mL	[64]
HPLC	Human plasma	1000	SPE	13	5 ng/mL	[65]
HPLC	Diluted formulations	100	-	15	0.05 µg/mL	[66]
HPLC	Diluted formulations	5	-	35	0.2 µg/mL	[67]
HPLC	Diluted formulations	20	-	31	0.5 µg/mL	[68]
HPLC	Diluted formulations	10	-	35	0.24 µg/mL	[69]
HPLC	Diluted formulations	5	-	9	3 µg/mL	[36]
LC-MS/MS	Human plasma	50	PP	5	5 ng/mL	[70]
LC-MS/MS	Human plasma	1000	SPE	4	0.2 ng/mL	[71]
LC-MS/MS	Human plasma	2000; 250	Ultra filtration and LLE	10	0.4 ng/mL	[72]
LC-MS/MS	Human plasma	200	LLE	9	0.5 ng/mL	[73]
LC-MS/MS	Human plasma	50	LLE	3	5 ng/mL	[74]
LC-MS/MS	Human plasma	450	On-line SPE	10	0.14 ng/mL	[75]
LC-MS/MS	Human plasma	100	PP and on-line SPE	3	0.15 ng/mL	[76]
MEKC	Human plasma	1000	SPE	25	0.08; 0.22 µg/mL	[77]

HPLC - high performance liquid chromatography; LC-MS/MS - liquid chromatography-tandem mass spectrometry; MEKC - micellar electro-kinetic chromatography; LLE - liquid-liquid extraction; SPE - solid-phase extraction; PP - protein precipitation; LLOQ - lower limit of quantification.

Current methods for quantification of taxanes

A number of methods have been published for the quantification of taxanes in biological and diluted preparations, namely high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and micellar electro-kinetic chromatography (MEKC), which are summarized in Table 1. Considering biological samples, analysis of taxanes used as monotherapy or in combination with other drugs was performed mainly in plasma although urine, whole blood and serum were also occasionally reported. For comparison purposes, only plasma samples were considered.

These methods typically use some sort of sample pretreatment to remove proteins from blood or plasma samples prior to the analysis, as they can block frits and injectors. Matrix effects should also be minimized or eliminated to ensure proper analysis [78, 79]. Therefore, samples are usually pretreated using protein precipitation (PP), ultra filtration, liquid-liquid extraction (LLE), solid-phase extraction (SPE) or a combination of two of these methods, followed by an evaporation step, to prepare and concentrate the sample before analysis. PP is a very simple technique that involves precipitation of the proteins present in the sample with organic solvents, being able to be automated and performed in 96-well plates. However, it yields a dirtier extract after centrifugation that is also diluted with the solvent, which can create assay sensitivity issues. Ultra filtration is another simple technique that involves the retention of larger molecules such as proteins in a small pore membrane. LLE uses immiscible organic solvents, for example ethyl acetate, diethyl ether and hexane, and is thought to be the most effective technique to remove matrix effects, since ionized compounds, such as some phospholipids, do not readily partition into the organic phase, where the drug present in the sample will be extracted. Despite the high efficiency of LLE, it is difficult to automate, requires solvent extraction equipment that may not be available in clinical laboratories, can be time consuming and is environmentally unfriendly. SPE comprises the binding of an analyte to a stationary phase packed into a column, eliminating proteins and matrix interferences. The analyte is recovered by elution with an organic solvent, typically methanol or acetonitrile. SPE has a wider range of applications than LLE, but it is also more expensive. It can also be used in two distinct ways: off-line and on-line. Columns supplied as individual units and in a 96-well plate format for manual and automated use, respectively, are named off-line SPE, where samples are prepared separately from the LC-MS/MS equipment. In on-line SPE, the analyte is bound to a small column, washed with a weak solvent and finally eluted with a strong solvent, after switching the column onto an analytical one. PP is usually performed before on-line SPE to prevent instrument obstructions if the system does not include specifications that perform this step. Advantages of SPE and LLE over PP are the achievement of a cleaner extract and the inclusion of an additional concentration step, improving the method sensitivity [58, 80]. Quantification of taxanes in pharmaceutical diluted formulations did not include any preliminary treatment, despite the presence of CrEL and polysorbate 80 as concentrated formulation vehicles.

High performance liquid chromatography

HPLC is the most common analytical method employed to quantify taxanes. This technique provides precise and highly reproducible quantitative analysis, crucial features for quality control purposes, is flexible and customizable [81] with satisfactory specificity and sensitivity. The principle behind this technique consists on the passage of a sample in a liquid mobile phase under high pressure through a column, packed with the stationary phase. Separation is achieved according to the different affinities of the analyte with the mobile and stationary phases. This method is currently used for the analysis of DNA, proteins, glycosides, polyphenols, drugs and toxins, among many others [82-86]. Routine use of this technique involves quantification of analytes in biological samples, such as blood, urine and other body fluids. For taxanes analysis, reversed phase HPLC with ultraviolet (UV) detection is usually employed, due to the nature of these compounds.

Liquid chromatography-tandem mass spectrometry

To overcome the limitations in terms of selectivity and sensitivity of HPLC, a solution was found by coupling liquid chromatography to mass spectrometry, offering information rich detection [87]. LC-MS/MS has been routinely used in some clinical laboratories for over 10 years and has gained importance in the quantification of various drugs. This technique provides high specificity, sensitivity and separation capability, being the only alternative to immunoassay for compounds without natural chromophores or fluorophores [88]. LC-MS/MS can be useful in several fields such as TDM, pharmacology and toxicology [89-91]. On the other hand, the high instrument costs and technical complexity eventually limit the clinical use of LC-MS/MS to situations where no viable alternatives exist [87]. LC-MS/MS quantification of taxanes in biological matrices represent an increasing number of published methods, that normally employ HPLC-MS/MS, in positive ionization mode with an C18 analytical column [58].

The advances in LC technology allowed the development of new techniques and the combination of some previously created. Corona et al [76] reported a LC-MS/MS method with an on-line sample purification by a two column switching system using 100 μ L of plasma, obtaining an impressive LLOQ of 0.15 ng/mL, with runtimes as low as 3 min. Another example are ultra high pressure liquid chromatography (UHPLC) systems, that made possible the recent trend to use HPLC columns with sub-2 μ m particles, with much higher back pressures, obtaining increased peak resolution and sensitivity and reduced run times [88]. Turbulent flow liquid chromatography-tandem mass spectrometry (TFLC-MS/MS) is another hyphenated technique, which has been proposed as an alternative to LC-MS/MS, as it allows the on-line purification of biological samples, decreasing the amount of manual manipulation while keeping sensitivity levels [92]. It is also worth mentioning that LC-MS/MS methods for determination of cabazitaxel plasma levels are now starting to be published [93, 94], as well as an HPLC method for the quantification of felotaxel [95], a natural consequence of the development and clinical use of new taxanes.

Micellar electro-kinetic chromatography

MEKC has been gaining importance and instrumental advances over the last decades. In this capillary electrophoresis-based method, small diameter capillaries are used to perform highly efficient separations of small, large, charged and uncharged molecules [96]. Micelles function as a pseudostationary phase (PSP) and the aqueous buffer that surrounds them is considered the mobile phase. Separation is based on the different partitions of the analytes in the PSP and mobile phase, as neutral analytes can migrate into the micellar hydrophobic core [97]. MEKC has been applied to drug [98-101] and food/environmental analysis [102-104]. It is regarded as a simple, efficient and convenient technique, with low sample, solvent volumes and analysis time, being as an attractive alternative to conventional HPLC [105]. In the particular case of taxanes, and to date, quantification of paclitaxel using this method has been reported in plasma [77] and in urine [106] with UV detection by the same research group, which indicates that this is a relatively new application for this technique. MEKC can also be coupled to MS allowing the identification of drug impurities [107], extending the applicability of this technique.

Comparison of analytical methods

Considering the data from Table 1, it is clear that HPLC presents higher LLOQ than LC-MS/MS. This is particularly evident when on-line extraction methods are used. This reason alone would be an indication of the preference for the later for quantification of taxanes plasma levels. Other reasons for this assumption would also be the shorter runtimes (varying between 13 to 30 min and 3 to 10 for HPLC and LC-MS/MS), increased specificity and the large quantity of published work that would help in the implementation process of the technique in a hospital laboratory. However, investing in a LC-MS/MS equipment is very expensive and highly specialized personnel are needed to operate it and interpret results. On the other hand, both HPLC and LC-MS/MS require plasma samples pretreatment, which can be time consuming, effortful, require higher

sample volumes, and have incomplete recovery rates, resulting in quantification errors. Plasma volumes can vary between 100 to 2000 μL and 50 to 2000 μL , for HPLC and LC-MS/MS, respectively, which means that volumes needed for the two methods are essentially the same. HPLC methods reported were applied to 24 hours PK studies to quantify taxanes in patient's plasma after recommended doses and infusion times. In these studies, the plasmatic levels of taxanes at the 24 hour time point could still be accurately quantified, which means that they were above the LLOQ proposed. On the other hand, it has been stated that the high sensitivity of LC-MS/MS allows quantification of taxanes in patients plasma for more prolonged time periods than HPLC [108]. MEKC seems to be a promising technique for drug analysis purposes, as it is recognized to provide a fast and efficient separation, at competitive costs [109, 110]. However, the lack of published work for taxanes plasma analysis also limits its comparison with the former techniques and shows that more optimization and efforts are needed in order to achieve full method potential.

Regarding diluted formulations, HPLC is definitely the method of choice. This can be explained given the method sensitivity (LLOQ ranging from 0.05 to 3 $\mu\text{g}/\text{mL}$), since the final concentrations of taxanes preparations are required to be from 0.3 to 1.2 mg/mL and up to 0.74 mg/mL for paclitaxel and docetaxel, respectively [42, 54]. The use of LC-MS/MS would be inappropriate, as it would be more expensive. These samples do not require pretreatment, preventing analyte loss and allowing the method to be less time consuming and easier to perform. The lack of this step also enables the use of small sample volumes. This is an advantage, considering that it eliminates the need to prepare a larger volume of the diluted formulation for the analysis, so that the prepared intravenous bags can be directly sampled, without implying further monetary costs. However, for clinical applications, a runtime improvement would be important for a quick quality control check immediately after drug preparation. Interestingly, Delmas et al. [111] reported the development of a flow injection analysis (FIA) coupled with a HPLC-UV method, leading to extremely fast run times, for cytotoxic preparation control in a hospital setting.

MEKC has also been reported to be useful for the analysis of pharmaceutical formulations [99, 112] and to evaluate their stability [98]. However, applications for the particular case of diluted formulations of taxanes have not been published, to date.

CONCLUSION

The use of analytical methods to routinely quantify taxanes concentrations, in clinical practice, would ideally be easy to perform, inexpensive, require small sample volumes and minimal sample preparation or on-line extraction methods. None of the analytical methods currently found in use are ideal, however development efforts are being focused on these fields.

LC-MS/MS would be an excellent method of choice to quantify taxanes plasma levels. However, since it is not available in most hospital laboratories and the instrument costs are still very high, HPLC seems to be a reasonable and versatile alternative to both quantification of taxanes plasma levels in oncologic patients and to perform quality control of the diluted intravenous formulations, prior to administration. Nevertheless, development and validation of HPLC methods with on-line sample extraction would be an important asset. This would allow both an increase in sensitivity and a reduction of sample manual manipulation. MEKC seems to be a promising technique for taxanes analysis, although needing further methodological and instrumental improvement. Moreover, at present, studies concerning the development of methods for quantification of new-generation taxanes are also crucial.

These methods can also be extended to other cytotoxic agents, contributing to the development of PK/PD models to individualize and/or adjust cytotoxic doses within treatment schemes and the establishment of a safe cytotoxic supply chain in the hospital setting, by assuring content accuracy and stability.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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