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# High sensitivity ELISA determination of taxol in various human biological fluids

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**Abstract**

Taxol (paclitaxel)-the natural product isolated from Pacific yew (*Taxus brevifolia*) is a novel agent with high activity in the treatment of patients with several malignant tumors including those resistant to other cytotoxic drugs. The therapeutic index of this promising anticancer drug could be further increased by the exploration of its pharmacokinetic–pharmacodynamic relationship in cancer patients. Since taxol is highly protein bound, a very specific and highly sensitive analytical method is required in order to determine free, protein unbound and biologically active taxol species in human physiological fluids: plasma; plasma ultrafiltrate; and salivary fluids. In order to accomplish this, a new indirect competitive enzyme-linked immunosorbent assay (ELISA), for quantitating such a low bioactive taxol concentration level, has been developed in our laboratories. This method uses taxol competitive inhibition of mouse anti-taxol antibodies binding to the solid phase coated antigen 7-succinyltaxol-bovine serum albumin. This indicates recognition of the active taxol in the solution phase, where a diluted horseradish peroxidase labeled goat anti-mouse enzyme conjugate is used. While employing this technique, after systematic optimization of the experimental conditions, we are able to detect the anticipated taxol in plasma ultrafiltrate and salivary fluids at the concentration level of subpicogram per milliliter. The working range of the assay is approximately five orders in magnitude, i.e. from pg $ml^{-1}$ to 100 ng $ml^{-1}$. The clinical part of this study verified the working range of the ELISA method using samples of physiological fluids from a cancer patient treated with 3 h intravenous (IV) infusion of this drug. Our results of taxol determination in plasma, plasma ultrafiltrate and saliva demonstrate the applicability of the newly developed ELISA method for further pharmacokinetic studies of free, biologically active taxol species in cancer patients. 

*Keywords:* Taxol detection; Enzyme-linked immunosorbent assay (ELISA); Physiological fluids; Cancer; Anticancer drug

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## 1. Introduction

Paclitaxel (taxol) is one of the most active anticancer agents introduced in clinical oncology practice in the last decade. As a natural product, taxol was detected as the active component of the bark of the Pacific and Western yew in 1971, by the National Cancer Institute drug development program [1]. Further clinical development of taxol was delayed for many years due to difficulties in large-scale isolation and extraction. In the late 1970s the mechanism of action of taxol was described as unique among plant alkaloids, and other antineoplastic agents. Taxol affects the stabilization of microtubules, which are intracellular structures vital to mitosis and other critical cell functions [2,3]. Preclinical studies revealed experimental antitumor activity including tumors resistant to prior therapy but also with several hypersensitivity reactions. However, the results from the initial clinical studies revealed that hypersensitivity reactions could be prevented in cancer patients by pretreatment with antihistamines. Myelosuppression, particularly neutropenia, has been the major dose-limiting toxicity [4]. Several tumors have been found to be highly responsive to taxol treatment, with response rates over 30% for ovarian cancer [5–7], higher than 50% for breast cancer [8], and over 20% for non-small cell lung cancer [9,10], all resistant to previous chemotherapy. Since taxol is highly protein bound (about 98%), analytical methods to detect the free, unbound and bioactive fractions of taxol have to be very specific and highly sensitive. Current analytical methods for detection of taxol (taxanes) include high performance liquid chromatography-HPLC (Rizzo et al., 1990 [11]; Cardellina 1991 [12]; Harvey et al., 1991 [13]; Auriola et al., 1992 [14]; Willey et al., 1993 [15]), multimodal thin layer chromatography-MTLC (Stasko et al., 1989 [16]), a tubulin-dependent biochemical assay (Hamel et al., 1982 [17]), and micellar electrokinetic chromatography-MEKC (Chan et al., 1994 [18], Hempel et al., 1996 [19]). The chromatographic methods for quantitating taxol in different samples containing closely related taxanes (cephalomannine, baccatin III, and 10-deacetylbaccatin III) are relatively insensitive, laborious and time consuming and most of all, they cannot detect the biologically active forms of taxol in physiological fluids in ultra-low concentrations, i.e. pg $ml^{-1}$.

This would leave the immunoassay as the preferred method for quantifying analysis of taxol [20–23] at the required concentration levels. These assays provide highly sensitive and precise methods for the estimation of biological parameters, with the advantage that they can handle a large number of samples, which may be then analyzed more rapidly. Among the various immunoassay formats, the most common is the enzyme-linked immunosorbent assay (ELISA), which includes a separation step to remove the free antibody portion from the bound antibody. Several assay configurations can be used in ELISA, depending on the analyte and the availability and purity of the antibodies and the corresponding enzyme labels. In the 'sandwich-type' ELISA technique a 'capture' antibody is immobilized on a solid support of the microplate, sample is added and the analyte is allowed to bind to the antibody. After a washing step a second enzyme labeled 'detection' antibody is used to detect this complex. The assays are very selective because of two analyte–antibody binding steps. The competitive ELISA, used in this study, requires that the antibody compete for the immobilized and free analyte. The detection limit depends upon antibody affinity. ELISAs, performed in microtiter plates, are sensitive and can analyze multiple samples.

In this paper we are presenting the results on the development of a specific and sensitive ELISA method for the detection of taxol in physiological fluids and its application to determine the taxol level in samples obtained from one cancer patient following administration of taxol at 250 mg $m^{-2}$ doses by 3 h of intravenous infusion.

## 2. Experimental

### 2.1. Materials

7-succinyltaxol (7-SucTax) used in this study was purchased from Hawaii Biotechnology

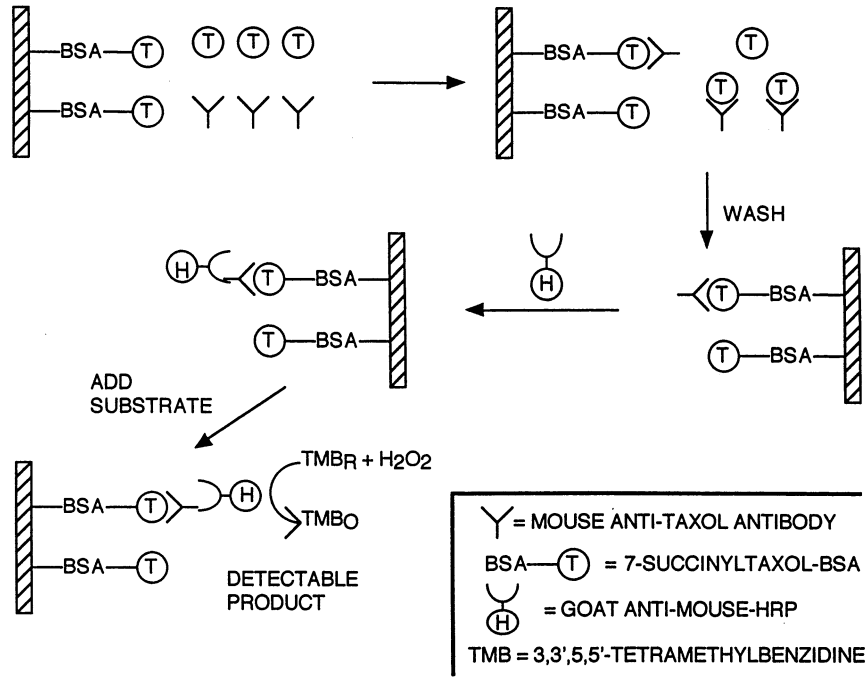


Fig. 1. A scheme of the ELISA method for taxol.

Group, Inc. (Aiea, HA) and partially obtained as a kind donation from Dr Richard R. Himes, Department of Biochemistry, University of Kansas, Lawrence, KS 66045. Taxol (*Taxus brevifolia;* Baccatin III *N*-benzyl-(-phenylisoserine ester; purity > 99% by HPLC) was purchased from Calbiochem (La Jolla, CA). Anti-taxol monoclonal antibody (Tax-Ab) was purchased from Hawaii Biotechnology Group, Inc. (Aiea, HA). Bovine serum albumin (BSA) and (EDAC) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-hydroxysulfosuccinimide (NHS) was purchased from Pierce Chemical Co. (Rockford, IL). Goat anti-mouse horseradish peroxidase (Gt x Mo-HRP) was purchased from Jackson Laboratories (West Grove, PA) and Spectrapor membrane tubing from Spectrum Medical Industries, Inc. (Los Angeles, CA). All other chemicals were purchased from Fisher Scientific (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI).

### 2.2. Methods

#### 2.2.1. Synthesis of BSA-7-succinyltaxol conjugate (BSA-7-SucTax)

To a 500 μl solution of 7-SucTax (0.6 mg, 0.63 μmol) in anhydrous *N*,*N*-dimethyl formamide (DMFO) were added 700 μl phosphate buffered saline (PBS, pH 7.0; 0.01 M), 300 μl of a 1.0 M EDAC solution (191.4 mg in 1 ml water) and a solution of NHS (0.2 mg, 0.92 μmol) in 500 μl PBS (pH 7.0; 0.01M). The reaction was stirred at room temperature for 10 min and then a solution of BSA (4 mg, 60 nmol) in a 1.00 ml of PBS (pH 7.0; 0.01 M) was slowly added. After stirring for 24 h at room temperature the dialysis was performed using Spectrapor membrane tubing and the concentration of BSA-7-SucTax protein was calculated on the basis of BSA concentration.

#### 2.2.2. ELISA for taxol

Competitive inhibition ELISA was performed on polystyrene ELISA 96 well microtiter plates

(Corning). A simplified scheme of the ELISA method is illustrated in Fig. 1. Microtiter high binding plate wells were coated for 2 h at 37°C with 100 μl per well of BSA-7-SucTax conjugate (5 μg $ml^{-1}$) in sodium bicarbonate buffer (pH 9.5; 0.1M). The plates were then washed four times with PBS (pH 7.4; 0.01M) containing 0.5% (v/v) Tween 20 (PBS-T-20) and blocked for 1 h at 37°C with 100 μl of PBS-T-20, containing 0.2% of ELISA grade (98–99%) BSA. After washing the plates four times with PBS-T-20 and drying them by the air at room temperature, the solid taxol (Paclitaxel, 99% purity) was dissolved in HPLC grade methanol (Tax-MeOH, 0.5 mg $ml^{-1}$). Physiological fluids (whole and parotid saliva, plasma, and plasma ultrafiltrate) were spiked with Tax-MeOH solution in order to obtain a serial dilution in a concentration range from 667 ng $ml^{-1}$ (0.13 μl Tax-MeOH per well) to 1 pg $ml^{-1}$. After the specific monoclonal Tax-Ab of the optimal concentration (78 ng $ml^{-1}$) of 0.2% BSA in PBS-T-20 (PBS-T-20-BSA, pH 7.4, 0.01 M) was added in the amount of 100 μl per well, the plates were again incubated for 1 h at 37°C. Microtiter plates were washed four times with PBS-T-20 (pH 7.4) and 100 μl of a 1:15000 dilution of horseradish peroxidase labeled Gt x Mo-HRP in PBS-T-20-BSA were added to each well and incubated at 37°C for 1 h. The plates were then washed four times with nanopure water and 100 μl of microwell peroxidase substrate system was added to each well. The reaction was stopped after 10 min by adding 50 μl of 1 N HCl to each well, and the absorbance was measured on Kinetic Vmax Microplate Reader (Molecular Devices Corp., Menlo Park, CA) using a sample wavelength of 450 nm and a reference wavelength of 650 nm.

## 3. Results

The main experimental variables used for the optimization of the assay were concentrations of BSA-7-SucTax sodium bicarbonate buffer (pH 9.50; 0.1 M), concentration of Tax-Ab, and the dilution of Gt x Mo-HRP conjugate in PBS-T-20-BSA. The pH level of the samples might be also considered as another variable. The sensitivity of the ELISA slightly increased as the alkalinity of our samples decreased (from pH 8.2 to the final pH 7.0) but this effect also improved the linearity of the absorbance (and the inhibition) plot, where the absorbance was plotted against the measured range of the concentration of Tax-MeOH in the physiological fluids. Optimal conditions, which lead to the lower limit of detection (LOD), were determined by optimizing of the previous factors. They are as follows.

The concentration of BSA-7-SucTax [5 μg $ml^{-1}$], the concentration of Tax-Ab [78 ng $ml^{-1}$] and a 1:15000 dilution of Gt x Mo-HRP enzyme conjugate in PBS-T-20-BSA.

Samples of taxol-spiked individual and pooled human physiological fluids (pH 7.0) were then analyzed under these optimal conditions. The following Table 1 describes our results.

In this part of the study, a very specific and highly sensitive ELISA was developed. While employing this new technique, we are able to detect taxol in human physiological fluids as shown in Table 1. The results indicate detection of taxol at subnanogram concentration levels. The implications of these results are two fold. First, the limit of detection in plasma is much lower than previously cited literature. Second, multiple physiological fluids (saliva and plasma ultrafiltrate) have been investigated. The range for quantitation is

Table 1
Limit of detection of taxol in human physiological fluids for different matrices

| Sample | Matrix | Detection |
|---|---|---|
| Saliva (whole) | Individual | Subnanogram (<50 pg $ml^{-1}$) |
| Saliva (whole) | Pooled | Subpicogram (<1 pg $ml^{-1}$) |
| Saliva (parotid) | Pooled | Subpicogram (<1 pg $ml^{-1}$) |
| Plasma ultrafiltrate | Individual | Subnanogram (<2 pg $ml^{-1}$) |
| | Pooled | Subnanogram (<3 pg $ml^{-1}$) |
| Plasma | Individual | Subnanogram (<350 pg $ml^{-1}$) |
| | Pooled | Subnanogram (<200 pg $ml^{-1}$) |

Table 2
Taxol recovery from spiked samples of physiological fluids (% mean recovery and % RSD)

| Concentration (ng ml$^{-1}$) | Saliva % (mean RSD) | | Plasma %(mean RSD) | | Ultrafiltrate % (mean RSD) | |
|---|---|---|---|---|---|---|
| 0.2 | 99.0 | 13.6 | 117 | 31.6 | 97.5 | 11.3 |
| 2.0 | 100.5 | 38.8 | 97.5 | 4.1 | 106 | 17.5 |
| 20 | 97.0 | 11.3 | 96.5 | 15.5 | 107 | 9.8 |

approximately five orders in magnitude (from pg ml$^{-1}$ to 100 ng ml$^{-1}$). Fig. 2 represents the inhibition curve for taxol concentration in plasma ultrafiltrate at pH 7.0 on the logarithmic scale revealing the sensitivity of about five orders of magnitude with the subnanogram detection limit. Fig. 3 shows the corresponding inhibition curve for the taxol concentration in the salivary fluids at pH 7.0. Also in this medium the broad sensitivity range exceeds five orders of magnitude with the subpicogram detection limit. This assay was applied then to the taxol determination of blood and salivary samples obtained from a cancer patient treated with taxol that was administered at 250 mg m$^{-2}$ doses in 500 ml of 5% dextrose by 3 h intravenous infusion. The samples were collected at the predetermined time intervals during the I.V. infusion (0, 0.5, 1, 2, and 3 h) and after the I.V. infusion (0.5, 1, 2, and 12 h). Samples of saliva and plasma ultrafiltrate were diluted in 1:100 ratio in PBS buffer solution. Plasma samples with an anticipated higher concentration of taxol were then diluted in 1:1000 ratio in the same solution.

Quality control of the ELISA method was performed with three measurements of known concentration per each sample type. The samples were spiked with taxol to final concentration of 0.2, 2.0, and 20 ng ml$^{-1}$ and applied into first twelve wells on the microtiter plate. Recovery results (% mean and RSD) are summarized in Table 2.

Concentration vs. time curves of taxol measured in the whole saliva, plasma and plasma ultrafiltrate samples from a cancer patient are shown in Fig. 4. They reveal the feasibility of this assay for the further clinical pharmacokinetic studies including the free, bioactive taxol species.

It is important to consider the possible interference of Taxol metabolites on the assay. Biliary excretion and hepatic metabolism have been described as the main elimination routes for taxol clearance [24]. 6α-hydroxytaxol is the principal

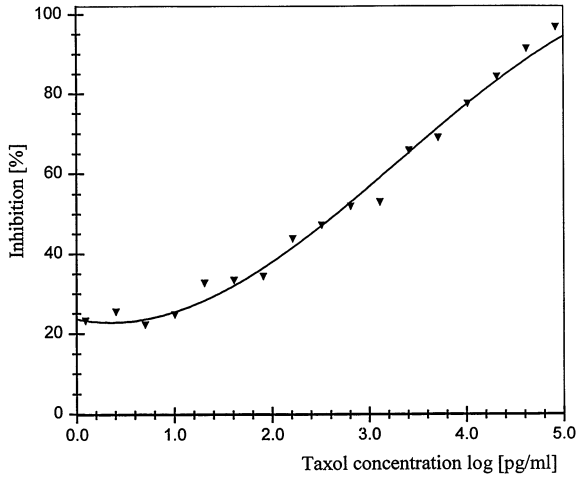


Fig. 2. Taxol calibration curve in pooled plasma ultrafiltrate matrix (pH 7.0).

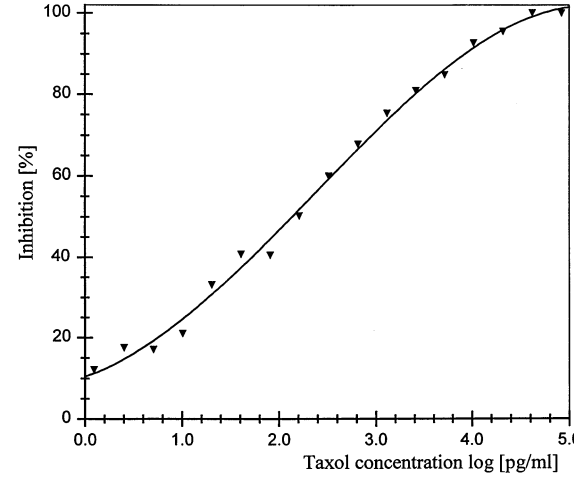


Fig. 3. Taxol calibration curve in pooled saliva matrix (pH 7.0).

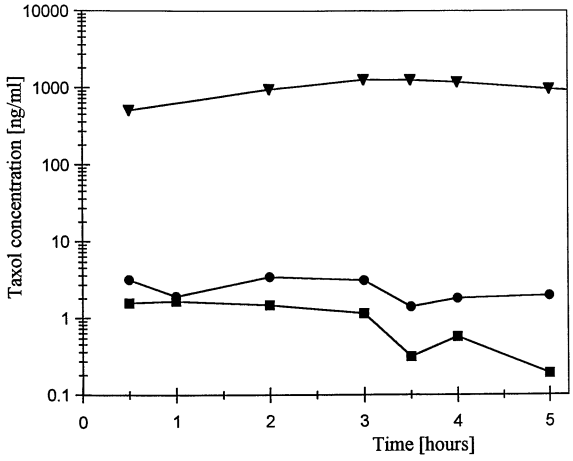


Fig. 4. Detection of taxol in human biological fluids by ELISA (▼, plasma; ●, plasma ultrafiltrate; ■, whole saliva).

human metabolite of taxol. The concentration versus time curve of this metabolite in plasma follows the same general pattern as taxol [25], but metabolite concentrations are far below the corresponding concentrations of the parent drug (10% or less). Since the formation of the taxol major metabolite (6α-hydroxytaxol) accounts for only a fraction of the total taxol concentration in human plasma, the effect of the metabolite on the assay results can be considered minimal even if metabolite binding is strong.

## 4. Discussion

Taxol is the most active anticancer drug introduced in the cancer chemotherapy in the last ten years. Although the pharmacokinetic-pharmacodynamic relationship of this drug has been firmly established [25,26], the free, protein unbound and biologically active taxol could not be used for such studies due to the lack of highly specific analytical methodology. Since taxol is highly protein bound and protein binding may be variable among cancer patients, it is anticipated that pharmacokinetic studies directly evaluating the free, bioactive taxol species could further increase the therapeutic index of this promising drug [27]. This paper presents the first evaluation of the free, bioactive taxol species in a cancer patient using the highly specific ELISA method developed in our laboratories. Development of the ELISA method with such a low detection limit (subnanogram $ml^{-1}$) was essential, since the HPLC methods available for the analysis of taxol concentrations in the physiological fluids have detection limits far above the levels required for the accurate determination of the taxol free, protein unbound forms. The very low detection limit, specificity, and a broad range of sensitivity of our new ELISA method not only enable the determination of the free biologically active taxol species, but also would enable pharmacokinetic studies of taxol administered at low dose continuous infusion [28], which until now could not be performed using the current HPLC methods. The feasibility of our new ELISA method was clinically tested on the samples from a cancer patient treated with this drug. Although our clinical results are limited, they indicate the potential of this method for further clinical pharmacokinetic studies using biologically active taxol species. The results suggest higher protein binding of taxol at lower plasma concentration that will require additional studies already planned in our laboratories.

In summary, a new original, sensitive and highly specific ELISA analytical method has been developed in our laboratories, with the determination limits of subnanomoles taxol concentration in physiological fluids. Our preliminary results indicate its feasibility for clinical pharmacokinetic studies using free, biologically active taxol species. This method, currently tested in clinical studies involving larger number of cancer patients treated with taxol, is aimed in the direction of further improvement of the therapeutic index of this drug.

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