

Short Communication

Measurement of Fraction Unbound Paclitaxel in Human Plasma

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ABSTRACT:

The clinical pharmacokinetic behavior of paclitaxel (Taxol) is distinctly nonlinear, with disproportional increases in systemic exposure with an increase in dose. We have recently shown that Cremophor EL, the formulation vehicle used for i.v. administration of paclitaxel, alters drug distribution as a result of micellar entrapment of paclitaxel, and we speculated that the free drug fraction (fu) is dependent on dose and time-varying concentrations of Cremophor EL in the central plasma compartment. To test this hypothesis, a reproducible equilibrium dialysis method has been developed for the measurement of paclitaxel fu in plasma. Equilibrium dialysis was performed at 37°C in a humidified atmo-

sphere of 5% CO₂ using 2.0-ml polypropylene test tubes. Experiments were carried out with 260- μ l aliquots of plasma containing a tracer amount of [G -³H]paclitaxel with high-specific activity against an equal volume of 0.01 M phosphate buffer (pH 7.4). Drug concentrations were measured by both reversed-phase HPLC and liquid scintillation counting. Using this method, fu has been measured in three patients receiving three consecutive 3-weekly courses of paclitaxel at dose levels of 135, 175, and 225 mg/m² and found to range between 0.036 and 0.079. The method was also used to define concentration-time profiles of unbound drug, estimated from the product of the total plasma concentration and fu.

Paclitaxel (Taxol; Fig. 1) is a naturally occurring taxane diterpenoid first extracted from the bark of the Western yew tree, *Taxus brevifolia* (Wani et al., 1971). The compound is a potent inhibitor of cell replication in malignant cells, a property attributed to its ability to stabilize the microtubule cytoskeleton and to block the transit of cycling cells from the G₂-phase to the M-phase (Verweij et al., 1994; Sparreboom et al., 1998c). The clinical pharmacokinetics of paclitaxel (administered as a 3-h i.v. infusion) have been well documented, and revealed a striking nonlinear disposition profile in plasma with disproportional increases in systemic exposure resulting from a given increase in dose (Schiller et al., 1994; Sonnichsen et al., 1994; Gianni et al., 1995; Bhalla et al., 1999; Karlsson et al., 1999).

It has recently been shown that Cremophor EL (CrEL¹), the vehicle used for i.v. drug administration, profoundly influences the cellular distribution of paclitaxel in human blood (Sparreboom et al., 1999a). Cellular kinetic experiments indicated that erythrocyte uptake of paclitaxel was significantly reduced by polyoxyethyleneglycerol tricristoleate (Fig. 1), the major compound present in CrEL, as a result of binding to CrEL micelles which, in turn, can reduce the free drug fraction (fu) available for cellular partitioning (Sparreboom et al., 1999a). Furthermore, we found that the altered blood distribution is highly dependent on dose and time-varying concentrations of CrEL in the central blood compartment during paclitaxel administration (Sparreboom et al., 1999b). This latter aspect of a concentration-dependent binding of paclitaxel to CrEL micelles occurring after therapeutic

doses suggests that the total plasma concentration (C_p), which is routinely measured, is not reflective of the unbound drug concentration (C_u). The rationale for monitoring C_u is founded on the basic pharmacologic tenet that drug bound to protein, or other (macro)molecules, is unable to cross cell membranes and interact with the active site. Although this relationship is intuitive, little has been published on this topic regarding anticancer drugs, with the notable exception of etoposide (Stewart et al., 1991). Knowledge of the extent of binding of paclitaxel is of crucial importance for understanding the clinical pharmacologic behavior of this drug, and could have significant clinical relevance in view of the fact that relationships between drug exposure and effect (i.e., toxicity and efficacy) are still poorly defined. To address these issues, we set out to define a reliable equilibrium dialysis method and demonstrate its application to binding measurements of paclitaxel in plasma samples of cancer patients receiving multiple courses of the drug administered by a 3-h i.v. infusion.

Materials and Methods

Chemicals. Paclitaxel powder (batch 484034; purity 98.3% by reversed phase HPLC) was obtained from Bristol-Myers Squibb (Woerden, The Netherlands). [G -³H]Paclitaxel (batch 227-163-0024; radiochemical purity 99.7%), with a specific activity of 2.4 Ci/mmol was obtained from Moravek (Brea, CA). The majority of the tritium label is in the *m*- and *p*-positions of the aromatic rings, with minor amounts in the 10-, 3'-, and 2-positions of the taxane ring system. Ethanol absolute was purchased from Merck (Darmstadt, Germany), and phosphate-buffered saline from Oxoid (Unipath LTD, Basingstoke, Hampshire, UK). The CrEL reference material was obtained from Sigma Chemicals Co. (St. Louis, MO). Emulsifier-safe scintillation cocktail was purchased from Packard Instruments Co. (Groningen, The Netherlands).

Equilibrium Dialysis. Paclitaxel fu was measured by equilibrium dialysis at 37°C in a humidified atmosphere of 5% CO₂ using test vials made from 2.0-ml polypropylene Safe-Lock vials (Eppendorf, Hamburg, Germany), carrying a 260- μ l inside recess in the lids (Reinard and Jacobsen, 1989). Before incubation, 2 μ l of a [G -³H]paclitaxel solution (500-fold diluted in ethanol) was added to 300 μ l of plasma, followed by mixing for 10 s. Dialysis was

¹ Abbreviations used are: CrEL, Cremophor EL; fu, fraction unbound; C_p, total plasma concentration; C_u, unbound concentration; AUC, area under the concentration versus time curve; CL, clearance.

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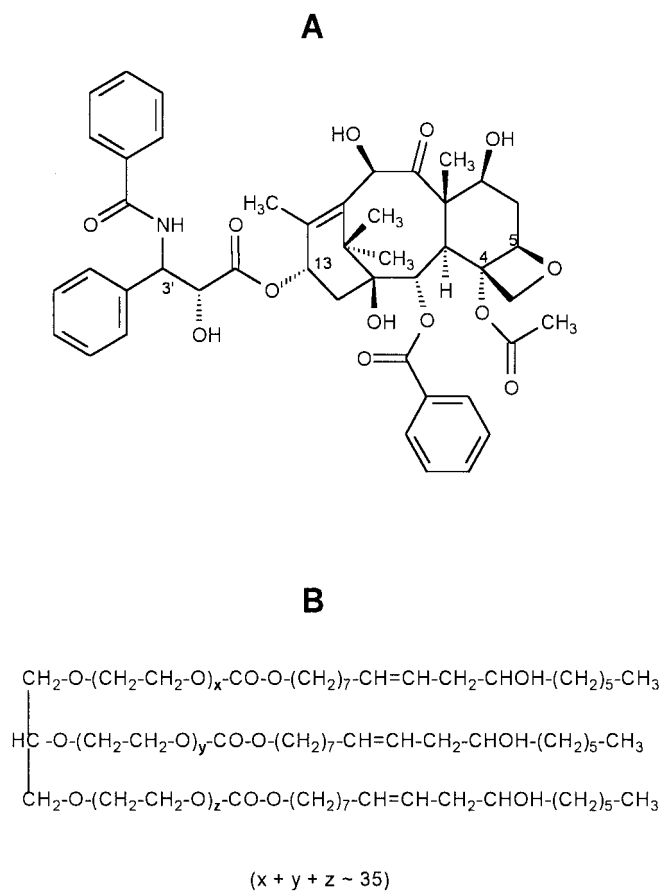


Fig. 1. Chemical structures of paclitaxel (A) and the major component of CrEL, polyoxyethyleneglycerol triricinoleate (B).

carried out with 260 μl of this sample against an equal volume of phosphate-buffered saline (pH 7.4) for 24 h in a moist chamber, which was shown previously to be sufficiently long to attain equilibrium (Kumar et al., 1993; Sparreboom et al., 1999a). Spectra/Por 3 dialysis tubing with a 12,500 molecular weight cut-off (Spectrum Medical, Kitchener, Canada) was soaked in phosphate-buffered saline (pH 7.4) before use. After establishment of the equilibrium, 150 μl of the buffer solution, containing only unbound paclitaxel, and 150 μl of the plasma fraction, containing both bound and unbound drug, were transferred to separate 2-ml vials (Eppendorf) and 1.9 ml of Emulsifier-Safe scintillation cocktail were added. After manual mixing for 30 s, the ^3H -labeled paclitaxel was quantified by liquid scintillation counting using a Wallac 1409 liquid scintillation counter (Turku, Finland). All samples were counted until a preset time of 20 min was reached. The ratio of drug concentrations measured in the buffer and plasma after dialysis was taken as an estimate of paclitaxel fu. Because the volume shift during dialysis was negligible (<10%), the results were used directly without applying a correction factor.

Pharmacokinetic Studies. The three patients studied were a 64-year-old female with non-small cell lung cancer, a 62-year-old male with bladder cancer, and a 51-year-old female with a malignant solid tumor of unknown primary origin for whom paclitaxel as monotherapy was a viable therapeutic option (Table 1). Before treatment, the patients had a World Health Organization performance status of <2, did not receive previous anticancer treatment with taxanes, and had adequate bone marrow function (white blood cell count, $>3.0 \times 10^9/\text{liter}$; platelet count, $>100 \times 10^9/\text{liter}$), renal function (serum creatinine, $\leq 140 \mu\text{M}$ and creatinine clearance $>60 \text{ ml/min}$), and hepatic function (serum bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase levels within normal limits).

Paclitaxel [Taxol; formulated in a mixture of CrEL and dehydrated ethanol USP (1:1, v/v) provided by Bristol-Myers Squibb] was diluted into 500 ml of isotonic sodium chloride before dosing. The drug was administered every three

TABLE 1

Characteristics of patients undergoing blood and plasma sampling during and after paclitaxel infusion

Characteristic	Patient 1	Patient 2	Patient 3
Baseline screening			
Age (years)	64	62	51
Gender	Female	Male	Female
Height (cm)	164	167	168
Weight (kg)	69.7	64.4	61.4
BSA (m^2)	1.76	1.72	1.70
WHO PS	1	1	0
Primary tumor	NSCLC	Bladder	ACUP
Pretherapy clinical chemistry values			
Serum creatinine (μM)	82	93	69
Total bilirubin (μM)	8.0	8.0	3.0
ASAT (units/l)	10	18	26
ALAT (units/l)	16	12	31
Total protein (mg/ml)	78	74	72
Serum albumin (mg/ml)	43	36	45
Worst observed toxicity (according to NCI-CTC grading)			
135 mg/m^2			
175 mg/m^2			
225 mg/m^2	Nausea 2	WBC 3/ANC 2	Nausea 1

BSA, body surface area; WHO PS, World Health Organization performance status; NSCLC, non-small cell lung cancer; ACUP, adenocarcinoma of unknown primary origin; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; NCI-CTC, National Cancer Institute common toxicity criteria; WBC, white blood cell count (leukocytopenia); ANC, absolute neutrophil count (neutropenia).

weeks as a 3-h i.v. infusion at consecutive (decreasing) levels of 225, 175, and 135 mg/m^2 . Premedication consisted of dexamethasone (10 mg i.v.), clemastine (2 mg i.v.), and ranitidine (50 mg i.v.), all given 30 min before start of the drug administration. During administration, no other comedication was given that might interfere with paclitaxel disposition. The clinical protocol was approved by the Rotterdam Cancer Institute Review Board, and the patients gave written consent before entering the study.

Blood samples (~6 ml) were collected in Vacutainer glass tubes containing 143 USP units of lithium heparin as anticoagulant at 1, 2, and 3 h during infusion, and at 5, 15, and 30 min, and 1, 2, 4, 6, 8, 12, and 21 h after end of the infusion. After agitation, 2-ml aliquots of whole blood were snap-frozen at -20°C at the patient site, and the remaining sample was centrifuged for 5 min at 4000g. Plasma was transferred into a polystyrene container and snap-frozen at -20°C . All samples were stored at -80°C until analysis. The concentration of paclitaxel in total plasma (C_p) and whole blood (C_b) was determined by isocratic reversed-phase HPLC with UV detection at $\lambda = 230 \text{ nm}$, as described (Sparreboom et al., 1998a). The unbound paclitaxel concentration (C_u) was estimated from the product of C_p and fu in each individual pharmacokinetic sample. The analytical procedure for CrEL in plasma samples was based on a colorimetric dye-binding microassay using Coomassie Brilliant Blue G-250 (Sparreboom et al., 1998b), with modifications as described (Brouwer et al., 1998).

Paclitaxel concentration-time profiles of C_u , C_p , and C_b were analyzed using the Siphar software package (version 4.0; SIMED, Créteil, France), by determination of slopes and intercepts of the plotted curves with multiexponential functions. The program determined initial parameter estimates, and these were improved using an iterative numerical algorithm based on Powell's method. Model discrimination was assessed by a variety of considerations including visual inspection of the predicted curves, dispersion of residuals, minimization of the sum of weighted squares residuals, and the Akaike information criterion. Final values of the iterated parameters of the best-fit equation were used to calculate pharmacokinetic parameters, including the terminal disposition half-life ($t_{1/2, z}$), area under the concentration-time curve (AUC) from zero to infinity, and clearance (CL, defined as dose divided by AUC). The peak concentration (C_{max}) was put on par with the observed drug level at the end of infusion. Noncompartmental analysis of CrEL plasma concentration data was performed as described previously (Sparreboom et al., 1998d). All statistical tests were calculated using the Number Cruncher Statistical System package (version 5.X; Dr. J. Hintze, Kaysville, UT).

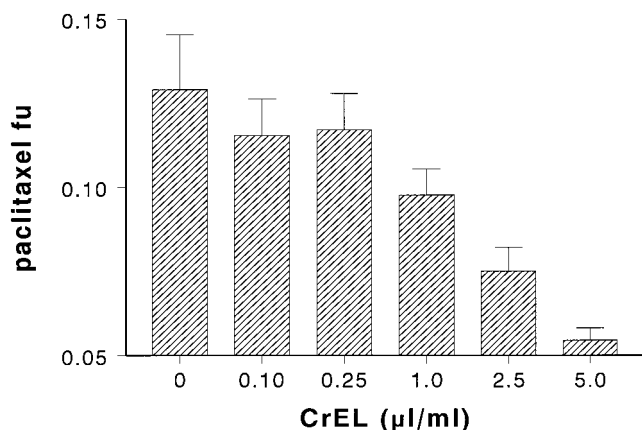


FIG. 2. Extent of binding of paclitaxel to human plasma, expressed as the unbound fraction (f_u), as a function of the spiked CrEL concentration.

Data are presented as mean values \pm S.D. of fourteen independent observations.

Results and Discussion

In Vitro Binding Experiments. We initially investigated ultrafiltration, as measurement of paclitaxel in the ultrafiltrate would provide a direct measure of Cu (Bowers et al., 1984). This technique, using a standard micro system (Amicon Centrifree, Danvers, MA), however, gave nonreproducible results and posed serious hurdles owing to a problem of sensitivity with currently available analytical methods for the determination of paclitaxel (reviewed in Sparreboom et al., 1998a). As a next step, we evaluated the possibility of using equilibrium dialysis to determine the effects of CrEL on paclitaxel f_u in human plasma. The major prerequisite in determining paclitaxel Cu is that the condition of the in vitro measurement should simulate those existing in vivo when the blood sample was taken. Hence, all measurements were carried out with undiluted plasma and at 37°C to give meaningful results. It was confirmed in all equilibrium dialysis experiments that the total drug recovery from all the fractions was equal to the amount of [G - 3 H]paclitaxel added to the plasma samples ($P > .29$ versus hypothesized mean of initial value).

In the absence of CrEL, paclitaxel was found to bind extensively to human plasma, probably due to nonspecific hydrophobic binding to both serum albumin and α_1 -acid glycoprotein (Kumar et al., 1993), with a mean (\pm S.D.) f_u of 0.13 ± 0.016 ($n = 14$). This value is within the same range as described previously in human plasma samples as determined by both equilibrium dialysis and ultrafiltration techniques (Longnecker et al., 1987; Wiernik et al., 1987; Jamis-Dow et al., 1993; Van Tellingen et al., 1999). Experiments demonstrated that the radioactivity seen in the protein-free fraction was not derived from any radioactive material that might have been associated with the polypropylene tube wall or the membrane. Very small amounts of 3 H-labeled water were formed, however, during the course of the 24-h incubation experiments. This was determined in selected buffer samples as the difference before and after drying (at 37°C) following equilibrium dialysis in the absence or presence of 5.0 μ l/ml (paclitaxel, 1.2 μ M). It accounted for $\leq 4.7\%$ of total radioactivity and was independent of the CrEL concentration applied. The buffer solutions collected after dialysis of paclitaxel (12 μ M) were also analyzed by a specific HPLC method (Sparreboom et al., 1998a) to exclude an artifact due to alteration of the fraction of total radioactivity associated with unchanged compound (not shown).

As f_u measurements were to be made on patient samples that contained variable amounts of paclitaxel, f_u was also determined in blank plasma samples over the entire anticipated concentration range (0.03, 0.12, 0.30, 0.60, and 1.2 μ M). Paclitaxel concentration had no

TABLE 2

Within-run and between-run variability for analysis of paclitaxel f_u in spiked human plasma samples in the absence and presence of CrEL

CrEL	GM	WRP	BRP	n
μ l/ml		%	%	
0	0.129	4.1	3.0	14
0.1	0.115	5.4	3.3	14
0.25	0.117	7.2	2.5	14
1.00	0.0977	4.5	2.9	13
2.50	0.0750	9.2	0.77	14
5.00	0.0545	5.8	1.4	13

GM, grand mean of paclitaxel f_u ; WRP, within-run precision; BRP, between-run precision; n , total number of observations.

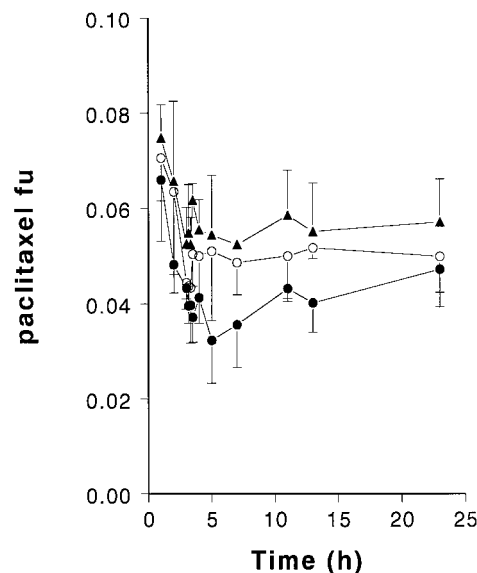


FIG. 3. Fraction unbound paclitaxel (f_u) versus time curves in three patients receiving three 3-weekly courses of the drug formulated at 6 mg/ml in a mixture of CrEL-dehydrated ethanol USP (1:1, v/v) at dose levels of 135 mg/m² (▲), 175 mg/m² (○) and 225 mg/m² (●).

Data are presented as mean values (symbol) \pm S.D. (error bar).

influence on f_u as determined by unweighted ANOVA ($P = .19$; $n = 36$), with an overall coefficient of variation of 4.2%, indicative of a lack in saturation of paclitaxel binding to human plasma. In the presence of CrEL, however, a clear and statistically significant decrease in f_u of paclitaxel (1.2 μ M) was observed, which was distinctly concentration-dependent within the clinical range ($P < .00001$, one-way ANOVA). At the highest CrEL concentration tested, i.e., 5 μ l/ml, a decrease of (about) 60% was noted in f_u as compared to f_u in the absence of CrEL (Fig. 2).

During the establishment of the method, duplicate or triplicate plasma samples with differing paclitaxel f_u values depending on the spiked CrEL concentrations were subject to repeated analysis on 6 consecutive days, to assess reproducibility. The mean relative deviation of these samples was 9.2% ($n = 82$), assuring high discriminatory power in the detection of changes in paclitaxel f_u in the presence of CrEL. With the final method, the within-run and between-run variability, expressed as the percentage relative standard deviation calculated by one-way ANOVA (Shah et al., 1991), were always less than 9.2% at the six CrEL concentrations analyzed (Table 2). These values are comparable with that obtained for a variety of other compounds using equilibrium dialysis or ultrafiltration techniques (Verbeek and Cardinal, 1985; Legg and Rowland, 1987), and were considered to be

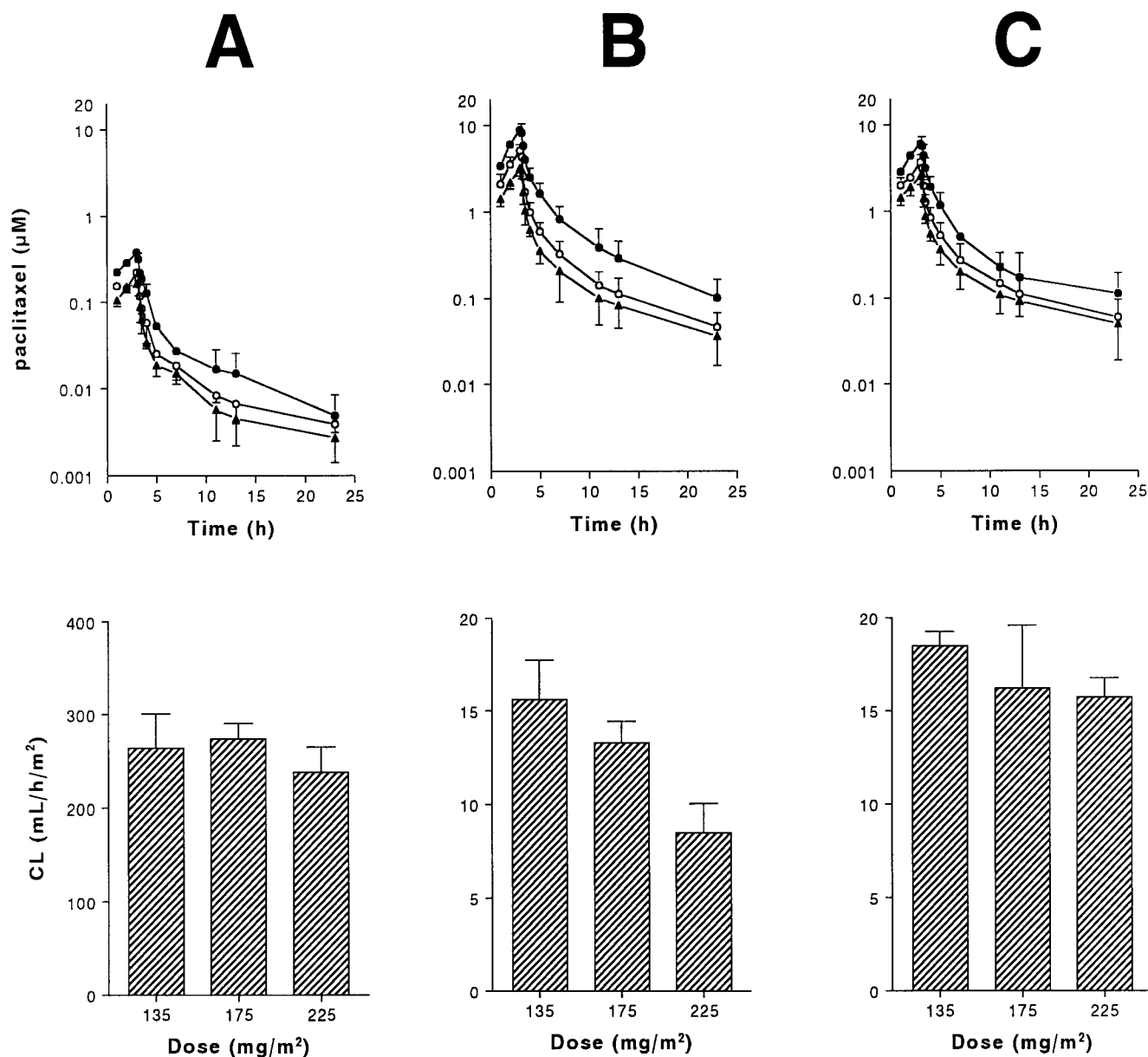


FIG. 4. Concentration-time curves of paclitaxel and paclitaxal clearance.

Upper panel, concentration-time curves of paclitaxel displayed as unbound concentration (A), total plasma concentration (B), and whole blood concentration (C) in three patients receiving three 3-weekly courses of the drug formulated at 6 mg/ml in a mixture of CrEL-dehydrated ethanol USP (1:1, v/v) at dose levels of 135 mg/m^2 (\blacktriangle), 175 mg/m^2 (\circ) and 225 mg/m^2 (\bullet). Lower panel, paclitaxel clearance of unbound drug (A), total plasma concentration (B), and whole blood concentration (C) as a function of the total dose administered. Data are presented as mean values (symbol) \pm S.D. (error bar).

acceptable for analysis of patient samples in support of pharmacokinetic studies.

Patient Studies. The developed method was applied to plasma samples of three patients treated with paclitaxel at three different dose levels. Similar to our in vitro experiments, a distinct CrEL concentration and time dependence was noted for paclitaxel fu (Fig. 3). Logarithmic concentration-time profiles of paclitaxel Cu, and total paclitaxel in plasma and whole blood are shown in Fig. 4 (upper panel). Plasma AUC values of total paclitaxel increased disproportional with dose from 10.2 ± 1.34 to 15.5 ± 1.38 and 31.8 ± 5.40 $\mu\text{M} \cdot \text{h}$ at dose levels of 135, 175, and 225 mg/m^2 , respectively, which is in excellent agreement with earlier studies (Gianni et al., 1995; Bhalla et al., 1999). Disproportionality was less pronounced with data based on fu and whole blood, as indicated by the respective clearance

values as a function of the dose administered (Fig. 4; lower panel). The overall mean values for paclitaxel fu and whole blood clearance [255 ± 33.1 $\text{l}/\text{h}/\text{m}^2$ (coefficient of variation: 13.0%) and 16.0 ± 3.22 $\text{l}/\text{h}/\text{m}^2$ (coefficient of variation: 20.1%), respectively] were relatively consistent in the three patients, suggesting minor interindividual variability. In addition, preliminary analysis showed that a linear two-compartment model could adequately describe the Cu versus time curves based on the Akaike information criterion ($r^2 = 0.99 \pm 0.01$; $P < .0001$), whereas linear models for total paclitaxel plasma data were significantly biased (not shown). The terminal disposition half-life of paclitaxel was similar between dose levels and analyzed matrices, with mean values of 6.54 ± 1.43 h (Cu), 7.10 ± 1.01 h (C_p), and 6.91 ± 0.97 h (C_b). In all, these findings, although preliminary, corroborate our hypothesis that the AUC of unbound paclitaxel should

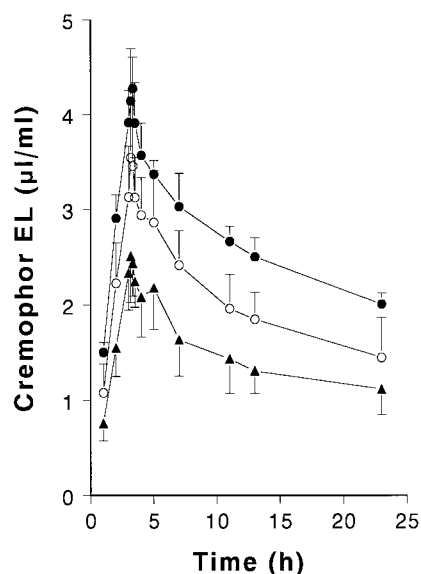


FIG. 5. Plasma concentration-time curves of CrEL in 3 patients receiving three 3-weekly courses of the drug formulated at 6 mg/ml in a mixture of CrEL-dehydrated ethanol USP (1:1, v/v) at dose levels of 135 mg/m² (▲), 175 mg/m² (○), and 225 mg/m² (●).

Data are presented as mean values (symbol) \pm S.D. (error bar).

be a linear function of the dose administered, in spite of the nonlinear disposition profile when only total paclitaxel plasma levels are considered (Sparreboom et al., 1999a). Mean plasma concentration-time profiles of CrEL following paclitaxel administration are shown in Fig. 5. As expected, the apparent plasma clearance of CrEL was dose-independent in each patient and averaged 331 ± 48.8 l/h/m², with C_{max} levels progressively increasing from 2.56 ± 0.48 to 3.60 ± 0.67 and 4.14 ± 0.55 µl/ml at the three consecutive dose levels, which is within the range described earlier (Sparreboom et al., 1998d).

In conclusion, we have demonstrated, by using a reliable and reproducible equilibrium dialysis method, that paclitaxel fu in human samples is significantly influenced by the presence of CrEL in vitro and in vivo. This effect of CrEL has been shown to be concentration-dependent, leading to changes in the pharmacokinetic behavior of paclitaxel. It is proposed that paclitaxel Cu should be monitored to further define relationships between drug exposure measures and pharmacodynamic outcome of treatment. Future studies will focus on this aspect in addition to the mechanism for the increased paclitaxel binding in the presence of CrEL.

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