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Determination of the docetaxel vehicle, polysorbate 80, in patient samples by liquid chromatography–tandem mass spectrometry

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Abstract

A new simple method was developed for the quantitative determination of the docetaxel (Taxotere) vehicle, polysorbate 80 (Tween 80), in human plasma. Calibration curves were constructed in the range of 1–100 μg/ml, using paclitaxel (0.01 m*M*) as internal standard, and were analyzed using a power fit with equal weighting. Sample pretreatment involved a one-step extraction with acetonitrile–*n*-butyl chloride (1:4, v/v). The analytes were separated on a Waters X-Terra MS column (50×2.1 mm I.D.) packed with 3.5-μm ODS material, and eluted with methanol–water (9:1, v/v) containing 0.1% formic acid. The column effluent was monitored by tandem mass spectrometry with electrospray ionization. The overall extraction efficiency was 50–60%, with values for precision and accuracy of ≤16% and <15% relative error, respectively. Our current method is ~60–100-fold more sensitive than previous assays, and will be used to define Tween 80 disposition in patients receiving Taxotere.

Keywords: Docetaxel; Polysorbate 80

1. Introduction

Docetaxel (Taxotere) is a highly lipophilic antineoplastic agent that is prepared by chemical manipulation of 10-deacetyl baccatin III, an inactive precursor isolated from the needles of the European yew tree, *Taxus baccata* L. [1]. The compound belongs to the class of taxane diterpenoids, and is a potent inhibitor of cell replication by stabilization of the microtubule cytoskeleton [1]. In clinical trials, docetaxel has proven to be active against various tumor types, including breast, lung, head and neck, and ovarian carcinomas, and has become standard second-line chemotherapy for patients with metastatic breast cancer [1]. For clinical use, the drug is formulated in the polyoxyethylated surfactant polysorbate 80 (Tween 80; polyoxyethylene (20) sorbitan monooletate) (Fig. 1) [2]. The amount of this vehicle that can be safely administered to patients is seriously limited due to the occurrence of various side-effects, including hypersensitivity reactions [3,4], vesicular degeneration [5] and dermatitis [6]. In addition, Tween 80 is known to alter membrane

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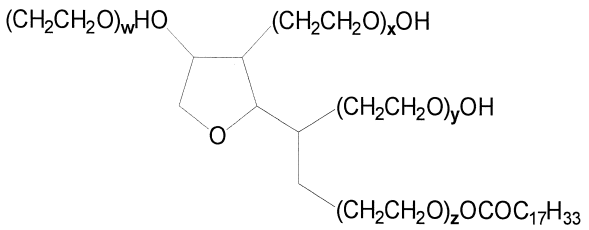

Fig. 1. Chemical structure of Tween 80 (polysorbate 80; polyoxyethylene (20) sorbitan monooleate). Chemical formula, $C_{64}H_{124}O_{26}$ (approximate); molecular weight, 1309.68 Da; density, 1.064 g/ml; specific gravity, 1.1.

fluidity leading to increased membrane permeability [7], and this may, at least in part, explain the observed cumulative-fluid retention observed with docetaxel therapy [8]. To further assess the impact of Tween 80 on the pharmacology and toxicity profile of docetaxel, defining of the pharmacokinetic behavior of this compound is mandatory [2]. To enable such studies, various analytical procedures have been developed in recent years. Initially, attention was directed toward measurement of the polyoxyethylated portion of the molecule, since it would be impossible to distinguish the fatty acid moiety, viz. oleic acid, from endogenous compounds [9]. The so-called polyol moiety is detectable by a wide variety of methods including a resorcinol-glucose precipitation, a colorimetric method using ammonium cobaltothiocyanate, turbidimetric or gravimetric procedures and complex formation with a barium phosphomolybdic reagent [10,11]. The ammonium-cobaltothiocyanate complexation has also been used in combination with HPLC and UV detection for analysis of Tween 80 in urine and ascites fluid, using either post-column [12,13] or on-line complexation [14]. More recently, a bioassay has been developed based on the ability of Tween 80 to modulate MDR1 P-glycoprotein-mediated daunorubicin efflux in multidrug-resistant T-cell leukemia VLB_{100} cells [15], as well as a method based on binding of the molecule to Coomassie brilliant blue G-250 in protein-free extracts [16]. Because of the complexity, limited sensitivity, and time-consuming nature of these assays, clinical applicability to large-scale pharmacokinetic studies is seriously limited. We now describe the development and validation of a novel assay for the determination of Tween 80 based on HPLC with tandem mass spectrometric detection (MS–MS). The method is rapid, relatively inexpensive, and easily utilized for processing large numbers of human plasma samples, as well as being adaptable to automation. To ensure the usefulness of the method in relevant clinical studies, Tween 80 was also determined in plasma samples from a cancer patient treated with Taxotere.

2. Experimental

2.1. Chemical and reagents

Tween 80 (lot no. 99H01002) and the internal standard, paclitaxel (lot no. 26H0284) were supplied by Sigma (St. Louis, MO, USA). Methanol and acetonitrile (both HPLC grade) were from EM Science (Gibbstown, NJ, USA), *n*-butyl chloride from AlliedSignal (Muskegon, MI, USA), and formic acid (88%, v/v, in water) from J.T. Baker (Phillipsburg, NJ, USA). Deionized and distilled water obtained from a Milli-Q-UF system (Millipore, Milford, MA, USA) was used throughout in all aqueous solutions. Drug-free (blank) human plasma originated from Pittsburgh Blood Plasma (Pittsburgh, PA, USA) or was obtained from healthy volunteers.

2.2. Sample extraction

Prior to extraction, frozen plasma samples were thawed in a water bath, and were homogenized by vortex mixing. The sample-pretreatment procedure was a modification of the method described for extraction of docetaxel from human plasma by Loos et al. [17]. Briefly, a 1-ml aliquot of plasma was added to a screw-cap culture tube (16×125 mm) containing 7.0 ml of a mixture of acetonitrile–*n*-butyl chloride (1:4, v/v) and 10 μl of a methanolic solution of paclitaxel (0.01 m*M*), used as internal standard. The tube was capped and mixed vigorously for 10 s on a vortex-mixer, and for 5 min on an automated-multitube shaker, followed by centrifugation at 2000 *g* for 10 min at ambient temperature. The top organic layer was transferred to a disposable borosilicate glass culture tube (13×100 mm). Next, the organic fraction was evaporated to dryness at 60 °C under a gentle stream of nitrogen. The residue was redissolved in 100 μl of methanol–water (1:1,

v/v) by vortex mixing (10 s) and ultrasonication (5 min), followed by an additional centrifugation step (2000 *g* for 8 min). Next, the sample was transferred to a 250-μl polypropylene autosampler vial, and a volume of 10 μl was injected into the HPLC instrument for quantitative analysis using a temperature-controlled autosampling device operating at 10 °C.

2.3. Equipment and conditions

Chromatographic analyses were performed using a Waters Model 2690 system (Waters, Milford, MA, USA) equipped with a Waters Model 996 photodiode-array detector. Separation of the analytes from potentially interfering material was achieved using a Waters X-Terra MS column (50×2.1 mm I.D.) packed with a 3.5-μm ODS stationary phase, protected by a guard column packed with 3.5-μm RP18 material at a column temperature of 40 °C. The mobile phase used for the chromatographic separation was composed of methanol–water (9:1, v/v) containing 0.1% formic acid, and was delivered isocratically at a flow-rate of 0.15 ml/min. The column effluent was monitored using a Micromass Quattro LC triple-quadrupole mass spectrometric detector (Beverly, MA, USA). The instrument was equipped with an electrospray interface, and controlled by MassLynx version 3.4 software (Micromass), running under Microsoft Windows NT on a Compaq AP200 Pentium III computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a collision energy of 35 eV and a cone voltage of 38 V. Samples were introduced into the interface through a heated nebulizer probe (350 °C). Initially, MS data were collected over *m/z* 300–1400, and eventually the spectrometer was programmed to allow the $[MH]^+$ ion of Tween 80 at *m/z* 1309.8 and that of the internal standard, paclitaxel, at *m/z* 854.9 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The daughter ions for Tween 80 (*m/z* 419.4) and the internal standard (*m/z* 509.4) were monitored through the third quadrupole (Q3). Argon was used as collision gas at a pressure of 0.0027 mBar, and the dwell time per channel was 0.3 s for data collection.

2.4. Calibration

Stock solutions of Tween 80 were prepared by dissolving ~10 mg, accurately weighed, in 1.00 ml of methanol–water (1:1, v/v), and were stored in polypropylene vial at −80 °C for up to 4 weeks without any significant degradation. One of the stock solutions was diluted further in blank human plasma on each analysis day to prepare calibration samples containing Tween 80 at concentrations of 1, 2, 5, 10, 50, and 100 μg/ml. Calibration curves were computed using the ratio of the peak area of Tween 80 and the internal standard by using a weighted (1/[nominal concentration]) non-linear power-regression analysis.

2.5. Method validation

Method validation was performed according to the guidelines recorded elsewhere [17]. All validation runs were performed on 3 consecutive days, and included a calibration curve processed in triplicate, and sets of quality control (QC) samples in duplicate. QC samples were prepared independently in blank human plasma at Tween 80 concentrations of 4, 25, and 80 μg/ml. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$DEV = 100 \times \{([\text{Tween 80}]_{\text{mean}} - [\text{Tween 80}]_{\text{nominal}})/[\text{Tween 80}]_{\text{nominal}}\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using the NCSS v5.0/1992 software package (J.L. Hintze, East Kaysville, UT, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$BRP = 100 \times \{\sqrt{((MS_{bet} - MS_{wit})/n)}/GM\}$$

where *n* represents the number of replicate observa-

tions within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$WRP = 100 \times \{\sqrt{(MS_{wit})}/GM\}$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from four different donors for the presence of interfering (endogenous or exogenous) peaks. The extraction efficiency of the assay was measured by comparison of extracted plasma samples and aqueous samples of Tween 80 at concentrations of 4, 25, and 80 μg/ml. The stability of Tween 80 in plasma was tested at the concentrations of the QC samples by three freeze–thaw cycles, for 4 h at 0 and 37 °C.

2.6. Patient samples

The patient studied participated in a clinical phase I study of Taxotere given in combination with irinotecan (CPT-11). The clinical preparation of Taxotere contained 40 mg of the active drug per milliliter of Tween 80, and a dose of 35 mg/m^2 was delivered as a 37-min intravenous infusion. The protocol was approved by the Institutional Review Board of The Johns Hopkins Oncology Center (Baltimore, MD, USA), and written informed consent was obtained prior to treatment.

Blood samples were collected in heparin-containing tubes before the start of infusion, immediately before the end of infusion (34 min), and at 14 and 25 min, and at 3.75, 7.77, 25.1, 49.2, and 164.5 h after the start of docetaxel infusion. The samples were centrifuged immediately for 10 min at 3000 *g* at ambient temperature, and the plasma supernatant was collected and frozen at −80 °C until later analysis.

3. Results and discussion

3.1. Analytical procedure

The mass spectrum of Tween 80 showed a protonated molecular ion ([MH$^+$]) at *m*/*z* 1309.8, in accordance with the NTP chemical repository database, and prominent fragment ion peaks at *m*/*z* 375.4, *m*/*z* 419.4, *m*/*z* 463.4, and *m*/*z* 507.2 (Fig. 2). The characteristic polymer-like pattern with a mass difference of 44 a.m.u. can also be seen in other polyoxyethylated-type surfactants (e.g. Cremophor EL, polyethylene glycols, and octyl or nonyl phenolethoxylates), since the repeating unit is also an ethoxylate group in these cases. From this series, the fragment daughter ion of Tween 80 at *m*/*z* 419.4 was selected for subsequent monitoring in the third quadrupole.

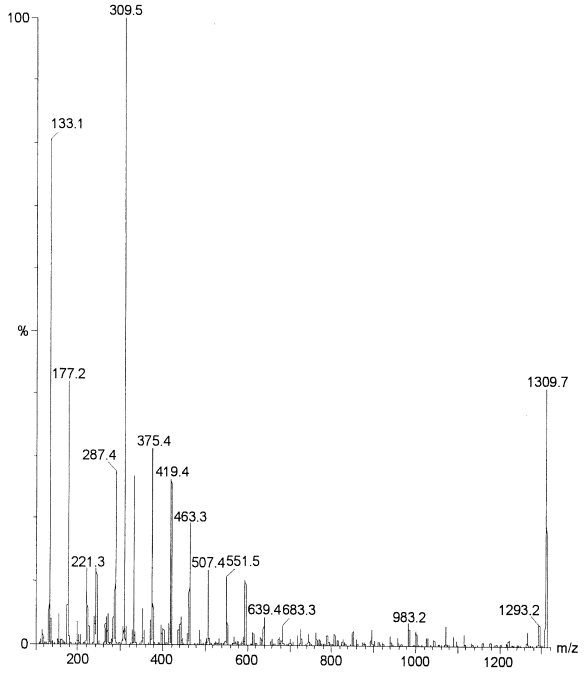

Fig. 2. Mass spectrum of a crude aqueous solution of Tween 80.

Since Tween 80 is freely soluble in short-chain alcohols (aqueous solubility, 50–100 mg/ml at 23 °C), sample pretreatment was initially performed by a single protein precipitation step with methanol or ethanol, with direct injection into the HPLC system. However, this resulted in unusual chromatographic behavior with separation into several peaks of various peak–area proportions (not shown). Protein-precipitation procedures using mixtures containing (strong) acids or bases also resulted in peak separation, probably related to acid-induced hydrolysis or saponification, a known characteristic of polyoxyethylates. Among various other procedures tested, Tween 80 was eventually efficiently extracted

with optimal elimination of endogenous interference using a mixture of acetonitrile–*n*-butyl chloride (1:4, v/v). This sample handling had been developed initially for the quantitative determination of docetaxel [17] and paclitaxel [18] in human plasma samples, and thus allowed the use of a known internal standard with high extraction efficiency for the current Tween 80 analysis. In the final procedure, only a small fraction of the sample after extraction was injected (i.e. 10 μl of 100 μl used for reconstitution) on the column to maintain high efficiency and resolution, and assay sensitivity was thus compromised. Although increased injection volumes could achieve higher response factors, overloading of the small column resulted in various kinds of distorted separation artifacts, including asymmetric sample bands.

In developing the present analytical assay for Tween 80 in human plasma samples, our previously published procedure for determination of sub-nanogram levels of docetaxel by HPLC with MS–MS detection was used as the point of departure [19]. In order to ensure sufficient selectivity in our new assay, we have slightly modified the mobile phase composition for the isocratic elution by increasing the organic modifier content to 90%, which resulted in reduced tailing bands arising from secondary retention effects.

Our previous data from stability studies indicated that Tween 80 was unstable in freshly-obtained human plasma samples after storage at 37 °C as a result of an esterase-mediated hydrolysis of the fatty-acid side chain on the core structure [16], necessitating rapid freezing of clinical sample after blood collection to prevent continuing degradation. In view of the relative stability of Tween 80 in plasma samples pretreated at −80 °C (see below), all calibration and QC samples were always prepared in a pre-frozen plasma matrix.

3.2. Method validation

Chromatograms of blank and spiked human plasma samples are shown in Fig. 3. The selectivity for the analytes is shown by the sharp and symmetrical resolution of the peaks, with no significant interfer-

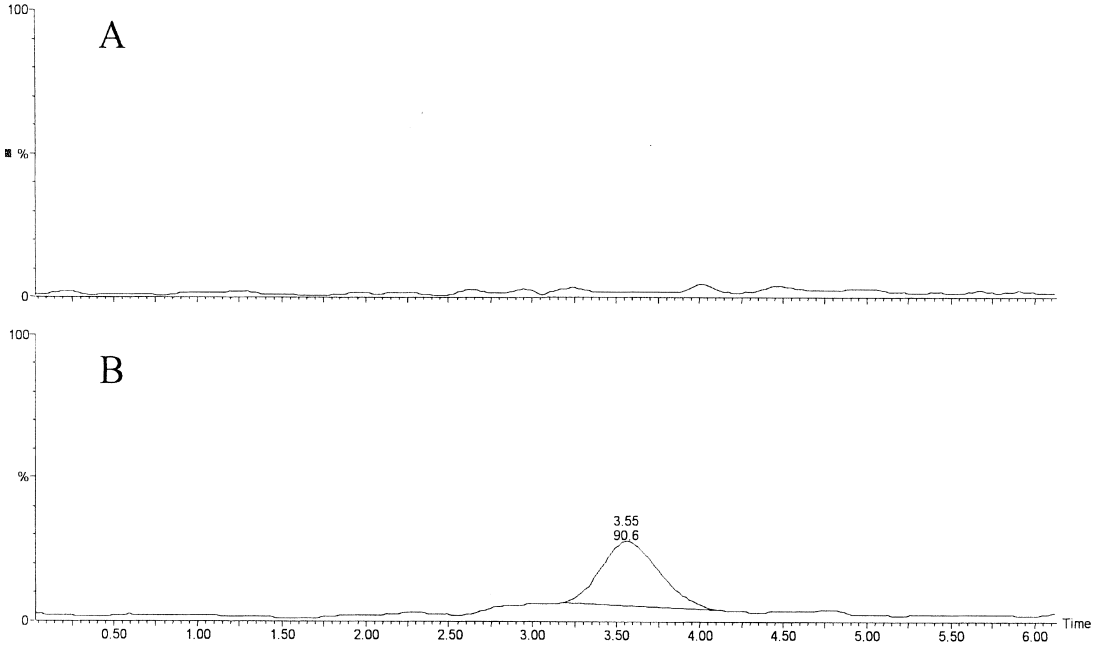

Fig. 3. Typical HPLC chromatograms of (A) a blank human plasma sample, and (B) a spiked human plasma sample containing Tween 80 at 1 μg/ml. Based on chromatographic behavior and spectrometric properties relative to pure reference standards, the major peak was identified as Tween 80 (t_R = 3.55 min).

ing peaks for both Tween 80 and the internal standard in drug-free specimens, obtained from four different individuals. The retention times for Tween 80 and paclitaxel under the optimal concentrations were 3.59±0.036 and 1.94±0.041 min, respectively, with an overall chromatographic run time of 10 min.

Calibration curves of Tween 80 in human plasma over the range of 1–100 μg/ml were best fitted using a power regression analysis, applying the peak height in combination with a weight factor (1/[nominal Tween 80 concentration]), as a result of a non-linearity in the response pattern (Fig. 4). This type of non-linearity has previously been observed with calibration curves of the related surfactant, Cremophor EL, constructed in human plasma [20]. However, regardless of the causes for this concentration-dependent behavior, it did not present any significant limitation with regard to the overall assay performance, as correlation coefficients for the calibration curves of ≥0.990 were observed throughout the validation. In addition, deviations from the interpolated Tween 80 concentrations of the standard plasma samples were all within the acceptable 80–120% of the nominal values (range, 95.3–106.7%). The mean regression equation was:

$$y = (0.0296 \pm 0.0151) \cdot x^{(0.778 \pm 0.068)} \quad (n = 9)$$

The lower limit of quantitation, defined as the spiked Tween 80 concentration at which at least 80% of the samples assayed were within 20% deviation from the nominal concentration, was established at 1 μg/ml. At this concentration, the values for accuracy, within-run and between-run precisions were 102.1, 3.01 and 4.33%, respectively. This represents an increase in sensitivity of ~60–100-fold as compared to previously published chemical assays based on HPLC with on-column derivation [12], colorimetric dye-binding in protein-free extracts [16], or (semi-quantitative) biological assays based on the ability to modulate MDR1 P-glycoprotein activity in cell culture systems [15]. The within-run and between-run variabilities, expressed as the percentage relative standard deviations, were always less than 20%, whereas the mean predicted concentration was within 14% of the nominal value at the three concentrations analyzed (Table 1). In addition, there appeared to be no evidence of analyte degradation in human plasma at various time periods and temperatures of storage (Table 1). By comparing the peak area ratios of Tween 80-containing plasma samples with those for non-processed samples prepared in the mobile phase, the mean extraction efficiency was found to be slightly concentration-dependent with mean values of 51.7% at 4 μg/ml, 55.1% at 25 μg/ml, and 61.6% at 80 μg/ml. An improvement in extraction recovery (~80%) could be accomplished by using 4-ml volumes of acetonitrile–*n*-butyl chloride (1:4, v/v) for primary isolation, followed by a repeat of the entire extraction procedure. However, in view of the relative consistency in the generated data, and the rapidity and ease of use, all further

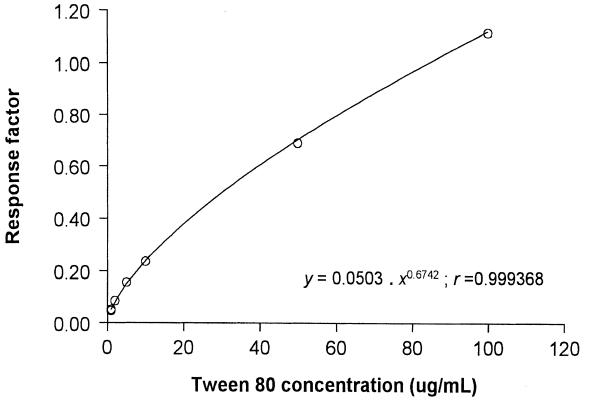

Fig. 4. Representative concentration (x)–response (y) curve for Tween 80 in human plasma fitted by a power regression analysis with equal weighting (mean equation: $y=(0.0296\pm0.0151)\cdot x^{(0.778\pm0.068)}$; $r=0.997$; number of calibration curves (n)=9).

Table 1
Validation characteristics of Tween 80 in human plasma

Nominal concentration (μg/ml)	4.0	25	80
Accuracy (%)	95.2	86.0	98.8
Precision (%)			
Within-run	14.8	10.0	15.8
Between-run	14.9	[a]	[a]
Stability (% of initial)			
Freeze–thaw cycles	101	ND	108
4 h at 0 °C	ND	104.8	ND
4 h at 37°	ND	142.4	ND
Extraction recovery (%)	51.7	55.1	61.6

[a] No significant additional variation was observed as a result of performing the assay in different runs. ND, not done.

experiments were performed using a one-step extraction. On the basis of the generated validation parameters, the method was considered acceptable for the analysis of plasma samples in support of clinical pharmacokinetic studies [21].

3.3. Plasma concentration–time profile

The suitability of the developed method for clinical use was demonstrated by the determination of Tween 80 in plasma samples from a single cancer patient treated with Taxotere at a dose level of 35 mg/m^2 (docetaxel dose, 65.8 mg; Tween 80 dose, 1.75 g). The plasma–concentration versus time curve of Tween 80 showed a distinct bi-exponential profile with a peak level of 304 μg/ml (Fig. 5), which is in agreement with previously published values that measured Tween 80 in plasma using a biological assay [15]. The total area under the plasma concentration–time curve (AUC), calculated up to the last sampling point with detectable levels (3.75 h) extrapolated to infinity, was 321.7 mg.h/ml. The disappearance of Tween 80 from the central plasma compartment was characterized by a short terminal disposition half-life of 1.07 h and a total plasma clearance of 5.44 l/h, which is within the same range as described for this compound previously in mice [16]. This relatively fast clearance, very distinct from that of the related vehicle Cremophor EL [22], likely reflects rapid esterase-mediated breakdown of the molecule within the systemic circulation [2]. The volume of distribution at steady-state was similar to the total blood volume (4.16 l), suggesting that Tween 80 circulates as large micelles and does not significantly distribute outside the central compartment.

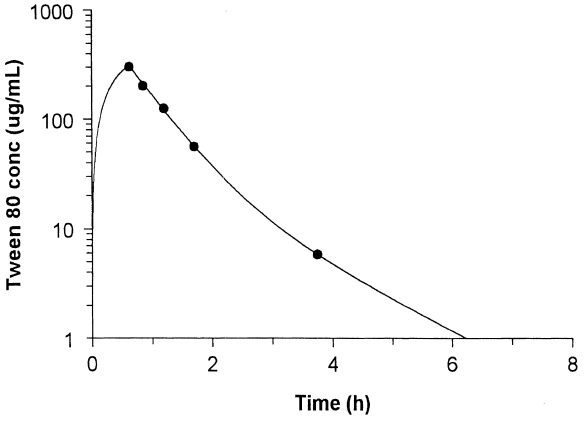

Fig. 5. Plasma concentration–time profile of Tween 80 in a single patient given a 37-min intravenous infusion of Taxotere at a dose level of 35 mg/m^2 (docetaxel dose, 65.8 mg; Tween 80 dose, 1.75 g).

In conclusion, we have developed and evaluated a novel assay for measuring Tween 80 levels in human plasma. The method was shown to meet the current requirements as to validation of bioanalytical methodologies, providing good accuracy and precision. The described method permits the analysis of patient samples to concentrations of 1 μg/ml, which is sufficiently sensitive to allow pharmacokinetic monitoring after intravenous administration of Taxotere at low doses. Knowledge of Tween 80 pharmacokinetics in cancer patients is of particular importance, as it may extend insight into a potential role of this vehicle in chemotherapy-induced toxicity profiles. To address this question, the assay is currently being used to measure Tween 80 concentrations in support of a project to develop a pharmacokinetic/pharmacodynamic model for this surfactant in patients receiving Taxotere.

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