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Adaptation of solid phase extraction to an automated column switching method for online sample cleanup as the basis of a facile and sensitive high-performance liquid chromatographic assay for paclitaxel in human plasma

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Abstract

An improved method for assaying paclitaxel in human plasma by high-performance liquid chromatography (HPLC) with UV detection at 227 nm has been developed by adapting previously reported sample preparation methods and chromatographic conditions to facilitate semi-automated sample cleanup using a column switching technique. Manual sample manipulations were limited to isolating the drug and internal standard from plasma (1.0 ml) by liquid–liquid extraction using tert-butyl methyl ether. The sample extract was initially loaded onto a short cartridge column containing a cyanopropyl stationary phase. During the predetermined time interval that the drug and internal standard eluted from the cartridge, 1.50–2.20 min, a fully automated 6-position switching valve was used to direct the effluent onto an octylsilica analytical column. The same mobile phase, composed of acetonitrile–methanol–ammonium acetate buffer (pH 5.0; 20 mM) (76:19:105, v/v/v) and delivered at flow rate of 1.0 ml/min, was used for both separations. The overall retention times of paclitaxel and the internal standard were 10.9 and 18.1 min, respectively. The analytical method was thoroughly validated for quantitating paclitaxel in plasma at concentrations ranging from 6 to 586 nM (5–500 ng/ml). The lowest concentration of paclitaxel measured with acceptable day-to-day accuracy (100.2%) and precision (RSD 11.7%, $n = 21$, 5 months) was 6 nM (5 ng/ml). The sensitivity and selectivity of the assay proved to be more than adequate for monitoring steady-state plasma concentrations of the drug when administered to cancer patients as a 96 h continuous intravenous infusion in combination with other anticancer agents, such as doxorubicin and topotecan. Moreover, the heart-cutting procedure prevented the problematic introduction of interfering nonpolar plasma components onto the analytical column, thereby enhancing sample throughput while decreasing the technical demands of the assay. The method was found to be extremely reproducible and robust during extended use for the routine analysis of plasma specimens acquired from several clinical trials.

Keywords: Paclitaxel; High-performance liquid chromatography; Column switching; Analysis; Plasma; Pharmacokinetics; Human, anticancer agents

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1. Introduction

Paclitaxel (Taxol®, Bristol–Meyers Squibb Co., Princeton, NJ) has shown encouraging clinical antitumor activity against breast, ovarian and non-small cell lung cancer [1,2]. It is most commonly administered at doses ranging from 135 to 250 mg/m^2 by a 3 or 24 h intravenous (i.v.) infusion once every 3 weeks. However, the therapeutically optimal dosing regimen has still not been established, even though the drug has been the object of intense clinical investigation throughout most of this decade [3,4]. The most promising alternative schedules appear to be a 1 h i.v. infusion repeated weekly and continuous i.v. infusion for periods longer than 24 h [1,5–9]. The rationale for a prolonged i.v. infusion derives from observations that the in vitro chemosensitivity of human carcinoma cell lines is substantially enhanced by extending the duration of exposure to paclitaxel beyond 24 h [10–12]. Furthermore, preliminary evidence of antitumor activity was obtained in several phase I/II trials of 96 h infusional paclitaxel in patients with breast cancer refractory to standard regimen chemotherapy, which includes doxorubicin, mitoxantrone, and taxanes given by short infusion [8,9,13]. These findings have generated interest at this institution to evaluate the use of paclitaxel in combination with other anticancer agents given as simultaneous 96 h i.v. infusions for treating women with recurrent ovarian cancer.

Provisions to monitor the plasma concentration of paclitaxel in patients during the infusion was considered to be a necessary component of these phase I combination studies. This was indicated by the demonstrated potential for pharmacokinetic interactions involving paclitaxel, as well asthe existence of a relationship between the severity of dose-limiting toxicities and duration of systemic exposure above a threshold plasma concentration of the drug [4,14]. It was previously shown that the maximum tolerated dose of single-agent 96 h infusional paclitaxel, 120–140 mg/m^2, provided steady state plasma concentrations ranging from 23 to 176 nM (mean, 50–60 nM) in breast cancer patients [1,9]. Since tolerated doses of paclitaxel could be lower when similarly administered concurrently with another cytotoxic drug, an assay with a limit of quantitation near 10 nM would be required to insure accurate measurement of drug levels during the infusion.

Although numerous assays for determining paclitaxel in plasma have been reported, as previously summarized [15,16], there are relatively few methods permitting specific quantitation of the drug at concentrations approaching 10 nM [17–20]. These more sensitive assays are invariably based upon reversed-phase high-performance liquid chromatography (HPLC) with UV detection at 227–230 nm. As a consequence of the low wavelength and relatively large sample volume required to maximize sensitivity, the sample preparation methods tend to be rather laborious, involving multiple manual liquid–liquid extractions (LLE), solid phase extraction (SPE), or a combination of both procedures, followed by extract concentration. However, co-isolated endogenous compounds that absorb prominently in the lower UV spectral region and which are more strongly retained than paclitaxel during HPLC have presented a recurrent problem for the analysis of plasma specimens [19,21–23]. The practice of flushing the analytical column between successive runs has been commonly employed to prevent detection interferences associated with the eventual elution of these compounds [19,21,23]. The objective of the presently described study was to identify a more efficient approach to resolve this problem, thereby enabling sample throughput to be substantially enhanced, without compromising assay sensitivity.

2. Experimental

2.1. Reagents and chemicals

The analytical reference sample of paclitaxel(M_r 853.92) was purchased from Sigma (St. Louis,MO). The following chemical were

used as supplied: 'Optima' grade acetonitrile and methanol, HPLC grade methyl tert-butyl ether (TBME) (Fisher Scientific, Fairlawn, NJ); reagent ACS ammonium acetate (Acros Organics/Fisher Scientific); ACS reagent acetic acid (99.7 + %), benzoyl chloride, octylamine and sodium hydroxide pellets (Aldrich, Milwaukee, WI). Distilled water was deionized and stripped of dissolved organics by passage through a Picosystem Ultrapure Water System consisting of mixed-bed resins and activated carbon (Hydro Service and Supply, Weymouth, MA). Outdated frozen human plasma was obtained from the Blood Transfusion Service, Massachusetts General Hospital (Boston, MA).

2.2. Internal standard synthesis

N-Octylbenzemide was synthesized by the reaction between octylamine and benzoyl chloride in the presence of 1.0 M sodium hydroxide according to the procedure described by Sharma et al. [24]. Recrystallization from methanol–water afforded 1.88 g (71% yield) of the product as a white solid (m.p. 43–45°C). Purity and structure confirmation were established by thin layer chromatography on silica gel and reversed-phase HPLC with electrospray ionization mass spectrometric detection.

2.3. Analytical solutions

Primary stock solutions of paclitaxel (0.10 mg/ml, 5 ml) and *N*-octylbenzemide (0.030 mg/ml, 25 ml) were prepared by accurately weighing the required amount of each compound on a Cahn C-34 analytical microbalance (ATI Orion, Beverly, MA). The samples were dissolved in methanol within class A borosilicate glass volumetric flask with PTFE-lined septum screw tops (Kontes, Vineland, NJ). These solutions were stored in a refrigerator (5°C) and used for up to one month. Standard solutions were made daily by serially diluting the paclitaxel stock solution with human donor plasma to provide concentrations of 500, 350, 200, 100, 50, 20, 10, and 5 ng/ml (586–6 nM).

2.4. Sample preparation

Plasma samples were prepared for analysis in disposable 10-ml borosilicate glass round-bottom culture tubes and conical-bottom centrifuge tubes with PTFE-lined phenolic screw caps (Fisher Scientific). The tubes were washed by overnight soaking in a 5% (v/v) solution of Contrad 70 Cleaning Agent (Fisher Scientific) in deionized water, then thoroughly rinsed with distilled water, and oven dried. All glassware was deactivated by treatment with a freshly prepared solution of 1% (v/v) Surfasil Siliconizing Fluid (Pierce Chemical, Rockford, IL) in HPLC grade hexanes (Fisher Scientific) and oven drying at 120°C.

Frozen plasma was thawed at 5°C, vortex mixed, and centrifuged for 10 min at 11 7500 × *g* to separate particulate matter and lipids. Plasma (1000 μl) and the internal standard solution (5 μl, 30 μg/ml *N*-octylbenzamide in methanol) were pipeted into a 10-ml glass centrifuge tube and mixed on a vortex stirrer. Following the addition of 5 ml of TBME, the tube was tightly capped and the contents mixed, initially by vortexing for 15 s, then by placing the tube horizontally in a model 6010 reciprocating shaker (Eberbach, Ann Arbor, MI) set at high speed for 10 min. Thereafter, the mixture was centrifuged (2500 × *g*, 5 min), upon which the upper organic phase was carefully removed and transferred into a glass culture tube using a silanized Pasteur pipet. This procedure was repeated once and the combined TBME extracts were evaporated using either an N-EVAP model 112 nitrogen evaporator (Organomation Associates, Berlin, MA) with a bath temperature of 40–45°C, or a HETO CS3 vacuum concentration system (Appropriate Technical Resources, Laurel, MD). The residue was dissolved by adding acetonitrile (100 μl), vortex-mixing for 15 s, then diluting with deionized water (200 μl). The reconstituted extract was clarified using a Costar polypropylene centrifugal filtration device with a 0.22 μm Nylon membrane (Corning Inc., Corning, NY) and centrifuging at 11 750 × *g* for 10 min. The filtrate was transferred into a silanized borosilicate glass insert which was sealed in an autosampler vial (12 × 32 mm) using a snap closure with a PTFE/silicone liner. A 250-μl

aliquot of the solution was injected into the chromatograph.

2.5. Chromatographic conditions

The chromatographic system consisted of two 1050 Series isocratic pumps and a 1050 Series autosampler fitted with a 500-μl sample loop and a 100-vial external tray (Hewlett–Packard, Wilmington, DE) A model 7000 six-port switching valve coupled to a model 5701 two-position pneumatic actuator was operated automatically with a type 7163 dual three-way solenoid valve (Rheodyne, Cotati, CA) controlled through the time-programmable + 24 V d.c. relay contact on each HPLC pump. The system was configured for heart-cutting as follows: port 1 of the switching valve (SV) was connected to a waste container; flow from pump A was directed to the autosampler then to SV port 2; a Brownlee Spheri-5 cyano cartridge column (30 × 4.6 mm i.d., 5 μm; Alltech Associates, Deerfield, IL) was connected between SV ports 3 (inlet) and 6 (outlet); pump B was connected to SV port 4; SV port 5 was connected to a NovaPak C_8 stainless steel column (15 cm × 3.9 mm i.d., 4 μm; Waters, Milford, MA). Both columns were preceded by 0.5 μm in-line filters (Upchurch Scientific, Oak Harbor, WA).

The same mobile phase, composed of acetonitrile–methanol–ammonium acetate buffer (pH 5.0; 20 mM) (76:19:105, v/v/v), was used for both the cleanup and analytical separations. The solution was degassed in an ultrasonic bath for 15 min before use. Chromatography was performed at ambient temperature with both pumps operating at a flow rate of 1.0 ml/min. The switching valve was initially positioned with ports 2–3, 4–5, and 1–6 connected to allow effluent from the cleanup column to flow to waste during sample injection. The valve was switched at 1.50 min postinjection to divert effluent from the cleanup column onto the analytical column for a period of 0.70 min, atwhich time the valve was switched back to the initial position. UV absorbance of the effluent from the analytical column was monitored at 227 nm (6.5 nm bandwidth), with a 1 s response time, using a Hewlett–Packard model 79853C Variable Wavelength Detector fitted with a 14 μl flow cell (8 mm pathlength). Operation of the chromatograhic system and data collection were completely controlled with HP ChemStation for LC software, rev. A.04.02, operating under Microsoft Windows95 on a Vectra XM 5/90 Series 3 computer (Hewlett–Packard). Chromatograms were integrated to provide peak areas using the data analysis functions of the software.

2.6. Assay validation

Accuracy, precision and linearity of the analytical method were evaluated by statistically analyzing the back-calculated drug concentrations and regression parameters from replicate calibration curves of paclitaxel in plasma. Within-day accuracy and precision were assessed from 5 sets of plasma standards, with drug concentrations of 0, 4.75, 9.50, 47.5, 95.0, and 475 ng/ml, that were prepared and analyzed on the same day. Data from a series of complete standard curves with eight concentration points that were assayed on different days, during a 5 month period, was used for calculating between-day accuracy and precision. The RSD of the mean predicted concentration for the independently assayed standards provided the measure of precision. The lower limit of quantitation (LOQ) was defined as the minimum drug concentration amenable to analysis with a between-day RSD not exceeding 20% [25]. Accuracy of the assay was determined by expressing the mean predicted concentration of paclitaxel as a percentage of the nominal concentration of the plasma standards.

Absolute recovery was calculated by comparing the chromatographic peak area of paclitaxel and the internal standard in extracted plasma samples to a paired reference solution. The reference solutions had drug and internal standard concentrations that were equivalent to the plasma standards following preparation for HPLC analysis, assuming quantitative recovery. They were made by spiking the paclitaxel stock solution into a vehicle composed of 0.5 μg/ml *N*-octylbenzemide in acetonitrile-water (1:2, v/v) to provide a drug concentration of 1.67 μg/ml, corresponding to the upper bound of the plasma standard curve (500 ng/ml). Serial dilution of this solution with the above

vehicle according the same scheme used to prepare the plasma standards afforded the remaining reference solutions. During the course of a single day, five aliquots from each of three different plasma standards, with drug concentrations of approximately 10, 50, and 100 ng/ml, were prepared for analysis and sequentially chromatographed in pairs with the corresponding reference solution.

2.7. Dosing and sample collection

Plasma specimens were acquired from patients with advanced ovarian cancer entered into phase I clinical trials designed to evaluate the administration of paclitaxel together with doxorubicin or topotecan by concurrent 96 h continuous i.v. infusions. The studies were approved by the Massachusetts General Hospital Institutional Review Board for Human Studies. All patients were fully counseled by the treating physician and signed an institutionally approved informed consent form prior to therapy. Blood specimens (7 ml), acquired daily during the infusion, were drawn from the arm opposing that used for dosing into Vacutainer Brand plasma tubes with freeze-dried sodium heparin (Becton–Dickinson, Franklin Lakes, NJ) The sample tubes were immediately placed on ice and centrifuged ($1500 \times g$, 10 min, 5°C) within 15 min after collection. The plasma was separated from the blood and stored in polypropylene cryotubes at -70°C until assayed.

2.8. Quantitation

A complete set of eight plasma standards with drug concentrations ranging from 5 to 500 ng/ml and a drug-free sample were prepared for analysis and run on a daily basis together with pharmacokinetic samples. Calibration curves were constructed by plotting the drug:internal standard chromatographic peak area ratio as a function ofthe nominal paclitaxel concentration of the plasma standards. Linear least squares regression was performed using a weighting factor of $1/y_{obs}$, without inclusion of the origin, to determine the slope, y-intercept, and correlation coefficient of the best-fit line. Results of the regression analysis were used to calculate the drug concentration in pharmacokinetic specimens.

3. Results and discussion

The isolation of paclitaxel from plasma and conditions for separating sample extracts by HPLC have been evaluated in considerable detail. TBME appears to be the solvent of choice for isolating paclitaxel directly from plasma by LLE [22]. Whereas the drug is quantitatively recovered from plasma upon extraction with TBME, the extraction efficiencies of ethyl acetate and chloroform are both substantially lower. Diethyl ether has also been used for extracting paclitaxel from plasma [16]. However, TBME has several definite advantages over diethyl ether, as it does not spontaneously form peroxides and is considerably safer to use due to its higher boiling point. Furthermore, unless removed shortly before using the solvent, the organic inhibitors of peroxide formation commonly used as additives to stabilize diethyl ether could prove to be significant sources of interference in an assay involving LLE as a method of sample preparation and low-wavelength UV detection. Paclitaxel can also be efficiently recovered from plasma by SPE using commercially available C-18 [19] and cyano [20] bonded-phase cartridges.

The chromatographic behavior of paclitaxel under isocratic reversed-phase conditions has been thoroughly studied [20,22]. The best separation of paclitaxel from degradation products, its principal metabolites and endogenous compounds extracted from biological fluids has been achieved on columns packed with an octylsilica stationary phase using a three component mobile phase composed of acetonitrile–methanol–ammonium acetate buffer (pH 5.0; 0.02 M) [16,20]. Chromatographic analysis of human plasma specimens prepared by LLE or SPE alone is complicated by the elution of endogenous compounds that are more strongly retained than paclitaxel under reversed-phase conditions [19,21,22]. Subjecting the material initially isolated from plasma by LLE to SPE prior to chromatographic analysis has been employed in an effort to remove these

lipophilic compounds [22,23]. However, this strategy has not satisfactorily resolved the problem, as indicated by the necessity to flush the analytical column with a stronger eluent than the analytical mobile phase between successive chromatographic runs when assaying clinical specimens [23]. Evidently, any potential selectivity advantage is lost because the strength of the eluent required to quantitatively recover the drug from the SPE cartridge in a relatively small volume (i.e., 0.5–2 ml) also promotes desorption of the more lipophilic endogenous compounds.

The chromatograms of human plasma shown in Fig. 1, determined according to the method initially described by Willey et al. [20] and modified by Sparreboom et al. [16,18], provide a more tangible understanding of the problem. This particular analytical method represents the most sensitive published procedure for measuring paclitaxel in plasma by HPLC with UV detection that has been sufficiently validated according to current guidelines [25]. The LOQ of the assay was reported to be 10 ng/ml for a 1.0 ml sample volume. To briefly summarize the procedure as reported, plasma samples were prepared for chromatography by initially extracting with diethyl ether, evaporating the solvent, then subjecting the reconstituted extract to SPE on a cyano Bond Elut cartridge. The material eluted from the cartridge with a solution of 0.1% (v/v) triethylamine in acetonitrile, upon evaporation and reconstitution, was loaded onto an octylsilica HPLC column, separated isocratically using a mobile phase composed of acetonitrile–methanol–ammonium acetate buffer (pH 5.0, 0.02 M) (4:1:5, v/v/v), and detected by UV absorption at 227 nm.

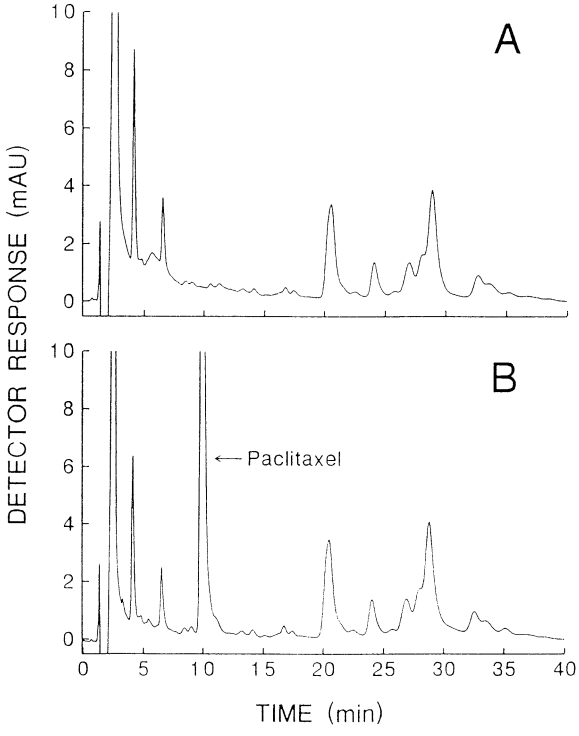

Fig. 1. Liquid chromatograms of drug-free human plasma (A) and plasma spiked with 1.0 μg/ml paclitaxel (B) prepared for chromatographic analysis by solid phase extraction as previously described [18], with minor modifications, depicting the elution of numerous endogenous peaks in the region from 20 to 35 min following paclitaxel (t_R 10.1 min).

The assay was implemented in our laboratory with several relatively minor modifications: substituting TBME for diethyl ether as the extraction solvent; using SPE cartridges and an analytical column from different manufactures, but with the same type of stationary phases; slight adjustment of the mobile phase composition. The appearance of the initial 20 min region of the chromatograms shown in Fig. 1 is very comparable to the chromatograms published by Willey et al. [20]. In both cases, paclitaxel eluted near 10 min with complete resolution from some relatively minor peaks that were also present in the chromatograms of drug-free plasma. Whereas the reported chromatograms were truncated at 20 min [20], we consistently observed numerous peaks of moderate intensity eluting between 20 and 40 min, as demonstrated in Fig. 1. Consequently, an effort was undertaken to modify this basic analytical method to prevent the introduction of lipophilic plasma components onto the analytical column and to simplify the technical demands of the sample preparation procedure. Online sample cleanup following TBME extraction of plasma using an automated column switching technique appeared to offer the most efficient approach to achieve these objectives. A substantial improvement in selectivity can very often be realized by adapting manual or semi-automated offline SPE to an online column switching method. This tech-

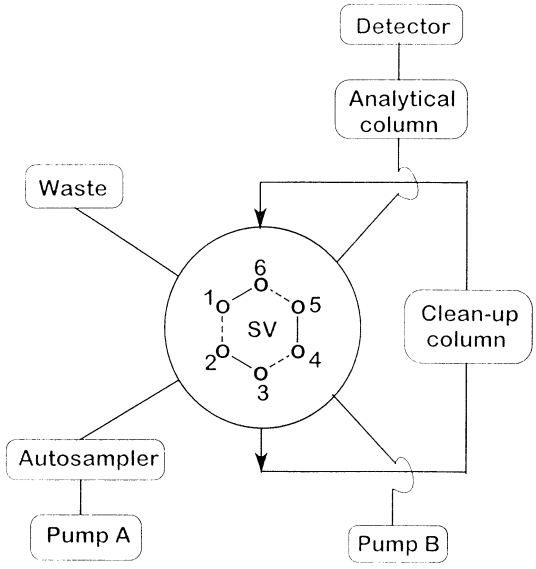

Fig. 2. Schematic representation of the chromatographic system and connections to the automated six-port switching valve (SV). Solid lines indicate the position of the switching valve during injection.

nique has been very satisfactorily implemented by our laboratories to facilitate the sensitive and specific determination of several drugs by HPLC with low-wavelength UV detection [26,27].

An attempt to use automated column switching for assaying paclitaxel in biological fluids has been described by Song and Au [17], but with limited success. In this method, ethyl acetate extracts of plasma were loaded onto a 7.5 cm octylsilica HPLC column. During the 8–15 min time interval that the drug and internal standard (cephalomannine) eluted from this column, the effluent was directed onto a 25 cm octadecylsilica column, from which paclitaxel eluted approximately 22 min after starting the run. A boxcar technique was used to increase sample throughput by injecting sequential samples onto the cleanup column every 15 min, before the separation of the previous sample on the analytical column had completed. However, newer HPLC instrumentation for which operation and data collection are controlled exclusively through computer software may not be able to accommodate this type of a run algorithm. Furthermore, separation of the sample is effectively achieved on the cleanup column, which is actually a short analytical column. Since the 25 cm analytical column does not significantly improve the separation of the peaks of interest, the apparent selectivity enhancement of the method results from the process of diverting flow from the cleanup column back to waste after the drug peak has completely eluted from it at 15 min. As a consequence, a considerable fraction, but not all, of the extractable plasma components that are more lipophilic than paclitaxel elute from the cleanup column to waste before the switching valve is again rotated 8 min after the next injection.

In our endeavor to adapt the sample preparation procedure described by Willey et al. [20] to an automated heart-cutting procedure, a 3 cm guard cartridge with a cyanopropyl stationaryphase was selected for use as the cleanup column. Aside from being consistent with the type of SPE cartridge used in the original assay, a cyanopropyl column should confer a greater degree of selectivity than a cleanup column with retention characteristics that were similar to the analytical column. The HPLC system consisted of a six-position automated switching valve, autosampler, two isocratic pumps, Speri-5 cyano cleanup column (3 cm × 4.6 mm i.d, 5 μm), a Nova-Pak C_8 analytical column (15 cm × 3.9 mm i.d., 4 μm), and a variable wavelength UV detector. A diagram depicting the manner in which these components were connected to the switching valve is shown in Fig. 2.

A series of chromatograms demonstrating the development and evaluation of the column switching method are shown in Fig. 3. The mobile phase used for this experiment was acetonitrile–methanol–ammonium acetate buffer (pH 5.0; 0.02 M) (76:19:105, v/v/v) at a flow rate of 1.0 ml/min. The mobile phase composition was based upon that developed by Willey et al. [20] with a minor adjustment of the organic strength to provide a retention time (t_R) for paclitaxel near 10 min on the analytical column. Under these conditions, paclitaxel eluted from the cleanup column as a very sharp peak, from 1.6–2.2 min baseline-to-baseline, with a t_R of 1.82 min (Fig. 3A). A chromatogram determined by separating a TBME extract of drug-free human plasma on the cleanup

column alone showed two sets of intense peaks, in the regions from 0.5 to 4 min and 6 to 10 min, with no peaks of significance eluting after 10 min (Fig. 3B). The remaining chromatograms shown in Fig. 3 were determined by loading TBME extracts of drug-free human plasma (Fig. 3C) or plasma spiked with 190 ng/ml of paclitaxel (Fig. 3D) onto the cyano cleanup column with the effluent flowing to waste. The switching valve was programmed to bring the cleanup column into the flow path between the analytical pump and column during the time interval from 1.50 to 2.20 min after starting the injection. In marked contrast to the chromatograms shown in Fig. 1, there were no significant peaks eluting either before or after paclitaxel (t_R 10.92 min) in these chromatograms. Thus, this procedure proved to be extremely effective in preventing the introduction of lipophilic endogenous components isolated from plasma onto the analytical column.

N-octylbenzemide was used as an internal standard for the method [24]. The primary factor for selecting this particular compound was that it co-eluted with paclitaxel on the cleanup column, thereby minimizing the time interval required to insure complete transfer of the drug and internal standard from the cleanup column to the analytical column. This was an extremely important

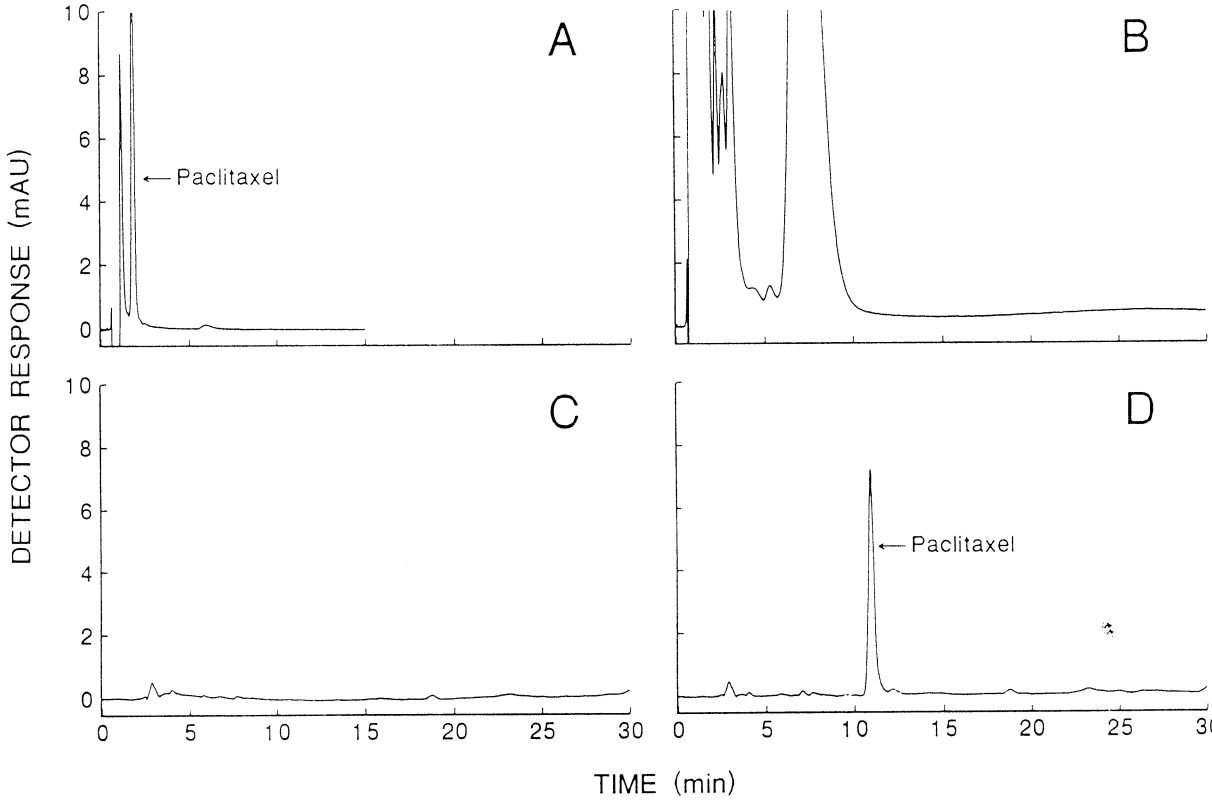

Fig. 3. Chromatograms illustrating the use of an online column switching technique to prevent the introduction of lipophilic plasma components onto the analytical column. The chromatograms shown in panels (A) and (B) were determined on a 3 cm cyano column: (A) 500 ng/ml paclitaxel in acetonitrile–water (1:2, v/v); (B) drug-free human plasma. Panels (C) and (D) depict chromatograms acquired by loading the sample onto the cyano column with effluent flowing to waste, then at 1.50 min after starting the run, flow from the cyano column was directed onto a 15 cm octyl–silica column for 0.70 min using an automated switching valve. Eluate from the octylsilica column was monitored by UV absorption at 227 nm: (C) drug-free human plasma; (D) 190 ng/ml paclitaxel in human plasma. Plasma samples were prepared for analysis as described in Section 2.4 and the chromatographic conditions are specified in Section 2.5.

consideration, because selectivity of the heart-cutting technique diminishes as a greater fraction of effluent from the cleanup column is introduced onto the analytical column. In addition, *N*-octylbenzamide was efficiently recovered from plasma by LLE with TBME and was stable in the solvent system used to reconstitute sample extracts. A solution of the internal standard was added directly to the plasma samples before processing for chromatographic analysis by LLE. Thus, in addition to correcting for variability in manual sample manipulations and the automatic injector, the internal standard served to monitor the performance of the cleanup column. Degradation in theperformance of the cleanup column would be indicated either by a trend toward decreasing area or greater variability in the area of the chromatographic peak for the internal standard during the sequential analysis of samples.

A reference solution containing paclitaxel (1.67 μg/ml) and the internal standard (0.5 μg/ml) in acetonitrile–water (1:2, v/v) was injected directly into the chromatograph to verify the performance of the system on a daily basis before running clinical samples. Chromatographic data compiled from these runs generally exhibited a high degree of consistency during routine use of the system over several weeks. For example, in the course of a typical month, when 270 plasma sample extracts were run on 11 days using the same cleanup and analytical columns, daily analysis of the reference solution afforded RSD values of 6.5% for the peak area of paclitaxel and 6.6% for the internal standard ($n = 10$). Retention times (mean ± SD) of paclitaxel and the internal standard were 10.99 ± 0.13 and 18.14 ± 0.27 min, respectively. Mean values of the peak width at half-height were 0.359 min with a 4.9% RSD for paclitaxel and 0.470 min with a 2.9% RSD for the internal standard. As indicated by this data, the cleanup column had a relatively long period of useful service and required infrequent replacement.

A total of 21 standard curves of paclitaxel in human plasma, consisting of eight calibration points spanning a 100-fold concentration range from 4.75 to 475 ng/ml (5.6–556 nM) plus a drug-free sample, were prepared and assayed on different days during a 5 month period by several different technicians. The paclitaxel-to-internal standard chromatographic peak area ratio increased proportionately to the nominal plasma concentration of drug throughout the range of the calibration curve. Calibration curves were sequentially analyzed by linear regression weighted according to y_{obs}^{-n} such that $n = 0, 1$ or 2. Residual analysis indicated that the weighting factor providing the best-fit of the calibration curves was the reciprocal of the observed peak area ratio [28]. The mean correlation coefficient for these standard curves was 0.999 ± 0.001 (SD). Mean values ($\pm$ SD) of the slope and *y*-intercept were 0.00402 ± 0.00048 and -0.0046 ± 0.0087, respectively. These results clearly indicate that the method is both linear and reproducible.

Absolute recovery of the internal standard from plasma, measured at the concentration used in the assay, was $87.8 \pm 3.6\%$ (mean ± SD, $n = 15$). The overall absolute recovery of paclitaxel determined at three concentrations ranging from approximately 10 to 100 ng/ml was $84.2 \pm 7.8\%$ ($n = 15$). Validation data depicting the accuracy and precision of the analytical method at concentrations encompassing the range of the standard curves are presented in Table 1. During the course of a single day, the drug was quantitated with a mean accuracy of $100.0 \pm 3.5\%$ (SD). Within-day precision, expressed as the RSD of five determinations, ranged from a minimum of 1.69% for the 475 ng/ml plasma standard to a maximum value of 8.91% at a concentration of 4.75 ng/ml. The mean predicted concentration of paclitaxel, calculated from the regression parameters of the 21 standard curves acquired over a 5 months, was similarly independent of the known concentration, ranging from 98.7 to 101.7%, with a grand mean of $99.9 \pm 1.2\%$. The plasma standard with the lowest concentration included in the standard curves, 4.75 ng/ml, was measured with an inter-day precision of 11.7%. This was established as the limit of quantitation for the assay since lower concentrations of paclitaxel could not be reliably measured [25]. These results serve to demonstrate that the assay is accurate, reproducible, and robust.

In confirmation of previously reported studies [17,20,22], both paclitaxel and the internal standard were found to be more than sufficiently

Table 1
Accuracy and precision of the method for the determination of paclitaxel in human plasma

Paclitaxel plasma concentration (ng/ml)	Within-day[a]		Between-day[b]	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
4.75	102.6	8.91	100.2	11.66
9.50	94.2	7.82	98.7	10.91
47.5	103.0	5.26	99.9	8.64
95.0	100.5	2.41	101.7	7.50
475.0	99.7	1.69	99.1	2.97

[a] Number of replicate determinations = 5.
[b] Determined from the back-calculated concentrations for 21 standard curves of paclitaxel in human plasma prepared and analyzed during a 5 month period.

stable in the reconstituted plasma extract at ambient temperature to permit overnight analysis using an automatic injector (data not shown). The stability of paclitaxel in human plasma at temperatures ranging from −20 to 37°C has already been thoroughly evaluated [20].

Typical chromatograms of plasma extracts of samples acquired from a cancer patient before and 22 h after starting treatment with 100 mg/m^2 paclitaxel given as a 96 h continuous i.v. infusion are shown in Fig. 4. Chromatograms of patient plasma specimens were extremely clean and highly reproducible, showing few peaks of significance other than the drug (t_R 10.9 min) and internal standard (t_R 18.1 min), even after numerous sequential runs. There were no interfering peaks in plasma specimens acquired from a group of 15 patients both before and during the concurrent administration of paclitaxel with other anticancer agents, including doxorubicin and topotecan, aswell as the usual spectrum of supporting medications. These observations serve to substantiate the excellent selectivity offered by this method. The average plasma concentration of paclitaxel achieved during the 96 h infusion of a 100 mg/m^2 dose to these 15 patients ranged from 21 to 70 ng/ml (24–80 nM). The mean total body clearance of the drug for the cohort, estimated as the ratio of the infusion rate to the average plasma concentration during the infusion, was 31.2 ± 10.7 l/h/m^2 This value was very similar to previously reported values of paclitaxel clearance when similarly administered as a single agent to breast

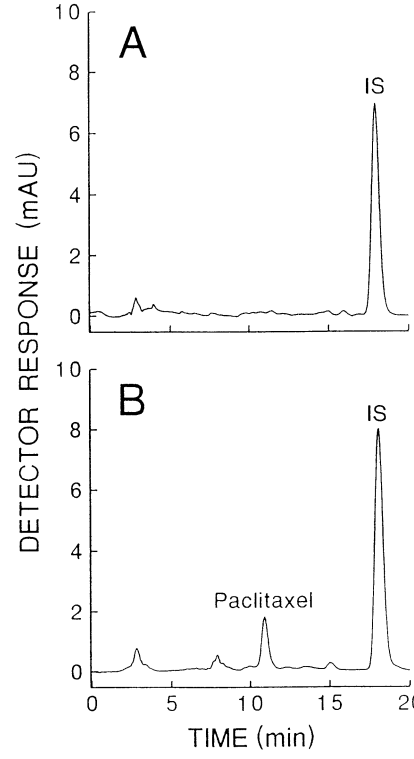

Fig. 4. Representative liquid chromatograms of plasma specimens obtained from a cancer patient prior to (A) and 22 h after (B) initiating treatment with 100 mg/m^2 paclitaxel and 2.0 mg/m^2 topotecan given as concurrent 96 h continuous i.v infusions. The concentration of paclitaxel in the sample shown in panel (B) was 48 ng/ml (56 nM). Chromatographic peaks: paclitaxel, 10.9 min; internal standard, 18.1 min.

cancer patients without metastatic liver involvement [8,9]. Thus, results based upon this analytical method are in excellent agreement with relevant pharmacokinetic data in the literature.

A relatively small endogenous peak eluting shortly after paclitaxel at about 12.3 min, but completely resolved from the drug peak, was consistently observed in chromatograms of patient plasma samples (Fig. 4). As a result, even though the drug and internal standard were separated by more than 5 min under these chromatographic conditions, the run time could not be significantly reduced by increasing the strength of the mobile phase without loss of the resolution between paclitaxel and the ensuing endogenous peak, thereby precluding quantitation of paclitaxel at concentrations approaching the limit of detection. Nevertheless, this assay represents a substantial improvement in both time and convenience, as compared to published analytical methods for determining paclitaxel in human plasma, which involve relatively long time delays or flushing the column with a strong eluent to remove lipophilic endogenous compounds between successive runs.

4. Conclusions

Numerous analytical methods for determining paclitaxel in biological fluids by HPLC with low-wavelength UV detection have been described in the literature. However, practical application of these procedures to quantitate paclitaxel in plasma at concentrations near 50 nM and lower has proven to be problematic due to lipophilic endogenous compounds that are invariably isolated from the sample together with the drug and which absorb prominently in the lower UV spectral region. Preventing interference with the detection of paclitaxel as these compounds eventually elute has required the use of a relatively long delay or flushing the analytical column with a strong eluent between successive chromatographic runs. The present study was undertaken to identify a more efficient approach to resolve this problem, which would enable sample throughput to be enhanced, without compromising assay sensitivity. These objectives were achieved by adapting previously reported sample preparation methods and chromatographic conditions to facilitate online sample cleanup using an automated column switching technique. This readily implemented procedure minimized manual sample manipulations, which were limited to those processes associated with the initial isolation of drug from plasma, and completely prevented the problematic introduction of interfering nonpolar plasma components onto the analytical column. Using a sample volume of 1.0 ml, the lowest concentration of paclitaxel quantified with acceptable day-to-day reproducibility was near 5 ng/ml (6 nM) (RSD 11.7%, $n = 21$, 5 months). The sensitivity and selectivity of the assay proved to be sufficient for pharmacokinetic drug level monitoring in cancer patients treated with paclitaxel in combination with other anticancer agents, such as doxorubicin and topotecan, given as simultaneous 96 h continuous i.v. infusions. It should be noted that the analytical method is highly selective for paclitaxel and does not permit the quantitation of its oxidative metabolises. However, plasma levels of the major metabolises achieved during prolonged infusion schedules of paclitaxel are very likely substantially lower than the parent drug and would not be detectable in any event.

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