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High-performance liquid chromatographic procedure for the quantitative determination of paclitaxel (Taxol®) in human plasma

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ABSTRACT

An isocratic high-performance liquid chromatographic method has been developed and validated for the quantitative determination of paclitaxel (Taxol®), a novel antimitotic, anticancer agent, in human plasma. The analysis required 0.5 ml of plasma, and was accomplished by detection of the UV absorbance of paclitaxel at 227 nm following extraction and concentration. The method involved extraction of paclitaxel from plasma, buffered with 0.5 ml of 0.2 *M* ammonium acetate (pH 5.0), onto 1-ml cyano Bond Elut columns. The eluent was evaporated under nitrogen and low heat, and reconstituted with the mobile phase, acetonitrile–methanol–water (4:1:5, v/v/v) containing 0.01 *M* ammonium acetate (pH 5.0). The samples were chromatographed on a reversed-phase octyl 5 μm column. The retention time of paclitaxel was 10 min. The validated quantitation range of the method was 10–1000 ng/ml (0.012–1.17 μM) of paclitaxel in plasma. Standard curve correlation coefficients of 0.995 or greater were obtained during validation experiments and analysis of clinical study samples. The observed recovery for paclitaxel was 83%. Epitaxol, a biologically active stereoisomer, and baccatin III, a degradation product, were also chromatographically separated from taxol by this assay. The method was applied to samples from a clinical study of paclitaxel in cancer patients, providing a pharmacokinetic profiling of paclitaxel.

INTRODUCTION

Paclitaxel (Taxol®), tax-11-en-9-one,5β,20-epoxy-1,2α,4,7β,10β,13α-hexahydroxy-4,10-diacetate-2-benzoate-13-(α-phenylhippurate), is a natural product extracted from the bark of the Western yew tree, *Taxus brevifolia* (Fig. 1). Paclitaxel was first isolated and identified as a potential antineoplastic agent in 1971 [1]. Paclitaxel has been licensed to Bristol-Myers Squibb Co. from the National Cancer Institute (NCI). Its development is a joint effort between the two institutions. Paclitaxel promotes microtubule assembly, and microtubules complexed with pacli-

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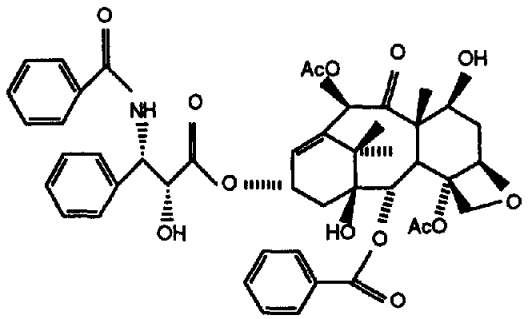


Fig. 1. Molecular structure of paclitaxel.

taxel appear to be stable against cold or calcium disassembly. Paclitaxel prevents cell division without affecting DNA, RNA, or protein synthesis. It is effective against a variety of cancers, such as resistant ovarian cancer and breast cancer [2–4]. Paclitaxel has shown significant antineoplastic activity in preclinical studies and is currently undergoing clinical evaluation. Since further pharmacokinetic and biopharmaceutic studies of paclitaxel would be beneficial, a rapid and sensitive high-performance liquid chromatographic (HPLC) method for the determination of paclitaxel in biological fluids was developed. This method has advantages over previously published assays described for paclitaxel [5–9]. It has a lower limit of quantitation, an extraction procedure that can be automated, a shorter run time, and no interference from either endogenous plasma constituents or common concomitant medications.

EXPERIMENTAL

Chemicals and reagents

Paclitaxel was obtained from the References Standard Laboratory of Bristol-Myers Squibb. Co. Epipaclitaxel, paclitaxel C, cephalomannine, and baccatin III were also prepared by Bristol-Myers Squibb Company. Control human plasma was obtained from Biological Speciality (Lansdale, PA, USA). HPLC-grade acetonitrile and methanol and glacial acetic acid (Reagent A.C.S. grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Absolute ethanol (U.S.P. dehydrated 200 proof) was obtained from Quantum Chemical, (Newark, NJ, USA). Ammonium acetate (Baker Analyzed reagent) was obtained from J. T. Baker (Philipsburg, NJ, USA). *n*-Hexane (high purity solvent) was obtained from American Burdick & Jackson (Muskegon, MI, USA). Triethylamine was obtained from Eastman Kodak (Rochester, NY, USA). All water was filtered and deionized with a Milli-Q system from Millipore (Bedford, MA, USA).

Preparation of standards and quality control samples

Standards were prepared from a 100.0 μg/ml stock solution of paclitaxel in ethanol. The high standard (1000 ng/ml) was prepared by diluting a portion of the paclitaxel stock solution with control plasma. The lower standards (10, 20, 50, 100, 200, 500, and 800 ng/ml) were prepared, immediately prior to each analytical run, by diluting the high standard with control plasma. Quality control samples, at concentrations of 10, 103, and 863 ng/ml, were prepared similarly from a different ethanolic stock solution prior to the start of sample collection.

Sample processing

Standard, quality control, and study samples were processed as a batch. All samples (0.5 ml) were buffered with 0.5 ml of 0.2 *M* ammonium acetate (pH 5.0) and mixed on a multi-tube vortex-mixer (Model 2600, Scientific Manufacturing Industries, Emeryville, CA, USA) for 20 s at a setting of 4. Paclitaxel was extracted from plasma by solid-phase extraction on 1-ml cyano Bond Elut columns (Analytichem International, Harbor City, CA, USA). The columns were first conditioned with 2 ml of methanol followed by 2 ml of 0.01 *M* ammonium acetate (pH 5.0). The columns were not allowed to dry. The samples were loaded onto individual Bond Elut columns and washed with 2 ml of 0.01 *M* ammonium acetate (pH 5.0), 2 ml of 20% methanol in 0.01 *M* ammonium acetate (pH 5.0), and 1 ml of *n*-hexane. The columns were dried under vacuum for 1 min. Paclitaxel was eluted from the columns into glass collection tubes using two 1-ml volumes of 0.1% triethylamine (TEA) in acetonitrile.

The eluents were evaporated to dryness under nitrogen gas at 30°C in a N-Evap (Organomation Assoc., Northborough, MA, USA). The residues were reconstituted in 200 μl of acetonitrile–methanol–water (4:1:5, v/v/v) containing 0.01 *M* ammonium acetate (pH 5.0 with glacial acetic acid) and vortex-mixed on a multi-tube vortex-mixer at a setting of 4 for 30 s. The reconstituted samples were transferred to WISP vials containing limited-volume inserts. The vials were randomized in the autosampler, and 100 μl were injected from each sample into the HPLC column.

HPLC instrumentation and conditions

Chromatography was performed using a Waters Model 6000A solvent pump and a Model 712 WISP autosampler (Waters Assoc., Milford, MA, USA). Detection was set at 227 nm with a Waters 484 tunable absorbance detector. An octyl 5 μm column (250 × 4.6 mm I.D., Jones Chromatography, Littleton, CO, USA) was used. Chromatograms were recorded on a Model BD41 flatbed recorder (Kipp and Zonen, Delft, Netherlands) and on a Model 3357 laboratory automation system computer (Hewlett-Packard, Palo Alto, CA, USA) for peak integration and quantitation.

The HPLC mobile phase was prepared by mixing 20 ml of 1.0 *M* ammonium acetate (pH 5.0 with glacial acetic acid) with 980 ml of deionized water, 200 ml of methanol, and 800 ml of acetonitrile. The resulting mixture was filtered through a 0.22-μm Durapore filter (Millipore, Milford, MA, USA). The flow-rate was 1.0 ml/min. The retention time of paclitaxel was *ca.* 10 min.

Data processing

The detector output was recorded on a strip-chart recorder and by a Hewlett-Packard HP 3357 laboratory automation system computer for peak integration and quantitation. The data management system used to acquire and process the analytical data has been described previously [10].

Validation procedures

Limit of detection/limit of quantitation (LD/LLQ). Plasma samples (0.5 ml) from twelve different volunteers were obtained in triplicate and divided into three sets; each set contained one plasma sample from each source. One set was spiked with ethanol and used as blanks. The second set was spiked with 5 ng/ml of paclitaxel. The third set was spiked with 10 ng/ml of paclitaxel. Samples were processed according to the stated procedure and subjected to HPLC analysis. The precision (% R.S.D.) and accuracy (% dev.) obtained from these samples were used to evaluate the LD/LLQ.

Intra- and inter-assay accuracy and precision. The intra-assay accuracy and precision of the method were determined by analyzing ten to twelve aliquots of three different concentrations (10, 103, and 863 ng/ml) on the same day. The inter-assay accuracy and precision were determined by assaying ten samples of two different concentrations (103 and 863 ng/ml) on three different days.

Recovery. The extraction recovery of paclitaxel was determined by comparing peak heights for standards obtained from a processed human plasma standard curve with the peak heights of corresponding standards obtained from a standard curve prepared in acetonitrile–methanol–water (4:1:5, v/v/v) without processing.

$$\% \text{ Recovery} = \frac{\text{peak height of processed standard}}{\text{peak height of non-processed standard}} \times 100$$

Stability. (a) The stability of paclitaxel in fresh plasma at 4°C and 37°C was determined by spiking fresh plasma with paclitaxel yielding a concentration of 875 ng/ml. The spiked plasma was stored at 4°C and 37°C. Aliquots (0.5 ml) were removed at various time intervals over a 24-h period and immediately frozen. The samples, along with standards, were processed and analyzed the next day.

(b) The stability of reconstituted samples was determined by spiking control human plasma with paclitaxel yielding a concentration of 750 ng/ml. Twenty-eight 0.5-ml aliquots were removed and processed. The reconstituted samples

were pooled, thoroughly mixed, and injected in sets of four over a 68-h time interval.

(c) The long-term stability of paclitaxel in frozen human plasma was determined by analyzing quality control (QC) samples (103 and 863 ng/ml), at selected time intervals, stored at −20°C for 2 years.

Specificity. (a) The specificity of this method was determined by examining the separation of paclitaxel from endogenous human plasma constituents in blank plasma chromatograms.

(b) The effect of concomitant medications on the performance of the assay was also determined by examining the separation of paclitaxel from interfering concomitant medications. Ethanolic solutions of 22 possible concomitant medications were injected to determine their retention times relative to paclitaxel. In addition, the maximum therapeutically observed concentration (in plasma) of each concomitant medication was added to 0.5 ml of plasma containing 1000 ng/ml of paclitaxel. The samples were processed and analyzed to determine the presence of interferences of the medications with paclitaxel in plasma.

(c) The specificity was also determined by examining the separation of paclitaxel from related compounds. Epipaclitaxel, paclitaxel C, baccatin III, and cephalomannine were prepared as ethanolic solutions and injected to determine if they coeluted with paclitaxel.

RESULTS AND DISCUSSION

Fig. 2 compares the chromatograms of a blank plasma sample and a plasma sample spiked with paclitaxel. The chromatographic profile indicated no interference from endogenous plasma substances. In addition, the paclitaxel peak for the 10 ng/ml standard was well resolved. Significant differences in the peak height of paclitaxel were obtained between the responses for the blanks and 5 ng/ml samples in 11 out of 12 instances, and in all 12 instances for the 10 ng/ml samples. The R.S.D. at 5 ng/ml was 55%, primarily because one sample gave no response for paclitaxel. However, the predicted concentrations were within 9% of the nominal concentration. At the 10 ng/ml level and under similar conditions, the

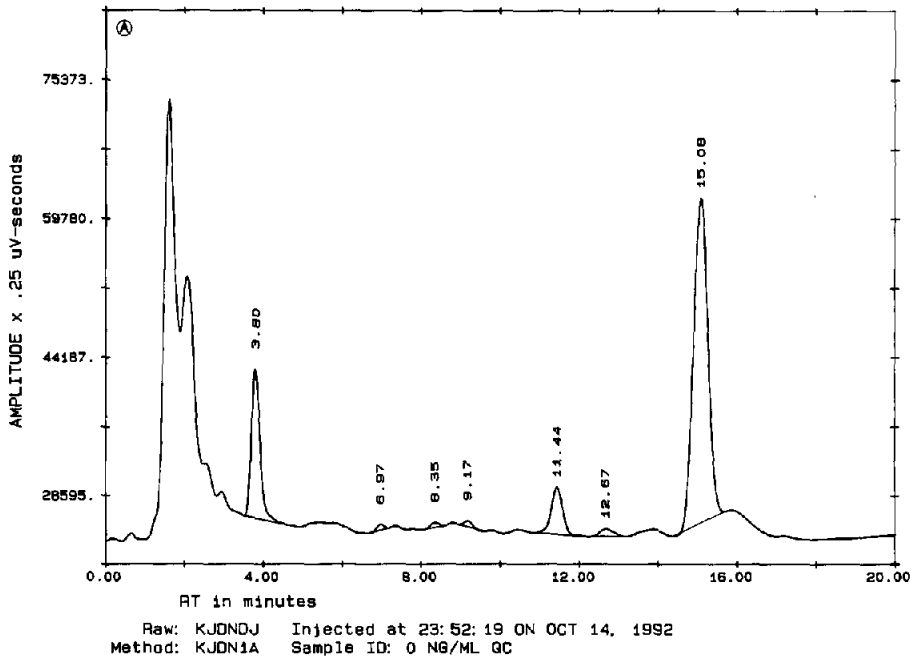
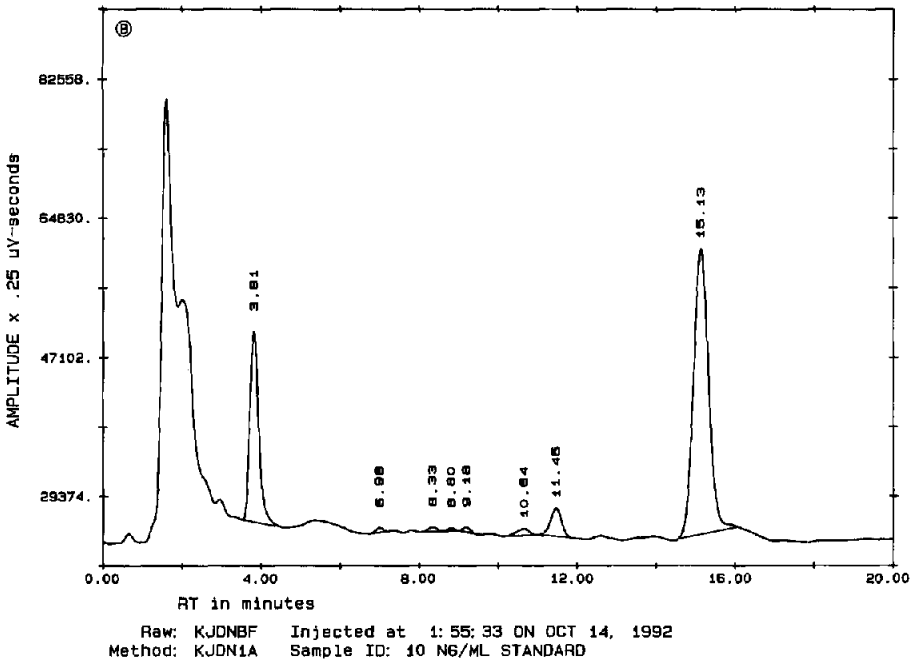


Fig. 2. Chromatograms of processed human plasma samples: (A) plasma blank; (B) 10 ng/ml paclitaxel standard; (C) 1000 ng/ml paclitaxel standard. Retention times of paclitaxel: 10.63–10.64 min; retention times of methyl paclitaxel: 15.07–15.13 min.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11.45
15.13
0.00
4.00
8.00
12.00
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20.00
RT in minutes
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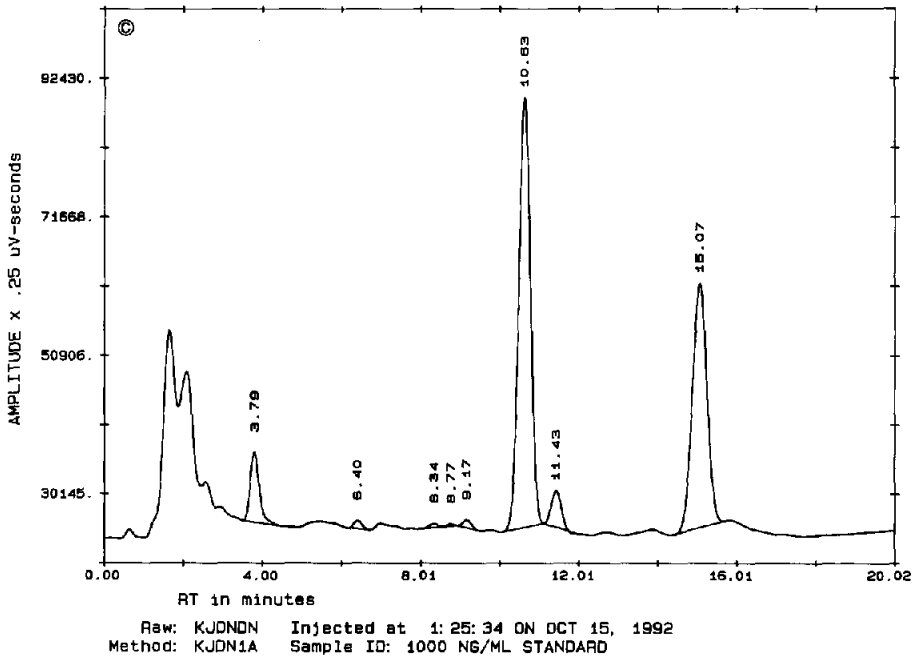
Method: KJDN1A Sample ID: 10 NG/ML STANDARD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0.00
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8.01
12.01
16.01
20.02
RT in minutes
Raw: KJDNDN Injected at 1:25:34 ON OCT 15, 1992
Method: KJDN1A Sample ID: 1000 NG/ML STANDARD

Fig. 2. Contnd.

TABLE I

INTRA- AND INTER-ASSAY ACCURACY AND PRECISION FOR PACLITAXEL IN HUMAN PLASMA

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	Deviation (%)	R.S.D. (%)
Intra-assay (n = 10–12)			
10.0	11.7	17.3	14.9
103	97.3	−5.5	2.9
863	826	−4.3	2.7
Inter-assay (n = 30)			
103	99.1	−3.7	3.4
863	841	−2.6	1.4

R.S.D. and dev. were 15% and 17%, respectively. Therefore, 10 ng/ml was used as the lowest limit of quantitation.

The assay was linear over the concentration range 10–1000 ng/ml in human plasma. Correlation coefficients of 0.997 or better were obtained throughout the validation. For a typical eight point standard curve extracted in duplicate, the R.S.D. were 5% or less, and the dev. was 10% or less, at all concentrations.

The intra-assay accuracy (dev.) was within 18% for all values, and the intra-assay precision (R.S.D.) varied between 2% and 15% (Table I). The inter-assay precision results show that the mean predicted concentrations were within 4% of the nominal values, with the R.S.D. less than 9% (Table I).

The absolute extraction recovery for each of eight different paclitaxel concentrations between 10 and 1000 ng/ml varied between 76% and 89%, based on the ratio of the peak height of a standard from a processed standard curve to the peak height of the corresponding standard from a non-processed standard curve (Table II).

Paclitaxel is stable in fresh human plasma for at least 24 h at 4°C and 8 h at 37°C. In 4°C plasma, there was only a 9% decrease from the original paclitaxel concentration after 24 h. In 37°C plasma, the paclitaxel concentration decreased to 65% of the original value after 24 h. This finding

TABLE II

RECOVERY OF PACLITAXEL FROM HUMAN PLASMA (n = 2)

Concentration (ng/ml)	Peak height		Recovery (%)
	Processed	Unprocessed	
10	493.4	648.4	76
20	1152	1486	78
50	3292	3862	85
100	6997	7882	89
200	12 536	15 903	79
500	34 305	40 724	84
800	54 106	64 954	83
1000	70 831	80 379	88

TABLE III

STABILITY OF RECONSTITUTED PLASMA EXTRACTS FOR THE HUMAN PLASMA ASSAY (n = 4)

Time (h)	Peak height (mean ± S.D.)	R.S.D. (%)
0	33532 ± 195	0.7
6	33191 ± 436	1.5
12	33158 ± 361	1.3
24	32276 ± 206	0.7
36	32480 ± 454	1.6
48	32227 ± 629	2.3
68	31764 ± 406	1.5

demonstrates that neither refrigeration nor room temperature storage causes degradation of paclitaxel in plasma samples for a reasonable amount of time subsequent to sample collection.

Reconstituted paclitaxel samples are stable in the autosampler for at least 68 h, as shown in Table III, where there was no significant decrease in mean peak height with time. This finding was important in optimizing the number of samples that could be analyzed during an analytical run without sacrificing sample integrity.

The stability of paclitaxel in frozen human plasma was established at 103 and 863 ng/ml for up to 2 years. After analysis on six different occasions, mean observed paclitaxel concentrations had dev. within ± 17% and R.S.D. values within 12% of the nominal concentrations. There does not appear to be a concentration dependence in the stability of paclitaxel.

None of the 22 compounds tested, including compounds routinely co-administered with paclitaxel in the clinic, interfered with the quantitation of paclitaxel (Table IV). All compounds, excluding daunorubicin and amsacrine, were not detected when injected as ethanolic solutions. In addition, no interference was found when each of the 22 substances was added to plasma containing paclitaxel. This finding demonstrates the applicability of this assay for the analysis of clinical study samples from patients who are usually treated with concomitant medication. Baccatin III, cephalomannine, paclitaxel C, and epipaclitaxel had retention times of 3.5, 8.8, 14.7, and 15.3 min, respectively, and did not interfere with the quantitation of paclitaxel.

TABLE IV

CHROMATOGRAPHIC RETENTION TIMES FOR PACLITAXEL AND POSSIBLE CONCOMITANT MEDICATIONS

Compound	Retention time (min)
Daunorubicin	6.9
Amsacrine	7.9
Paclitaxel	10.3

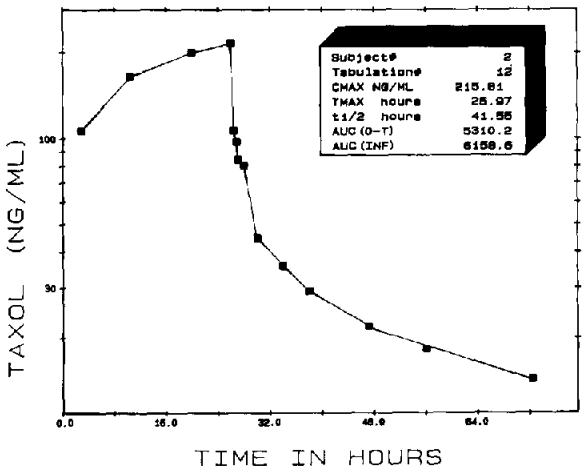

Fig. 3. Typical plasma concentration *versus* time profile of paclitaxel after a 24-h intravenous infusion of 135 mg/m^2 paclitaxel to a cancer patient.

The HPLC method described was used to assess the pharmacokinetics of paclitaxel during a clinical study in which paclitaxel was administered to cancer patients as a 24-h constant-rate intravenous infusion. Fig. 3 shows a typical plasma concentration–time profile for a patient receiving a 135 mg/m^2 dose of paclitaxel. After the end of infusion, the plasma levels declined exponentially, with a terminal half-life of 40 h.

The results from the validation of this procedure demonstrate that the lower limit of quantitation is lower than that for any of the published procedures for plasma [5–8]. Witherup *et al.* [9] published an assay that had a sensitivity as low as 5 ng/ml, but their method was applied to crude methanol extracts, not to biological fluids. Unlike the previous methods [7,8], which required a liquid–liquid extraction followed by solid-phase extraction, this method involves a rapid four-step solid-phase extraction procedure, including (1) conditioning of extraction cartridges, (2) addition of sample to cartridges, (3) washing endogenous materials through cartridges, and (4) eluting compounds off cartridges. In addition, this method requires only a 15-min run time *vs.* 20 min or more [5–9].

Subsequent to its validation, a modification of this method was validated using methyl paclitax-

el as an internal standard. Because addition of methyl paclitaxel to plasma samples is the only difference from the original procedure, validation involved only the determination of the range of reliable response, and the intra- and inter-assay accuracy and precision. Methyl paclitaxel elutes as a sharp peak at *ca.* 15 min and does not interfere with paclitaxel.

CONCLUSIONS

The procedure reported here is a reproducible, accurate, and precise isocratic method that allows the quantitation of paclitaxel concentrations of 10–1000 ng/ml in human plasma. Its sensitivity surpasses that of previous paclitaxel-specific assays and its simplicity allows the analysis of at least 100 samples per day. In addition, semi-automation of the solid-phase extraction procedure facilitates greater efficiency of sample analysis.

REFERENCES

1 M. E. Wall, M. C. Wani, H. L. Taylor, P. Coggon and A. T. McPhail, *J. Am. Chem. Soc.*, 93 (1971) 2325.
2 W. J. Slichenmyer and D. D. Von Hoff, *J. Clin. Pharmacol.*, 30 (1990) 770.
3 E. K. Rowinsky, L. A. Cazenave and R. C. Donehower, *J. Natl. Cancer Inst.*, 82 (1990) 1247.
4 D. G. Kingston, G. Samaranayke and C. A. Juey, *J. Nat. Prod.*, 53(1) (1990) 1.
5 S. M. Longnecker, R. C. Donehower, A. E. Cates, T. L. Chen, R. B. Brundrett, L. B. Grochow, D. S. Ettinger and M. Colvin, *Cancer Treat. Rep.*, 71(1) (1987) 53.
6 P. H. Wiernik, E. L. Schwartz, J. J. Strauman, J. P. Dutcher, R. B. Lipton and E. Paietta, *Cancer Res.*, 47 (1987) 2486.
7 J. L. Grem, K. D. Tutsch, K. J. Simon, D. B. Alberti, K. V. Willson, D. C. Tormey, S. Swaminathan and D. L. Trump, *Cancer Treat. Rep.*, 71 (12) (1987) 1179.
8 J. Rizzo, C. Riley, D. Von Hoff, J. Kuhn, J. Phillips and T. Brown, *J. Pharm. Biomed. Anal.*, 8(2) (1990) 159.
9 K. M. Witherup, S. A. Look, M. W. Stasko, T. G. McCloud, H. J. Issaq and G. M. Muschik, *J. Liq. Chromatogr.*, 12(11) (1989) 2117.
10 R. H. Farmen, J. F. Muniak and K. A. Pittman, *Drug Info. J.*, 21 (1987) 141.