ELSEVIER

Journal of Chromatography B, 703 (1997) 217–224

JOURNAL OF CHROMATOGRAPHY B

Simultaneous quantitation of plasma doxorubicin and prochlorperazine content by high-performance liquid chromatography

Caihong Mou, Neil Ganju, Kasi S. Sridhar, Awtar Krishan*

Division of Experimental Therapeutics, Department of Radiation Oncology and Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33136, USA

Received 18 February 1997; received in revised form 5 August 1997; accepted 5 August 1997

Abstract

A high-performance liquid chromatographic method has been developed and tested for simultaneous extraction, elution and determination of doxorubicin and prochlorperazine content in human plasma samples. The procedure consists of extraction through a conditioned C_{18} solid-phase extraction cartridge, elution from a Spherisorb C_8 reversed-phase column by an isocratic mobile phase (60% acetonitrile, 15% methanol and 25% buffer) followed by detection with electrochemical and fluorescence detectors. Recovery of doxorubicin and prochlorperazine from pooled human plasma samples ($n=3$) containing 100 ng/ml of the two drugs was 77.8±3.5% and 89.1±6.0%, respectively. The lower limits of quantitation for doxorubicin and prochlorperazine in plasma samples were 6.25 ng/ml and 10 ng/ml, respectively. A linear calibration curve was obtained for up to 2 μg/ml of doxorubicin and prochlorperazine. This combination method may be of particular value in clinical studies where phenothiazines such as prochlorperazine are used to enhance retention of doxorubicin in drug resistant tumor cells. © 1997 Elsevier Science B.V.

Keywords: Doxorubicin; Prochlorperazine

1. Introduction

Cellular resistance to cancer chemotherapeutic agents, such as doxorubicin is multifactorial [1–4]. Energy dependent drug efflux reduces cellular drug retention and chemosensitivity [5–7]. Several drugs can block efflux of a chemotherapeutic agent and increase cellular retention and chemosensitivity [8–10]. Laboratory and clinical studies have shown that phenothiazines (chlorpromazine, trifluoperazine and prochlorperazine) can block cellular doxorubicin efflux and modulate drug resistance [8,11,12]. In clinical trials prochlorperazine given as a 15 or 120 min infusion results in plasma concentrations high enough to block doxorubicin efflux in tumor cells [13,14].

In our earlier reported pharmacokinetic and phase I studies, separate extraction and elution methods were used for determination of prochlorperazine and doxorubicin content [13,14]. However, this duplicate process was time consuming and thus necessitated the need for a more rapid and cost effective method for simultaneous processing and analysis of phenothiazines and anthracyclines.

*Corresponding author.

0378-4347/97/$17.00
PII S0378-4347(97)00414-3

Several earlier papers have described the extraction of phenothiazines such as prochlorperazine and chlorpromazine from plasma between an organic and an aqueous phase [15,16], and through Sep-Pak C_{18} cartridges [17,18]. Smith et al. reported that extraction through a solid bonded phase cartridge resulted in improved recovery of chlorpromazines [19]. Prochlorperazine and chlorpromazine are heterocyclic compounds with good electrochemical reactivity. Doxorubicin and daunorubicin are highly fluorescent, but do not have strong EC reactivity. Simultaneous extraction and analysis of prochlorperazine and doxorubicin can minimize the use of expensive organic reagents as well as reduce the need for large samples. A combination method would also minimize errors as the compounds are extracted from the same aliquot and analyzed on a single HPLC system with two detectors.

In the present report, we describe a method for simultaneous extraction of prochlorperazine and doxorubicin with C_{18} bound solid-phase extraction cartridges and their quantitation by the electrochemical and the fluorescence detectors.

2. Experimental

2.1. Reagents and chemicals

Doxorubicin hydrochloride (Adriamycin, NSC-123127, Adria Labs, Columbus, Ohio, USA.), prochlorperazine edysilate (SoloPak Laboratories Inc. Elk Grove Village, IL, USA), daunorubicin hydrochloride (Cerubidine, NSC-821151, Wyeth Labs, Philadelphia, PA, USA.) and chlorpromazine hydrochloride (Thorazine, Smith Kline and Beecham Labs, Philadelphia, PA, USA) and other chemicals of analytical grade purity were purchased from commercial sources. All solvents including water used for extraction and in the mobile phase were of HPLC grade purity.

2.2. Extraction

Bakerbond spe* octadecyl 3-ml disposable extraction cartridges (J.T. Baker Inc., Philipsberg, NJ, USA) were conditioned by sequential washing with column volumes of 100% methanol, 25% methanol in HPLC grade water and 0.05 *M* phosphate buffer (pH 8.5). Plasma samples were thawed, vortexed and briefly sonicated. After addition of 20 μl of internal standards (100 ng/ml daunorubicin and chlorpromazine), 0.5 ml of the plasma was pipetted into the conditioned cartridge and washed with 2 ml of 10% methanol in HPLC grade water and 2 ml pure hexane. A Supelco vacuum manifold was used to control the flow-rate at 1–2 ml/min. The cartridge was eluted three times with 1 ml of chloroform–methanol (2:1, v/v) and the fraction was evaporated under nitrogen at 45°C. The residues were reconstituted in 200 μl methanol and 20–40 μl of the sample was injected into the HPLC column.

2.3. Calibration and sample preparation

Stock solutions (100 μg/ml) of doxorubicin, daunorubicin, prochlorperazine and chlorpromazine were prepared in absolute methanol and stored at −40°C in siliconized amber polypropylene microcentrifuge tubes (United Lab Plastics, St. Louis, MO, USA).

Plasma calibration samples were prepared by the mixing and serial dilution of doxorubicin and prochlorperazine stock solution in pooled human plasma. The concentration range used for calibration was 3.125 ng to 2 μg/ml for doxorubicin and 5 ng to 2 μg/ml for prochlorperazine. The lower limits of quantitation were 6.25 ng/ml for doxorubicin and 10 ng/ml for prochlorperazine.

The linear response range of fluorescence detector for doxorubicin was from 0.1 ng to 20 ng at 0.01 μAFS (micro amperes Full Scale). The linear response range for detection of prochlorperazine by the EC detector was from 0.2 ng to 10 ng at 1 nA/full scale and from 2 ng to 40 ng at 5 nA/full scale.

Human plasma samples containing two different concentrations (25 and 500 ng/ml) of doxorubicin and prochlorperazine with internal standards were analyzed at four different times during a day and on three consecutive days. The result were used to calculate coefficient of variation and relative error.

2.4. Instrumentation

A constaMetric IIIG metering pump (LDC/Milton Roy, Rivera Beach, FL, USA) in conjunction with a

six-port injector (Rheodyne 7125, Berkeley, CA, USA) was used with a Spectroflow 980 fluorescence detector (Kratos, Ramsey, NJ, USA) connected serially with a CC-4 electrochemical cell (Model LC-17A) in a LC-4B BAS amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). The output from the detectors was interfaced with an Axxiom multi-channel chromatography data system and controller (Model 747, Axxiom Chromatography Inc., Moorpark, CA, USA.). The analytical column used was a Spherisorb Octyl column (150× 4.6 mm, 5 μm; Keystone Scientific, Bellefonte, PA, USA). To protect the analytical column, an on-stream filter and a threaded conventional guard column packed with pellicular DELTABOND octyl (20×4 mm, 5 μm; Keystone Scientific, Bellefonte, PA, USA) were used. An extra pulse dampener was connected between the pump and the injector to smooth the pulse flow.

2.5. Measurement procedures

Drugs in the extracted samples were separated in a Spherisorb Octyl column at the isocratic flow-rate of 1.5 ml/min. Doxorubicin and daunorubicin were detected in a Kratos fluorescence detector using 230 nm excitation and 550 nm emission wavelengths. For prochlorperazine and chlorpromazine detection, the potential of the electrochemical detector was set at 850 mV in oxidizing mode versus the Ag/AgCl reference electrode. The working electrode used was a glassy carbon (1 or 5 nA/full scale). The isocratic mobile phase (60% acetonitrile, 15% methanol, 0.08% phosphoric acid and 0.08% diethylamine in HPLC grade water) was filtered through a 0.45 μm Nylon filter membrane (Millipore Corp., Marlborough, MA, USA) before use and degassed with helium.

2.6. Collection of patient samples

Peripheral blood samples from patients administered intravenous infusion of doxorubicin (60 mg/m^2 for 15 min) followed by continuous infusion of prochlorperazine (135 mg/m^2) for 120 min was collected in tubes containing sodium citrate as an anticoagulant. Plasma was separated by centrifugation at 1500 *g* for 10 min. Samples were collected prior to the start of infusion and 15, 30, 60, 90, 135 min after start of infusion and 4.5, 6.4 and 8.5 h after the end of infusion.

2.7. Data analysis

Plasma drug concentration (C_d) was calculated as the known concentration of the internal standard (C_{is}) multiplied by the ratio of area under the curve (AUC) of the analyte (A_d) and the internal standard (A_{is}), $C_d = C^*_{is} A_d / A_{is}$.

WinNonlin statistics program (Scientific Consulting, Inc., Apex, North Carolina) was used for modelling of the pharmacokinetic parameters and to determine the optimum fit from the diagnostics factors such as Akaike Information Criterion (AIC) and the Schwartz criterion (SC). The hybrid coefficients (A and B) and hybrid exponents (α and β) for the secondary parameters including the initial distribution and terminal elimination half-life, the total volume of distribution at steady state, the total volume of clearance and the area under the curve was also calculated by the WinNonlin program.

3. Results

The percentage recovery of the four drugs in 0.5 ml pooled plasma samples ($n=3$) extracted by our combination method was 77.87±3.51% for doxorubicin, 100.27±1.01% for daunorubicin, 60.60± 2.31% for chlorpromazine and 89.07±6.02% for prochlorperazine, respectively.

Calibration curves were established from triplicate samples of doxorubicin and prochlorperazine in pooled human plasma. Regression analysis gave a coefficient of determination (r^2) of 0.99563 for doxorubicin and 0.99348 for prochlorperazine concentrations of 10 to 2000 ng/ml.

Chromatograms shown in Fig. 1A are of doxorubicin (DOX), daunorubicin (DNR), chlorpromazine (CPZ) and prochlorperazine (PCZ) dissolved in methanol and injected directly into the column.

Fig. 1B shows chromatograms of prochlorperazine (PCZ) and chlorpromazine (CPZ) in human plasma with retention times of 5.7±0.2 and 7.4±0.2 min, respectively. The lower limit of quantitation for prochlorperazine was approximately 10 ng/ml. Fig.

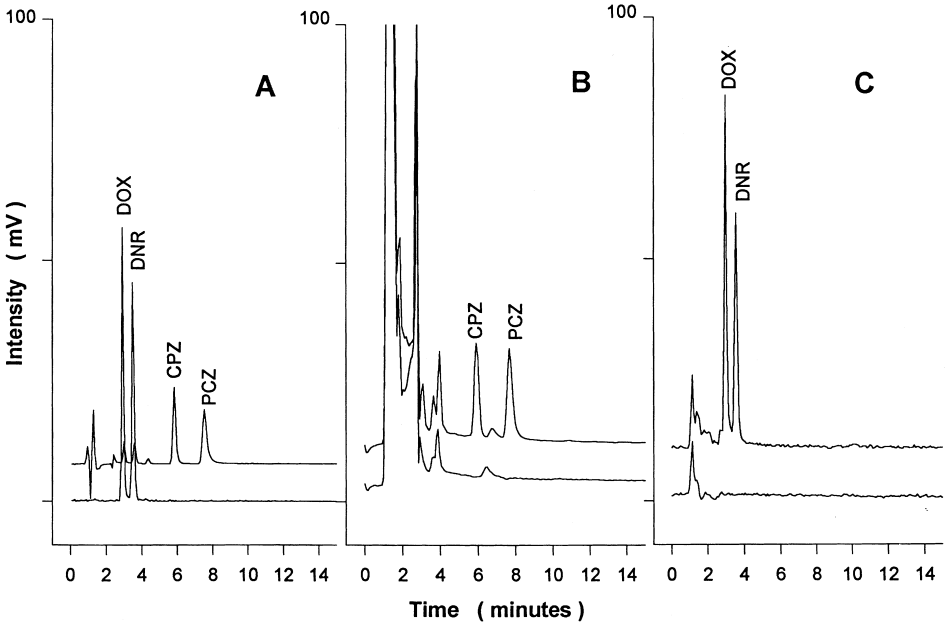

Fig. 1. (A) Chromatograms of doxorubicin (DOX), daunorubicin (DNR), chlorpromazine (CPZ) and prochlorperazine (PCZ) dissolved in methanol and directly injected into the column without extraction. (B) and (C) Human plasma samples spiked with chlorpromazine (internal standard), prochlorperazine, doxorubicin and daunorubicin (internal standard) and analyzed by our combination method.

1C shows the peaks for doxorubicin (DOX) and daunorubicin (DNR) in human plasma with retention times of 2.9±0.2 and 3.5±0.2 min, respectively. The lower limit of quantitation for doxorubicin in plasma was approximately 6.25 ng/ml.

Fig. 2A (pre-therapy) and Fig. 2B (after 2 h infusion) chromatograms of plasma samples from a patient show peaks of prochlorperazine and doxorubicin (Fig. 2B) with the internal standards of chlorpromazine and daunorubicin (Fig. 2A and Fig. 2B).

In Table 1 are listed the mean, standard deviation, coefficient of variation and relative error of plasma samples spiked with 25 or 500 ng/ml of prochlorperazine and doxorubicin and analyzed at four different times during a day or analyzed on three consecutive days at four different times. The coefficient of variation of the four plasma samples analyzed on the same day for doxorubicin and prochlorperazine content was 1.3–3.6% and on three different days varied from 1.5 to 7.9%. The relative error determined by the equation $(V_m - V_{ad})/V_{ad}$, where V_m is the measured value and V_{ad} is the added value was between 1.4 and 7.4%.

Plasma doxorubicin and prochlorperazine content of two patients on our doxorubicin and prochlorperazine protocol [13,14] shown in Fig. 3 were fitted into WinNonlin statistics program using weighted nonlinear least-square estimation regression analysis for the model discrimination and the parameter estimation. According to analysis of the residuals and AIC and SC, a two compartment model of intravenous bolus drug administration provided the best fit for the plasma doxorubicin concentration data,

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}$$

(C_t is the plasma doxorubicin concentration at t time point)

and a one compartment model of intravenous infusion gave the best fit for the plasma prochlorperazine

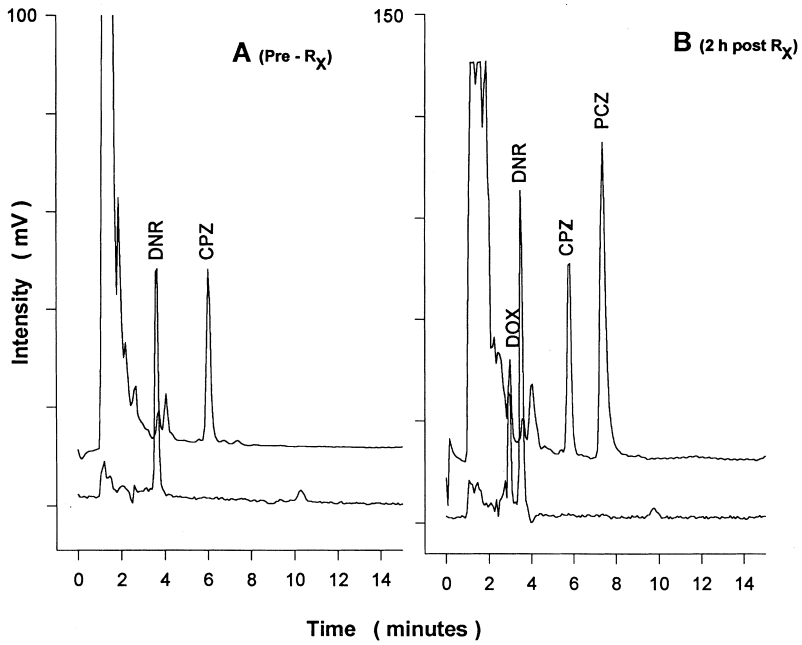

Fig. 2. Chromatograms of a pre-therapy plasma sample (A, spiked with internal standards) and a plasma sample after infusion of doxorubicin and prochlorperazine for 2 h (B) from the same patient extracted and measured by the combination method.

Table 1
Precision and accuracy of measurement within same day ($n=4$) and between-days ($n=3$) of low (25 ng/ml) and high drug concentrations (500 ng/ml)

Drug (ng/ml)	Mean concentration (ng/ml)	Standard deviation	Coefficient of variation (%)	Relative error (%)
Same day analysis				
PCZ				
25	25.42	0.93	3.6	1.7
500	510.24	7.40	1.5	2.0
DOX				
25	26.43	0.65	2.4	5.7
500	507.21	6.71	1.3	1.4
Between-days (n=12)				
PCZ				
25	25.67	2.03	7.9	2.7
500	508.78	8.33	1.6	1.8
DOX				
25	26.86	1.85	6.9	7.4
500	509.28	7.47	1.5	1.9

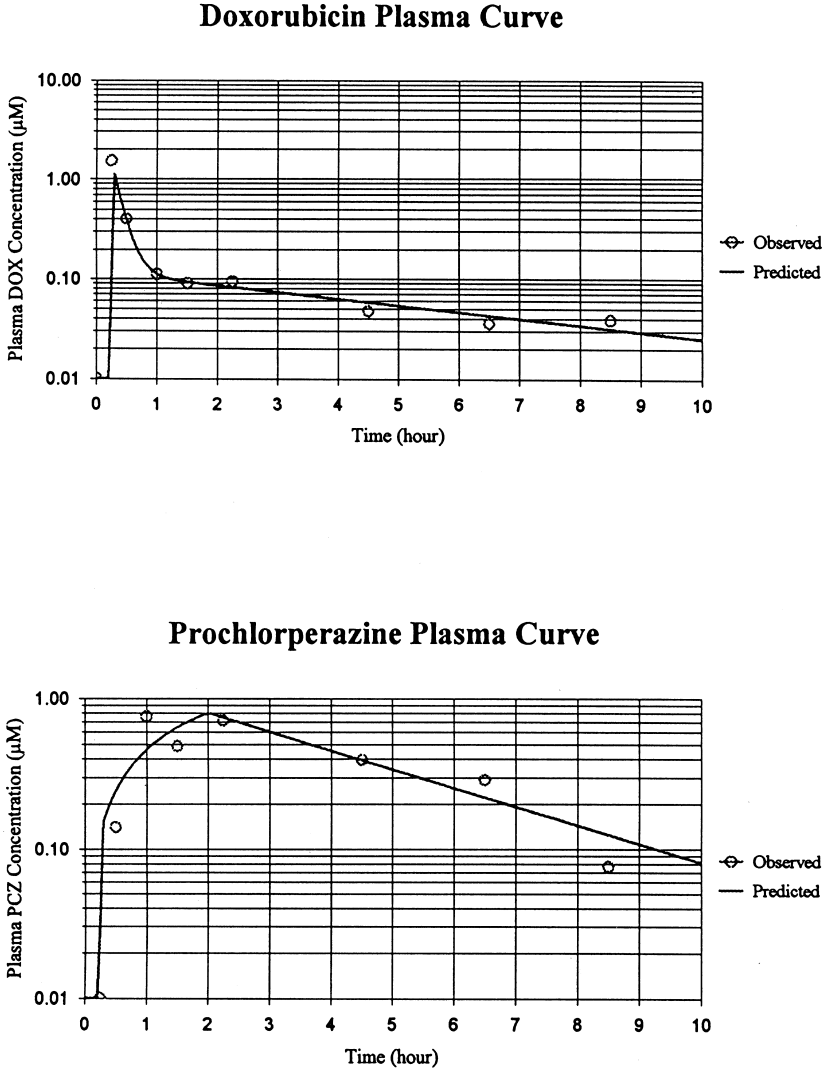

Fig. 3. Plasma doxorubicin and prochlorperazine decay curve in a patient following i.v. infusion of doxorubicin (60 mg/m^2) and prochlorperazine (135 mg/m^2).

data [20]. Results of pharmacokinetic parameters from this analysis are shown in Table 2.

4. Discussion

Data in the present report shows that the combination method for simultaneous extraction and analysis of anthracyclines (doxorubicin, daunorubicin) and phenothiazines (prochlorperazine, chlorpromazine) is reliable and can provide results similar to those obtained by separate elution and analysis of the individual drugs. With the combination method, we obtained good baseline separation for prochlorperazine, chlorpromazine, doxorubicin and daunorubicin without compromising sensitivity or linearity of the values obtained. Prochlorperazine and chlorpromazine with strong oxidation EC reactivity could be detected by the electrochemical detector without any interference from the serially connected

Table 2
Doxorubicin (60 mg/m^2) pharmacokinetics parameters after IV-bolus administration

	A	B	α	β	C_{max}	Cl
Patient#63	1.42	0.11	6.34	0.15	1.54	108.29
Patient#75	1.31	0.16	4.27	0.20	1.47	92.83
	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	V_{ss}	AUC	AUMC	MRT
Patient#63	0.11	4.54	546.03	0.96	4.82	5.04
Patient#75	0.16	3.51	346.18	1.11	4.16	3.73

PCZ (135 mg/m^2) pharmacokinetics parameters after IV infusion

	$t_{1/2\gamma}$ (h)	C_{max}	Cl	V_{ss}	AUC	AUMC	MRT
Patient#63	2.42	0.8	64.9	226.43	3.69	16.55	3.49
Patient#75	3.31	0.74	55.71	265.87	4.30	24.80	4.77

C_{max}: peak plasma concentration (μmole).
Cl: total body clearance (l/h).
V_{ss}: volume of distribution at steady state (l/kg).
AUC: area under the $C(t)$ curve. The area under the curve was calculated from the experimantal data by the trapezoidal rule and the terminal elimination (μmole×h/l).
AUMC: area under the moment curve. The area under the drug concentration–time versus time plot to infinity was calculated from time×concentration values by the trapezoidal rule and the terminal elimination (μmole*h/l).
MRT: mean residence time is the average amount of time a particle remains in a compartment or system (h).
$t_{1/2}$: distribution, intermediate, elimination half life (h).

fluorescence detector used for doxorubicin and daunorubicin detection. Simultaneous extraction and analysis of both drugs and the standards required only 0.5 ml of plasma samples instead of 2 ml samples needed for the separate extraction procedure.

The accumulation of non-polar organic compounds in the column can interfere with the electrochemical detection. Washing of the columns during extraction with 0.05 *M* Na_2HPO_4 does not remove the organic compounds which are subsequently eluted by chloroform–methanol (2:1, v/v) [21,22]. Washing with a small amount of hexane in our combination method removed these nonpolar organic compounds without affecting drug retention.

A comparison of the present combination method and the liquid–liquid extraction method used in our earlier study [13,14], gave comparable values for prochlorperazine ($r_2 = 0.89$) and doxorubicin ($r_2 = 0.992$) plasma content.

In conclusion, the combination method for simultaneous extraction and analysis of prochlorperazine and doxorubicin (and the internal standards of chlorpromazine and daunorubicin) by avoiding separate extraction, elution and analysis of the two drugs is convenient and economical. The sensitivity of the combination method is high enough to be used for pharmacokinetic analysis of doxorubicin and prochlorperazine in small plasma and tumor samples from cancer patients. This method can be especially useful in clinical studies seeking to increase cellular retention of doxorubicin in resistant tumor cells by blocking efflux with prochlorperazine.

Acknowledgements

This research was supported by NIH grant CA-57488 and CA-29360.

References

[1] A.M. Deffie, T. Alam, C. Seneviratne et al., Cancer Res. 48 (1988) 3595.
[2] J.G. Zijlstra, E.G.E. DeVries, N.H. Mulder, Cancer Res. 47 (1987) 1780.
[3] S. Nair, S.V. Singh, T.S.A. Samy, A. Krishan, Biochem. Pharmacol. 39 (1990) 723.

[4] M.M. Gottesman, I. Pastan, J. Clin. Oncol. 7 (1989) 409.
[5] D. Kessel, V. Botterill, I. Wodinsky, Cancer Res. 28 (1968) 938.
[6] K. Dano, Biochim. Biophys. Acta 323 (1973) 466.
[7] M. Inaba, H. Kobayashi, Y. Sakurai, R.K. Johnson, Cancer Res. 39 (1979) 2200.
[8] R. Ganapathi, D. Grabowski, W. Rouse, F. Riegler, Cancer Res. 44 (1981) 5056.
[9] L.M. Slater, P. Sweet, M. Stupecky, M.W. Wetzel, S. Gupta, Br. J. Cancer 54 (1986) 235.
[10] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Cancer Res. 41 (1981) 1967.
[11] A. Krishan, A. Sauerteig, L. Wellham, Cancer Res. 45 (1985) 1046.
[12] R.L. Miller, R.M. Bukowski, G.T. Budd et al., J. Clin. Oncol. 6 (1988) 880.
[13] K.S. Sridhar, A. Krishan, T.S.A. Samy et al., Cancer Chemother. Pharmacol. 34 (1994) 377.
[14] K.S. Sridhar, A. Krishan, T.S.A. Samy et al., Cancer Chemother. Pharmacol. 31 (1993) 423.
[15] A. Fowler, W. Taylor, D.N. Bateman, J. Chromatogr. 380 (1986) 202.
[16] A.O. Isah, M.D. Rawlins, D.N. Bateman, Br. J. Clin. Pharmacol. 32 (1991) 677.
[17] H. Hattori, S. Yamamoto, M. Iwata, E. Takashima, T. Yamada, O. Suzuki, J. Chromatogr. 579 (1992) 247.
[18] Y. Ishikawa, O. Suzuki, H. Hattori, Forensic Sci. Int. 44 (1990) 93.
[19] C.S. Smith, S.L. Morgan, S.V. Greene, J. Chromatogr. 423 (1987) 207.
[20] . J. Gabrielsson, D. Weiner, Pharmacokinetic and Pharmacodynamic Data Analysis, Concepts and Applications. Swedish Pharmaceutical Press, S-111 81 Stockholm, Sweden, 1994.
[21] J. De Jong, P.A. Maessen, A. Akkerdaas, S.F. Cheung, H.M. Pinedo, W.J.F. Van Der Vijgh, J. Chromatogr. 529 (1990) 359.
[22] P.A. Maessen, H.M. Pinedo, K.B. Mross, W.J.F. Van Der Vijgh, J. Chromatogr. 424 (1988) 103.