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Short communication

High-performance liquid chromatographic and pharmacokinetic analyses of an intravenous submicron emulsion of perillyl alcohol in rats

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ABSTRACT

Perillyl alcohol (POH) is currently in phase II clinical trials both as a chemopreventative and chemotherapeutic agent. The present report describes a simple, rapid and sensitive HPLC-UV method to quantify POH in rat plasma. After protein precipitation with acetonitrile, POH was separated using an Agilent Zorbax XDB C_{18} column (150 mm × 4.6 mm, 5 μm) with a mobile phase consisting of acetonitrile–water (40:60, v/v) at a flow rate of 1.0 ml min^{-1}, and detected at 210 nm. The method has been used successfully to determine trace levels of POH in plasma down to 0.015 μg ml^{-1}. The pharmacokinetics of POH after intravenous administrations in three formulations, i.e. POH solution (POH-SOL), negatively charged submicron emulsions (POH-SE) and positively charged submicron emulsions (POH-CSSE) were investigated. $AUC_{0-\infty}$, MRT, $t_{1/2\alpha}$ and $t_{1/2\beta}$ of POH-SE and POH-CSSE were significantly higher, while their total body clearance was lower than those of POH-SOL. In addition, $AUC_{0-\infty}$, MRT and $t_{1/2\beta}$ of POH-CSSE were significantly higher than those of POH-SE. The results indicate that the submicron emulsion formulation significantly increases POH blood concentrations and retention within the systemic circulation.

1. Introduction

Perillyl alcohol (POH) is a cyclic monoterpene (4-isopropenylcylcohex-1-ene carbinol) isolated from perillyl leaf, mandarin orange, cherry and peppermint. It has shown in animal studies therapeutic activity for pancreatic, mammary and liver tumors and chemopreventive activity for colon, skin and lung cancer [1–6].

POH has been successfully applied in patients with advanced solid tumors in phase I and II clinical trial [7–10]. Efforts have been made to avoid difficulties cased by the unpleasant taste and side effects including nausea, early satiety, eructation as well as fatigue [11]. Parenteral administration of drugs can bypass the gastrointestinal (GI) tract so as to cause less GI toxicity [12]. The intranasal formulation of POH was generally well tolerated and did not cause GI toxicity [10]. Efforts are now directed toward building intravenous submicron emulsions of POH free of GI toxicity [13]. In this study the pharmacokinetic properties of submicron emulsions were evaluated in rats in order to enable the prediction of the pharmacokinetics of these formulations in humans.

GC–MS methods have been used for pharmacokinetic studies of POH in dogs [14] and humans [15,16]. However, Phillips et al. [14] were unable to detect POH in plasma using the GC–MS method because of inadequate sensitivity. Moreover, the GC–MS method required complicated and time-consuming sample pre-treatment.

In the present study, a simple, sensitive, and rapid HPLC method was developed to quantify POH in rat plasma. Furthermore, the rat pharmacokinetic parameters of POH solution (POH-SOL), its negatively charged submicron emulsions (POH-SE) and its positively charged submicron emulsions (POH-CSSE) following intravenous administration were evaluated.

2. Experimental

2.1. Chemicals

POH (purity >96.0%) was purchased from Sigma Co. (USA). Poloxamer 188 was purchased from BASF Co. (Germany). Soybean phospholipid (Lipoid S75) was purchased from Tai Wei Pharmaceutical Co. (Shanghai, China). Chitosan was purchased from Ankang Biology Technology Co. (Shandong, China). Acetonitrile and methanol of HPLC grade were obtained from Siyou Chemical Reagent Co. (Tianjin, China). Heparin was from Tianjin Biochemical Pharmaceutical Industry (Tianjin, China). Triply deionized water

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was used for all preparations. All other reagents were of analytical grade.

2.2. Chromatographic conditions

The HPLC system consisted of Agilent 1100 modules, a quaternary pump, a diode-array detector, mobile phase degasser, auto-sampler with thermostat and a column heater compartment (Wilmington, DE, USA). The HPLC separation was performed using an Agilent Zorbax XDB C_{18} column (150 mm × 4.6 mm ID, 5 μm; Agilent Co., USA) with a C_{18} guard column (8 mm × 4 mm i.d., 5 μm). The mobile phase consisted of acetonitrile–water (40:60, v/v) with flow-rate of 1.0 ml min^{-1}. The mobile phase was filtered through 0.45 μm millipore filters before use. The injection volume was 20 μl. POH was detected at 210 nm. The column was operated at 30 °C. Agilent software, ChemStation, was used for data acquisition and analysis.

2.3. Preparation of stock solution and validation samples

The primary stock solution of POH (1.0 mg ml^{-1}) was prepared in methanol and stored at 4 °C. A series of POH standard solutions of plasma were prepared by spiking blank rat plasma with stock solutions to construct the standard calibration curves (SCC). The final concentrations were 25.0, 20.0, 10.0, 5.0, 2.5, 2.0, 1.0, 0.2 and 0.05 μg ml^{-1}. These standard solutions from SCC were also used for the validation of the method.

2.4. Sample preparation

All plasma, spiked plasma calibration standards and spiked plasma quality control samples were treated in the same manner described below. The plasma (0.1 ml) was mixed with 200 μl acetonitrile, and the mixture was vortexed vigorously for 3 min. After centrifugation of the mixture at 12,000 rpm for 10 min at 4 °C, and then a 20 μl aliquot of the supernatant was injected into the HPLC system.

2.5. Preparation of submicron emulsions of POH

POH-SE containing POH (2%, w/v), soybean oil (15%, w/v) as oily phase and Lipoid S75 as emulsifier (2.0%, w/v) were prepared as described recently [13]. Briefly, Poloxamer 188 (1.0%, w/v) and glycerol (22.5%, w/v) were dissolved in the aqueous phase. POH, soybean oil and Lipoid S75 were dissolved in soybean oil as oil phase. Both phases were heated separately to 70 °C, and then were mixed together and emulsified by a homogenizer at 12,000 rpm for 8 min. The pH of the crude emulsion was adjusted to 6.5 using citric acid (0.1 M). The coarse emulsion was ultrasonicated (power 25%) for 10 min. The pH of the emulsion was then adjusted to 7.0. POH-SE was packed in 15 ml sterile glass vials under nitrogen. The vials were sealed and POH-SE was sterilized by autoclaving at 115 °C for 30 min. POH-CSSE was prepared in a similar manner, but adding chitosan (0.5%) in the aqueous phase.

The droplet size and zeta potential were measured using a Malvern Zetasizer Nano-ZS90. The mean droplet size and zeta potential of POH-SE and POH-CSSE were 217.5 nm (−32.5 mV) and 205.4 nm (48.1 mV), respectively.

2.6. Linearity and quantifications

For the construction of calibration curve in plasma, nine different concentrations of POH were prepared and processed as described above. The calibration curve was constructed by performing linear regression analysis of the peak area (y) versus POH concentrations (x). The lower limit of detection (LLOD) was defined as the lowest concentration level resulting to a signal-to-noise ratio of 3:1, and the lower limit of quantification (LLOQ) was defined as that with the signal-to-noise ratio of 10:1.

2.7. Precision and accuracy

Validation samples were prepared and analyzed to evaluate the intra- and inter-day precision and accuracy. The intra-day precision of the assays performed in replicate ($n = 5$) were tested by using three concentrations of POH, 0.2, 2.5 and 20.0 μg ml^{-1}. The inter-day precision of the assays was also estimated on five consecutive days and the precision was evaluated as the relative standard deviation (R.S.D.). Accuracy was determined by comparison of the calculated mean experimental concentrations with theoretical concentrations.

2.8. Extraction recovery

Extraction efficiency of POH from plasma was evaluated ($n = 5$) at 0.2, 2.5 and 20.0 μg ml^{-1} by comparing the peak areas of an extracted sample containing a known amount of POH with the peak areas obtained from direct injections of the solution containing the same concentration of POH in pure solvent.

2.9. Stability

The short-term sample stability was examined by analyzing validation samples at room temperature for 24 h and long-term stability was examined by analyzing samples stored at −20 °C for 2 months. The samples were left at room temperature for 1 h to thaw and then refrozen at −20 °C for 24 h for the freeze-thaw stability study. This cycle was repeated three times and analysis was performed after the third freeze-thaw cycle. The POH stability in reconstituted extracts during run-time of HPLC was tested performing a second analysis of the same extract left for 8 h at room temperature.

2.10. Pharmacokinetic study

Eighteen Sprague–Dawley (SD) rats with the average body weight of 250 g (250 ± 20 g) were obtained from the Department of Laboratory Animal Science at Zhengzhou University (Zhengzhou, China). These rats were pathogen-free and kept in environmentally controlled quarters for at least 1 week before use, with free access to standard diet and water, except that food was withdrawn 18 h prior to the experiment.

For intravenous (i.v.) administration, POH was dissolved with 1,2-propanediol and then diluted with physiological saline as POH-SOL, with a POH final concentration of 20 mg ml^{-1}. The preparations were made immediately before drug administration. The SD rats were divided into three groups (6 rats/per group). POH-SOL, POH-SE and POH-CSSE were injected into the rat-tail vein at a dose of 65 mg kg^{-1} of body weight. Blood samples were collected from the eye ground vein before administration and at predetermined time intervals (0.08, 0.17, 0.25, 0.50, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 h) after intravenous administration. Plasma was obtained by centrifugation of the blood at 8000 rpm for 10 min, and prepared as described in Section 2.4.

The concentration of POH in rat plasma was determined as described above. Pharmacokinetic analysis of plasma POH concentration–time data was conducted using Kinetica Software (Version 4.4, Therom Electron Corporation).

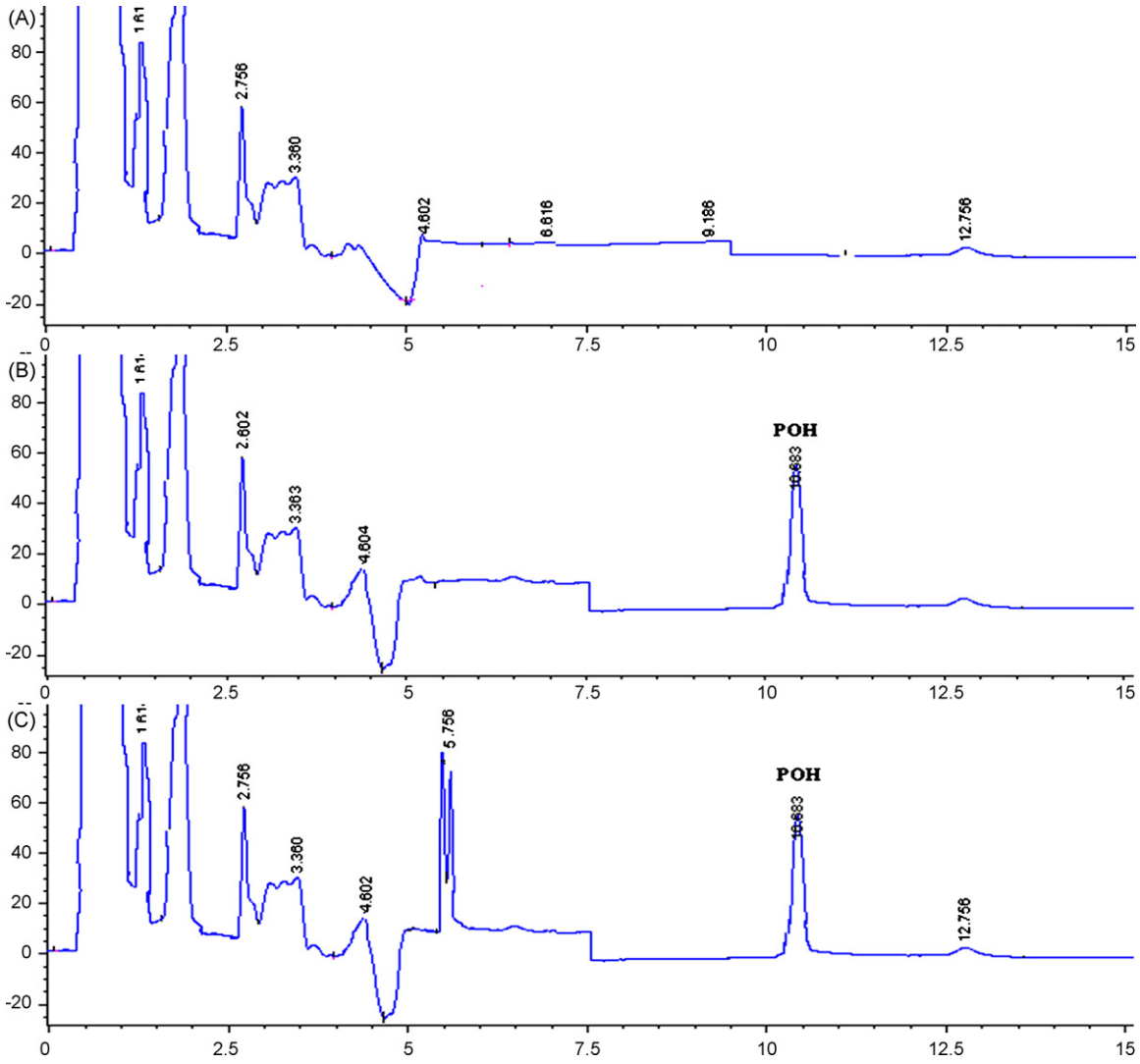

Fig. 1. Chromatograms of POH in rat plasma (A) blank plasma, (B) blank plasma spiked with POH 20 μg ml^{-1} and (C) plasma sample after i.v. administration of POH submicron emulsions at 0.5 h; R_t = 10.68 min; mobile phase: acetonitrile–water (40:60, v/v).

2.11. Statistical analysis

The pharmacokinetic results were analyzed statistically using an overall ANOVA. When comparisons between groups yielded a value of $P < 0.05$, the difference between these groups was considered statistically significant. All statistical analysis was performed using statistical package for social sciences (SPSS, Version 12.0).

3. Results and discussion

3.1. Method development

A straightforward and accurate HPLC method to determine POH in rat plasma was initially developed. The extracted samples of POH were successfully separated with a C_{18} column. Several mobile phases were tested and acetonitrile–water (40:60, v/v) was an appropriate mobile phase for separation of POH from endogenous compounds within a run-time of 15 min, with a symmetrical peak shape. The detection was at λ_{max} (210 nm) of POH in the mobile phase. Proteins in sample were precipitated by acetonitrile. The recovery was higher than 80%.

Typical HPLC chromatograms of POH after extraction from plasma are shown in Fig. 1. Fig. 1A shows the chromatogram of blank rat plasma. Comparing this chromatogram with that of rat plasma spiked with POH (Fig. 1B) the retention time of POH was found to be 10.68 min and no interfering peaks were observed within the time frame in which POH was detected. Fig. 1C shows the chromatogram of a POH sample obtained after intravenous administration to rats.

3.2. Linearity, LLOQ and LLOD

The calibration curve was found linear over the range of 0.05–25.0 μg ml^{-1}. The mean regression equation in plasma was $A = 17.00(\pm 0.87)C - 24.60(\pm 1.25)$ ($r = 0.9996$). The coefficient of variations of slope for POH in plasma was found lower than 10%, which indicates a high precision of the assay. The LLOD and LLOQ of POH were found to be 0.015 and 0.045 μg ml^{-1}, respectively.

3.3. Precision and accuracy

The precision and accuracy of the method were also defined by examining both intra- and inter-day assay variabilities. The results

Table 1
Accuracy and precision of the determination of POH in rat plasma ($n = 5$)

Theoretical concentration ($\mu g\,ml^{-1}$)	Experimental concentration (mean ± S.D.)	Precision (R.S.D.%)	Accuracy (%)
Intra-assay precision			
0.2	0.202 ± 0.009	4.46	100.84
2.5	2.431 ± 0.05	2.18	97.23
20.0	19.722 ± 0.3	1.53	98.61
Inter-assay precision			
0.2	0.199 ± 0.01	5.53	99.45
2.5	2.433 ± 0.08	3.45	97.31
20.0	19.834 ± 0.4	2.18	99.17

are shown in Table 1. These indicated that the values were within the acceptable range and the method was accurate, reliable and reproducible.

3.4. Recovery and stability

The mean extraction recoveries for POH ($n = 5$) at concentrations of 0.2, 2.5 and 20.0 $\mu g\,ml^{-1}$ from rat plasma were found to be 82.3 ± 3.2, 85.5 ± 5.4 and 87.6 ± 3.5%, and the R.S.D.s of extraction recoveries were 3.9, 6.3 and 4.0%, respectively.

The stability study of POH was conducted by comparing results under different storage conditions with those for validation samples freshly prepared at concentrations of 0.2, 2.5 and 20.0 $\mu g\,ml^{-1}$ in rat plasma ($n = 3$). The mean recoveries (%) were 96.8 ± 1.9 in short-term, 98.8 ± 3.1 in long-term, 98.2 ± 2.3 in freeze-thaw cycles, and 99.5 ± 1.8 in the extract stability test. The results indicated that POH was stable in rat plasma for at least 24 h when stored at −20 °C, and three freeze-thaw cycles and stability test of reconstituted extracts also indicated that POH was stable under these conditions.

3.5. Pharmacokinetic analysis

The method was successfully used to quantify POH in rat plasma samples after tail vein injection of POH-SOL, POH-SE and POH-CSSE at a dose of 65 $mg\,kg^{-1}$. Representative chromatograms are shown in Fig. 1. The plasma concentration vs. time profiles of POH obtained after the injection of the POH-SOL, POH-SE and POH-CSSE formulations are shown in Fig. 2. The concentration of POH in plasma declined biexponentially and POH concentrations were significantly higher for POH-SE and POH-CSSE treated rats than that of POH-SOL treated rats ($P < 0.05$) at all time points. In plasma, POH was detectable even after 10 h of injection in POH-SE and POH-CSSE treated rats while POH was not detectable in plasma of rats treated with POH-SOL.

The pharmacokinetic parameters that were obtained by fitting experimental data to a two-compartment model using Kinetica Software (Version 4.4) are listed in Table 2. The $AUC_{0-\infty}$, MRT, $t_{1/2\alpha}$ and $t_{1/2\beta}$ after intravenous administration of POH-SE and POH-CSSE to rats were significantly higher than those with POH-SOL ($P < 0.01$). The clearance of POH was slower in POH-SE and POH-CSSE treated rats than that obtained with POH-SOL ($P < 0.01$). These were in accordance with previous findings, where lipophilic drugs administered in lipid emulsions showed higher plasma concentration and longer retention time in the blood circulation following intravenous injection than in solution forms [17,18]. The most frequent toxic symptoms (nausea, eructation and early satiety) of POH were found in the GI tract. However, no correlation was observed between POH plasma levels and toxicity [11]. The GI toxicity of POH might be related with the fact that POH could potentially cause direct-contact gastric irritation. da Fonseca et al. [10] suggested that the intranasal formulation of POH did not cause GI toxicity.

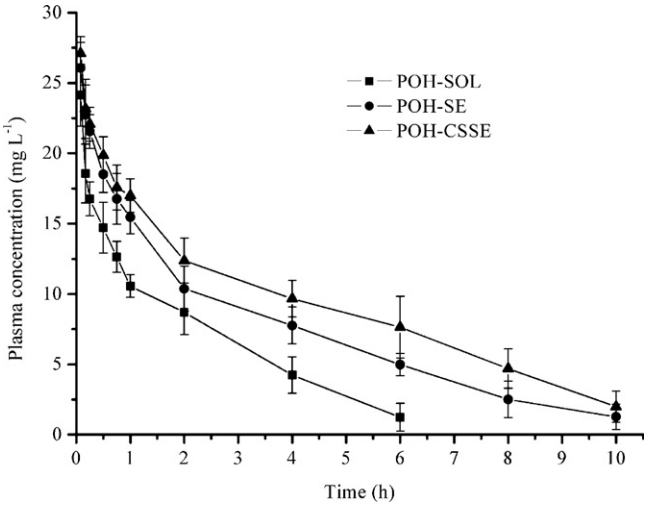

Fig. 2. Plasma concentration–time profiles of intravenous POH-SOL, POH-SE and POH-CSSE, respectively. Each point represents the mean ± S.D. of six rats.

By comparison, the $AUC_{0-\infty}$, MRT and $t_{1/2\beta}$ of POH-CSSE were significantly higher than those of POH-SE ($P < 0.05$). Kawakami et al. [17] and Shi and Benita [19] also suggested that the positively charged submicron emulsions altering the pharmacokinetics profile of incorporated drug submicron emulsions is more than that of the conventional negatively charged submicron emulsions. In order to further understand the disposition characteristics of the different submicron emulsion formulations, the tissue-distribution and tumor-targeting evaluation of POH-SE and POH-CSSE is under way.

4. Conclusion

A simple, rapid and sensitive HPLC method was developed and validated to investigate the level of POH in rat plasma. The pharmacokinetic profile of POH after intravenous administration of different formulations was evaluated. POH formulated in submicron emulsions significantly improved the pharmacokinetic profile of the drug administered to rats. These data may contribute to the evaluation of the tissue-distribution and tumor-targeting of POH-SE and POH-CSSE.

Table 2
Pharmacokinetic parameters after i.v. administration of POH-SOL, POH-SE and POH-CSSE

Parameter	Definitions	POH-SOL	POH-SE	POH-CSSE
$t_{1/2\alpha}$ (min)	Distribution half life	15.93 ± 1.21	19.16 ± 0.85*	18.95 ± 1.04*
$t_{1/2\beta}$ (min)	Elimination half life	81.65 ± 1.93	121.58 ± 2.64*	130.69 ± 3.22*,#
Cl (l/kg min)	Total body clearance	0.022 ± 0.0012	0.011 ± 0.00081*	0.011 ± 0.00062*
$AUC_{0-\infty}$ ($mg\,l^{-1}$ min)	Area under the curve	4944.2 ± 24.4	6716.2 ± 69.4*	7008.5 ± 48.9*,#
MRT (min)	Mean residence time	76.52 ± 1.35	131.32 ± 2.13*	142.53 ± 2.81*,#

The results are expressed as means ± S.D. ($n = 6$). *$P < 0.01$ *vs.* POH-SOL; #$P < 0.05$ *vs.* POH-SE.

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References

[1] M. Barthelman, W. Chen, H.L. Gensler, C. Huang, Z. Dong, G.T. Bowden, Cancer Res. 58 (1998) 711–716.
[2] P.L. Crowell, M.N. Gould, Crit. Rev. Oncog. 5 (1994) 1–22.
[3] C.E. Elson, T.H. Maltzman, J.L. Boston, M.A. Tanner, M.N. Gould, Carcinogenesis 9 (1988) 331–332.
[4] J.D. Haag, M.J. Lindstrom, M.N. Gould, Cancer Res. 52 (1992) 4021–4026.
[5] J.J. Mills, R.S. Chari, I.J. Boyer, M.N. Gould, R.L. Jirtle, Cancer Res. 55 (1995) 979–983.
[6] L.W. Wattenberg, Cancer Res. 43 (1983) 2448s–2453s.
[7] C.G. Azzoli, V.A. Miller, K.K. Ng, L.M. Krug, D.R. Spriggs, W.P. Tong, E.R. Riedel, M.G. Kris, Cancer Chemother. Pharmacol. 51 (2003) 493–498.
[8] H.H. Bailey, D. Levy, L.S. Harris, J.C. Schink, F. Foss, P. Beatty, S. Wadler, Gynecol. Oncol. 85 (2002) 464–468.
[9] H.H. Bailey, S. Attia, R.R. Love, T. Fass, R. Chappell, K. Tutsch, L. Harris, A. Jumonville, R. Hansen, G.R. Shapiro, J.A. Stewart, Cancer Chemother. Pharmacol. 62 (2008) 149–157.
[10] C.O. da Fonseca, G. Schwartsmann, J. Fischer, J. Nagel, D. Futuro, T. Quirico-Santos, C.R. Gattass, Surg. Neurol. 70 (2008) 259–266.
[11] G.H. Ripple, M.N. Gould, J.A. Stewart, K.D. Tutsch, R.Z. Arzoomanian, D. Alberti, C. Feierabend, M. Pomplun, G. Wilding, H.H. Bailey, Clin. Cancer Res. 4 (1998) 1159–1164.
[12] G.S. Banker, C.T. Rhodes, Modern Pharmaceutics, Fourth ed., Marcel Dekker, New York, 2002.
[13] H.Y. Hua, L. Liu, Y.X. Zhao, Z.Z. Zhang, Chin. J. Pharm. 39 (2008) 447–450.
[14] L.R. Phillips, L. Malspeis, J.G. Supko, Drug. Metab. Dispos. 23 (1995) 676–680.
[15] Z. Zhang, H. Chen, K.K. Chan, T. Budd, R. Ganapathi, J. Chromatogr. B: Biomed. Sci. Appl. 728 (1999) 85–95.
[16] G.R. Hudes, C.E. Szarka, A. Adams, S. Ranganathan, R.A. McCauley, L.M. Weiner, C.J. Langer, S. Litwin, G. Yeslow, T. Halberr, M. Qian, J.M. Gallo, Clin. Cancer Res. 6 (2000) 3071–3080.
[17] S. Kawakami, F. Yamashita, M. Hashida, Adv. Drug. Deliv. Rev. 45 (2000) 77–88.
[18] R.R. Patlolla, V. Vobalaboina, J. Pharm. Sci. 94 (2005) 437–445.
[19] C.Y. Shi, S. Benita, Drug Dev. Res. 50 (2000) 476–486.