

# Application of Validated RP-HPLC Method for Simultaneous Determination of Docetaxel and Ketoconazole in Solid Lipid Nanoparticles

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## Abstract

Docetaxel has significant single agent activity in prostate cancer and ketoconazole also has activity as a second line hormonal agent. *In vitro*, ketoconazole is synergistic with some chemotherapy agents by enhancing the intracellular retention of the cytotoxic agent. A potential drug-drug interaction exists though between docetaxel and ketoconazole because both agents are metabolized hepatically by the cytochrome P-450 system. Hence, a nanoparticulate system was formulated by loading both drugs for tumor targeting. Assay and *in vitro* release of the formulation were conducted by developing simple, precise, accurate, and validated analytical method for simultaneous determination docetaxel and ketoconazole using reversed-phase high-performance liquid chromatography (RP-HPLC). The RP-HPLC method was developed using Waters Symmetry C<sub>18</sub> column (25 cm × 4.5 mm, 5 μm) with a mobile phase consisting of acetonitrile and 0.2% triethylamine pH adjusted to 6.4 (48:52, v/v) at flow rate of 1 mL/min. Intra-day and inter-day variations were less than 2% over the linearity range, 0.5–20 μg/mL. The proposed two methods were successfully applied for the determination of docetaxel and ketoconazole in solid lipid nanoparticles.

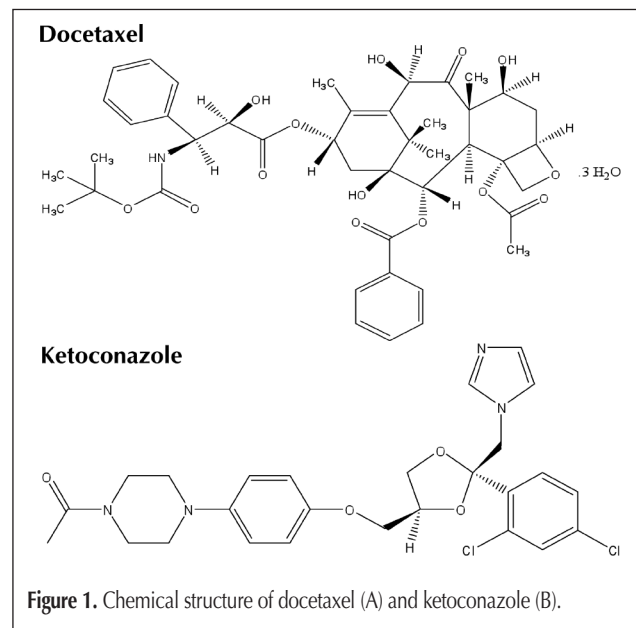
## Introduction

Docetaxel [4-acetoxy-2a-benzoyloxy-5b, 20-epoxy-1, 7b, 10b trihydroxy-9-oxotax-11-ene-13a-yl-(2R,3S)-3-tert-butoxycarbonylamino-2-hydroxy-3-phenylpropionate] is a novel anticancer agent of the taxoid family (Figure 1A). An analogue of paclitaxel, docetaxel, was obtained by semisynthesis from 10-deacetyl baccatin III, extracted from the needles of the European yew tree *Taxus baccata* L. (1,2). Docetaxel promotes tubulin assembly into microtubules, stabilizes microtubules and inhibits microtubules de-polymerization to free tubulin. This leads to disruption of the equilibrium within the microtubules system and

ultimately leads to cell death. Docetaxel has been demonstrated to be effective against wide range of tumors including breast, lung, prostate, ovarian, head and neck, gastric, pancreatic, and bladder cancers (3,4).

Ketoconazole (KTZ), *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl methyl)-1, 3-dioxolan-4-yl] methoxy] phenyl] piperazine (Figure 1B), is a synthetic imidazole antifungal. It is effective in the treatment of superficial and systemic infections and has been widely used in immune compromised patients and advanced prostatic carcinoma (5,6).

Docetaxel has significant single-agent activity in patients with prostate carcinoma, and ketoconazole has activity as a second-line hormonal agent. There have been trials that explored the combination of docetaxel with ketoconazole (7,8). Researchers found that pulse-treatment with microtubule-active drugs, like docetaxel, is more effective in inhibiting the growth of prostate cancer cells when it is followed by exposure to ketoconazole, and pharmacokinetic studies indicated that both docetaxel and keto-



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conazole are metabolized hepatically by the cytochrome P450 enzyme system (CYP3A4). The membrane transporter P-glycoprotein (ABCB1) also plays an important role in their disposition (9–12). Furthermore, ketoconazole has also proved to be a typical CYP3A4 inhibitor. Phase II clinical trials are currently being run for the appropriate use of this combination of drugs (13). An *in vivo* study suggested that concomitant administration of docetaxel and ketoconazole might result in a clinically significant drug–drug interaction leading to greater toxic effects (14). Hence, caution should be taken and substantial dose reductions are necessary if these two drugs need to be administered together. Therefore a nanoparticulate formulation for controlled release of ketoconazole and docetaxel for tumor targeting was developed. But, for inhibiting the cytochrome P-450 or p-gp, ketoconazole also should be released from the prepared formulation and the release of KTZ should be more and faster than docetaxel. To study the release of these two drugs from the nanoparticle formulation, simultaneous determination of these two drugs in PBS is needed.

Several chromatographic methods have been reported for the analysis of docetaxel or ketoconazole in biological matrices, including microbiological assays, high-performance liquid chromatography (HPLC) methods with UV, fluorescence, mass spectrometric (MS), and tandem mass spectrometric (MS–MS) detection (15–25). The liquid chromatographic (LC)–MS–MS method has been reported for simultaneous determination of docetaxel and ketoconazole in rat plasma (14). Even though an LC–MS–MS method is available in rat plasma, *in vitro* or *in vivo* method using HPLC with UV detection for simultaneous determination of both the drugs was not yet developed. In release samples, the drugs are present in phosphate buffer saline and these samples cannot be injected directly to LC–MS–MS. A method developed with LC–MS–MS requires again extraction procedures to determine the drugs.

Therefore, it is worthwhile to develop simple, precise, and accurate HPLC method for simultaneous determination of docetaxel and ketoconazole to study the controlled release of both drugs from solid lipid nanoparticles. In the current research, a reversed-phase (RP)-HPLC method for simultaneous determination of docetaxel and ketoconazole and applied for the assay and *in vitro* release studies of nanoparticulate system containing both drugs was developed.

## Materials and methods

### Chemicals and reagents

Docetaxel reference standard (purity: 100.7%) was from Therdose Pharma (Hyderabad, India). Ketoconazole (purity: 99.5%) reference standard was from Hetero Pharmaceuticals (Hyderabad, India). Glyceryl monostearate, Poloxamer 188 was purchased from Sigma (St. Louis, MO). Lecithin soya was obtained from Hi-media, India. The reference standards were used without further purification. HPLC grade acetonitrile and methanol were purchased from Merck (Mumbai, India). Triethylamine (HPLC grade) and chloroform were purchased from Merck. Deionized water was purified using Milli-Q system

(Millipore, Billerica, MA). All other reagents and solvents used in study were of analytical grade.

### Pharmaceutical preparation (docetaxel and ketoconazole loaded solid lipid nanoparticles)

Solid lipid nanoparticles were prepared by using emulsification and solvent evaporation method. An amount of 100 mg of glyceryl monostearate and 20 mg of lecithin were dissolved in 2 mL of chloroform and to this 5 mg of each of docetaxel and ketoconazole were added and vortexed to dissolve in the organic phase. An aqueous surfactant solution of Tween 80 (1% w/v) was prepared and emulsified with organic phase using Ultra Turrax high shear homogenizer (Ultra Turrax, Staufen, Germany) for 3 min and further sonicated (Sonics, Vibracell, Newtown CT) for 10 min to form nanoparticles. The obtained formulation was kept for stirring to evaporate any residual solvent present. Nanoparticles were separated by centrifugation at 70000 rpm for 30 min. Nanoparticles were washed by dispersing them in water followed by centrifugation, this procedure was repeated for two times. Docetaxel and ketoconazole loaded solid lipid nanoparticles obtained by previously described method were lyophilized and named as DK-SLN. Particle size of DK-SLN was measured using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

### Assay of docetaxel and ketoconazole present in DK-SLN solid lipid nanoparticles

Docetaxel and ketoconazole loaded solid lipid nanoparticles were prepared with particle size 112.9 with polydispersity index 0.21. An accurately weighed amount (10 mg) of docetaxel and ketoconazole loaded SLN was dissolved in 1 mL with chloroform–acetonitrile (1:3) and this sample was further diluted with acetonitrile to determine the total content using HPLC (Waters, Milford, MA).

### HPLC

#### Chromatographic conditions

Docetaxel and Ketoconazole were analysed using HPLC system consisting of Waters 2695–Alliance separations module with Millennium<sup>32</sup> software, auto injector and Waters 2996 photodiode array detector. Separation was carried out using a Waters Symmetry C<sub>18</sub> column (250 × 4.5 mm, 5 μm). Isocratic elution was carried with the mobile phase consisting of acetonitrile and 0.2% triethylamine pH adjusted to 6.4 with phosphoric acid (48:52, v/v) at flow rate of 1 mL/min. The mobile phase was filtered (Millipore system, 0.2 μm) under vacuum and degassed. Chromatographic separation was monitored at 230 nm. All the samples were analyzed at room temperature. Total run time for the analysis was 18 min.

#### Preparation of stock solution

Stock solution of binary mixture (docetaxel and ketoconazole) was prepared in acetonitrile to get a concentration of 1 mg/mL. Working standard solution was prepared by appropriate dilution of stock solution in acetonitrile to get 100 μg/mL. Further concentrations required for constructing calibration curve and quality control samples were prepared daily by dilution of 100 μg/mL working standard in phosphate buffer saline. All the solutions were stored at 2–8°C.

### *Calibration standard and quality control samples*

The standard calibration samples were prepared by diluting working standard solution (100 µg/mL) to yield seven different concentrations over a linearity range of 0.5–20 µg/mL (i.e., 0.5, 1, 2, 4, 8, 10, and 20 µg/mL). The quality control (QC) samples were prepared in same method as standard solutions to yield low, medium, and high concentrations (1.5, 7.5, and 15 µg/mL). Linearity of both drugs was evaluated over the concentration range of 0.5–20 µg/mL using least square linear regression analysis. Regression equations were used for determination of concentrations of QC samples containing both docetaxel and ketoconazole by replicate injections.

### *Linearity*

Linearity was performed by analysis of seven different concentrations in the range between 0.5–20 µg/mL for both drugs. Each concentration was injected and measured in triplicate. The relation between concentration (x) and its corresponding peak area ratio (y), that is, expressed by the equation  $y = mx + c$  for both drugs (m = slope, c = intercept).

### *Inter- and intra-day accuracy and precision*

A total of seven calibration standards were prepared on different days to evaluate inter- and intra-day accuracy and precision. In addition, in-house quality control standards were run in replicates of six to evaluate intra-day and inter-day accuracy and precision. Accuracy was determined as the absolute value of the ratio of the back-calculated mean values to their respective nominal values and expressed as percentage. Precision was determined by the percentage coefficient of variation (%CV) for QC concentrations.

### *Limit of detection and limit of quantitation*

The limit of detection (LOD) and limit of quantitation (LOQ) of the method were determined by replicate injections of binary mixtures. The LOD was defined as a minimum signal-to-noise of 3. The LOQ was determined by taking S/N ratio of 10.

### *System suitability*

System suitability parameters were measured to verify the system performance. Factors such as retention time, tailing factor, repeatability, resolution, and theoretical plate numbers were taken into consideration according to compendial specification for the testing of system suitability (26).

### *Robustness*

The robustness of a method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Minor changes in HPLC mobile phase composition, flow rate, pH, and different brands of C<sub>18</sub> columns were studied to evaluate the robustness of the developed method.

### *Stability*

The stock solution stability of binary mixture (docetaxel and ketoconazole) was evaluated at room temperature and 2–8°C for seven days by comparing with freshly prepared stock solution. Post-preparative stability of QC samples was evaluated by

injecting immediately after preparation and re-injecting after keeping in auto-sampler for 24 h.

### **In vitro release of docetaxel and ketoconazole from DK-SLN solid lipid nanoparticles**

In vitro release studies were performed using modified Franz diffusion cell. Dialysis membrane having pore size of 2.4 nm and with molecular weight cut off 12,000–14,000 was used. The membrane was soaked in double-distilled water for 12 h before mounting in a Franz diffusion cell. Lyophilized DK-SLN were dispersed in 10 mL of deionized water and a volume of one ml of docetaxel and ketoconazole loaded SLN formulation was placed in the donor compartment of each Franz Diffusion cell and the receptor compartments were filled with dialysis medium. An aliquot of 100 µl of sample was withdrawn from receiver compartment through side tube at time intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 h. Fresh medium was replaced each time to maintain constant volume. Samples were analyzed by HPLC as described previously. In vitro release studies were conducted in phosphate buffer saline pH 7.4.

## **Results and Discussion**

### **HPLC method development and validation**

The RP-HPLC method was developed to provide a specific procedure for the rapid separation of binary mixtures containing docetaxel and ketoconazole. To find the appropriate HPLC conditions for separation of docetaxel and ketoconazole, various reversed phase columns, isocratic and gradient mobile phase systems were tried. Separation of both drugs was attempted using phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>), ammonium acetate, and TEA with different organic solvents such as methanol and acetonitrile. With phosphate buffer and ammonium acetate buffer, both drugs were not eluted with good resolution. When methanol was used in the composition of the mobile phase, resulting peaks were not sharp and shapes of the peaks were also not good. Triethylamine buffer with acetonitrile has shown the elution of both drugs with good peak shapes and resolution.

Different columns such as RP-C<sub>8</sub>, RP-C<sub>18</sub>, RP-NH<sub>2</sub>, and RP-phenyl columns were used for the selection of appropriate column. Amino and phenyl columns were not eluted the both drugs and RP-C<sub>8</sub> column has shown less sensitivity compared with RP-C<sub>18</sub>.

To get optimum resolution between two peaks, different compositions of mobile phase were attempted. With the change of the mobile phase composition, the two drug peaks were merging and split peaks were obtained. At 48% of acetonitrile and 52% of TEA buffer, good resolution between the two peaks was obtained. The optimum wavelength for the estimation of both drugs was selected based upon the maximum area using Water 2996 PDA detector. Successful attempts were performed using reversed phase Waters C<sub>18</sub> symmetry (250 × 4.5 mm, 5 µm) with mobile phase of acetonitrile: 0.2% triethylamine pH adjusted to 6.4 (48:52, v/v). The optimum wavelength for detection was 230 nm at which better detector responses for both drugs were obtained. Decrease in concentration to 0.1% TEA has increased the tailing

as compared with high concentrations, but increased concentration from 0.2% to 0.5% has shown decreased peak area and increased peak width as compared with 0.2%. Effect of pH on the chromatographic conditions was studied to determine the optimum pH for the separation of drugs with good resolution and high sensitivity. Decrease in pH from 6.5 resulted in decreased peak area and lower resolution when compared with

pH 6.5. As shown in Figure 2, at a flow rate of 1 mL/min, the retention times of ketoconazole and docetaxel were found to be approximately 9.5 and 11.5 min, respectively.

#### Linearity

The developed HPLC method has shown the linearity over the range of 0.5 to 20  $\mu\text{g/mL}$  with regression coefficient of 0.9999 for ketoconazole and 0.9998 for docetaxel. The values of slope and intercept were 123130 and  $-60141$  for ketoconazole and 59410 and  $-28602$  for docetaxel respectively.

#### Intra- and inter-day variabilities

Intra- and inter-day accuracy and precision of the method was found to be 98.25–101.04 % and 0.48–1.14% for ketoconazole and 99.26–101.15% and 0.44–1.58% for docetaxel respectively (Table I). Thus, it was concluded that there was no significant difference for the assay, which was tested within day and between days.

#### LOD and LOQ

LOD of both drugs with the developed HPLC method was found to be 0.1  $\mu\text{g/mL}$  that yields S/N ratio of 3, and the LOQ value under the described condition was found to be 0.5  $\mu\text{g/mL}$  with S/N ratio of 10.

#### System suitability

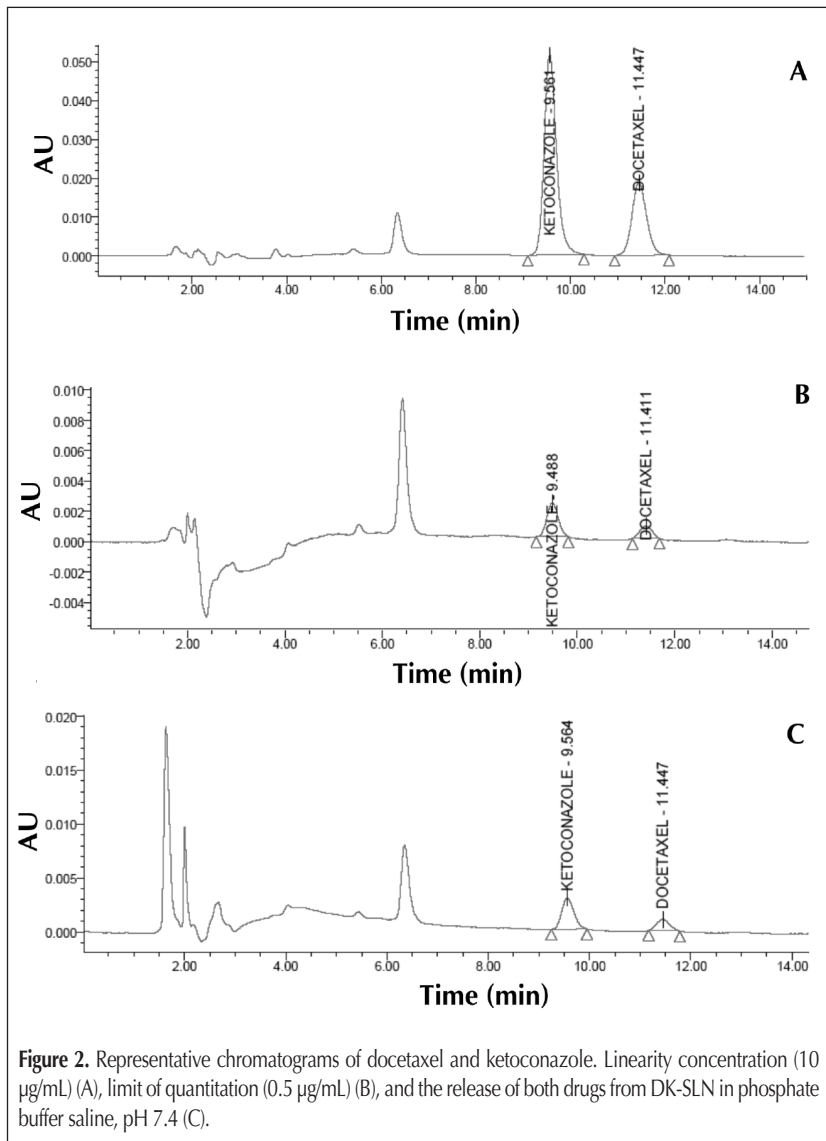
System suitability was performed by six replicate injections of QC samples. With the previously described conditions, resolution of peaks was found to be 3.99 and tailing factors were obtained as 1.01 for docetaxel and 1.16 for ketoconazole. Retention times of docetaxel and ketoconazole were 9.5 and 11.5 min, respectively. The theoretical plate count for ketoconazole and docetaxel are 7394.85 and 8307.82, respectively. The theoretical plate count was more than 5000 for both docetaxel and ketoconazole (according to USP).

#### Robustness

The minor change in composition of HPLC mobile phase ( $\pm 3\%$ ), flow rates at  $1 \pm 0.05$  mL/min and the effect of pH ( $\pm 0.2$  pH units) were studied to evaluate for the robustness of the method. The previously mentioned changes did not show any significant change in validation parameters, but more than 3% change in mobile phase and major changes in pH ( $\pm 1$  unit) had shown changes in retention time, resolution, and tailing factors.

#### Stability

Stability studies were performed for three QC concentrations. Stability was determined based on the peak areas and retention times in compar-



**Figure 2.** Representative chromatograms of docetaxel and ketoconazole. Linearity concentration (10  $\mu\text{g/mL}$ ) (A), limit of quantitation (0.5  $\mu\text{g/mL}$ ) (B), and the release of both drugs from DK-SLN in phosphate buffer saline, pH 7.4 (C).

**Table I. The Results of Inter- and Intra-day Accuracy and Precision of QC Samples**

QC Samples	Ketoconazole			Docetaxel			
	Nominal Conc. ( $\mu\text{g/mL}$ )	Mean Measured conc. ( $\mu\text{g/mL}$ )	Accuracy (%)	Precision (%)	Mean Measured conc. ( $\mu\text{g/mL}$ )	Accuracy (%)	Precision (%)
<i>Intra-day (n = 6)</i>							
1.5	1.48	98.68	0.56	1.51	100.93	0.48	
7.5	7.40	98.66	0.74	7.59	101.15	0.59	
15	15.16	101.04	1.05	14.99	99.92	0.82	
<i>Inter-day (n = 6)</i>							
1.5	1.49	99.19	1.14	1.52	101.04	0.48	
7.5	7.37	98.25	1.07	7.44	99.26	0.44	
15	15.01	100.10	1.07	15.05	100.33	0.90	

ison with freshly prepared solution of QC samples (initial concentration). The result showed that relative standard deviation was less than 1.5% for both drugs, indicating that solutions are stable within the given period (Table II).

### Application to assay and in vitro release studies of docetaxel and ketoconazole loaded solid lipid nanoparticles

Docetaxel and ketoconazole loaded solid lipid nanoparticles were prepared for targeting of both the drugs to specific tumor site. High encapsulation of both drugs in SLN helped to release both drugs at the targeted site and the release of ketoconazole can modulate the activity of CYP3A4 and inhibits the p-gp efflux. This phenomenon limits the CYP3A4 metabolism and p-gp efflux of docetaxel at the target site.

Applicability of the proposed method for the simultaneous estimation of docetaxel and ketoconazole was studied in docetaxel and ketoconazole loaded solid lipid nanoparticles (DK-SLN). Total content of docetaxel and ketoconazole present in

DK-SLN formulation was found to be  $4.79 \pm 0.05$  and  $4.63 \pm 0.12$  mg.

Drug release studies were performed as described previously. DK-SLN has shown about  $25.29 \pm 2.83\%$  of ketoconazole and  $18.87 \pm 1.48\%$  of docetaxel in 24 h. The release of both drugs has shown statistically significant difference ( $p < 0.05$ ) after 24 h. As shown in Figure 3, the release of docetaxel and ketoconazole was almost same up to 3 h, but release of ketoconazole was faster than docetaxel after 4 h. Controlled release of both drugs may also reduce the toxic effects associated with co-administration of both drugs. The release profile of docetaxel from DK-SLN was similar to the docetaxel loaded SLN reported by Zhenghong et al (27).

### Conclusions

The proposed RP-HPLC method is a suitable technique for the simultaneous determination of docetaxel and ketoconazole. The validated method is simple and reproducible which renders for routine analysis in quality control laboratories. The RP-HPLC method has shown good resolution between docetaxel and ketoconazole. The methods have shown good accuracy and precision. This quantitation was successfully applied for the assay and release studies of docetaxel and ketoconazole loaded solid lipid nanoparticles.

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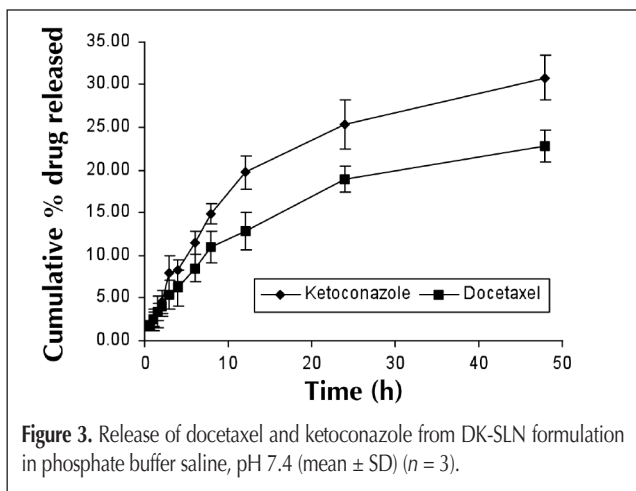


Figure 3. Release of docetaxel and ketoconazole from DK-SLN formulation in phosphate buffer saline, pH 7.4 (mean  $\pm$  SD) ( $n = 3$ ).

Table II. Stability Study of Docetaxel and Ketoconazole Spiked QC Samples\*

QC Samples	Ketoconazole			Docetaxel		
	Mean measured conc. ( $\mu\text{g/mL}$ )	Mean stability sample conc. ( $\mu\text{g/mL}$ )	% RSD	Mean measured conc. ( $\mu\text{g/mL}$ )	Mean stability sample conc. ( $\mu\text{g/mL}$ )	% RSD
<i>Stock solution stability at room temperature</i>						
1.5	1.48	1.49	0.34	1.51	1.49	0.33
7.5	7.40	7.54	0.19	7.59	7.57	0.07
15	15.16	15.12	0.05	14.99	15.20	0.05
<i>Stock solution stability at 2–8°C</i>						
1.5	1.48	1.47	1.43	1.51	1.53	0.91
7.5	7.40	7.53	0.27	7.59	7.65	1.04
15	15.16	15.17	0.18	14.99	15.29	0.17
<i>Autosampler stability</i>						
1.5	1.48	1.48	1.49	1.51	1.53	1.43
7.5	7.40	7.47	0.18	7.59	7.71	0.27
15	15.16	15.17	0.13	14.99	15.17	0.18

\* ( $n = 6$ )

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