Simultaneous Determination of Docetaxel and Celecoxib in Porous Microparticles and Rat Plasma by Liquid-Liquid Extraction and HPLC with UV Detection: *in vitro* and *in vivo* Validation and Application

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ABSTRACT - Purpose: A simple, rapid, sensitive, and reliable HPLC method with UV detection was developed and validated for simultaneous quantitation of docetaxel and celecoxib and paclitaxel for dissolution characterization and pharmacokinetic studies. **Methods:** The HPLC assay was performed isocratically on a reversed-phase C18 µ-Bondapack column using a mobile phase of acetonitrile:water (45:55, v/v) at a flow rate of 1.2 mL/min, and the analytes were detected at 230 nm. Paclitaxel was used as an internal standard for analysis of plasma samples following simple liquid-liquid extraction with n-hexane:isoamyl alcohol (97:3). The method was validated for specificity, linearity, sensitivity, precision, accuracy, robustness, and *in vitro-in vivo* application. **Results:** The retention times for docetaxel, paclitaxel, and celecoxib were 10.94, 12.4, and 16.81 min, respectively. The standard curves covering 0.1-1 µg/mL and 0.05-4 µg/mL were linear using dissolution medium and rat plasma, respectively. The limit of quantitation of the method was 50 ng/mL using 100 µL of rat plasma sample and injection of 50 µL of the residue. Within- and between-day precision and accuracy did not exceed 16.86% and 12.10%, respectively. This validated method was successfully used to quantify docetaxel and celecoxib simultaneously in the release study of docetaxel-celecoxib -loaded porous microparticles and pharmacokinetics studies. The methods were found to be simple, specific, precise, accurate, and reproducible. In this study, paclitaxel was used as the internal standard while dexamethasone, flutamide, and budesonide proved suitable alternative as an internal standard. **Conclusion:** Since docetaxel and celecoxib could be co-administered for the treatment of a wide range of cancers such as non-small cell lung carcinoma, the developed method is particularly advantageous for routine therapeutic drug monitoring and pharmacokinetic studies of these drugs.

Abbreviations: CXB, celecoxib; DTX, docetaxel; HPLC, high-performance liquid chromatography; LLE, liquid-liquid extraction; LOD, limit of detection; LOQ, limit of quantification; NSCLC, non-small-cell lung carcinoma; PP, protein precipitation; PTX, paclitaxel, SPE, solid phase extraction

INTRODUCTION

Lung cancer is one of the very common serious cancers accounting for the most cancer-related deaths in both men and women. In recent years many progresses have been made in the treatment of lung cancer, but the efficacy in NSCLC patients have been relatively low (1). Novel combination of chemotherapeutic agents with different mechanisms of action is considered as a new strategy to enhance anticancer efficacy of the drug, minimize emergence of resistance, and reduce adverse side effects. In this direction, combination of selective cyclooxygenase (COX)-2 inhibitors celecoxib with cytotoxic drugs docetaxel has shown synergistic antitumor effect in lung tumor models (2).

Docetaxel is a semi synthetic antineoplastic agent, which is isolated from the needles of the European yew tree *Taxus baccata L*. docetaxel has shown considerable antitumor effects on broad spectrum of cancers especially NSCLC as the first-line chemotherapeutic agent (1-4). Docetaxel inhibits microtubule depolymerization in the G2/M cell-cycle phases inducing apoptosis in cancer cells (3-7).

The cyclooxygenase-2 (COX-2) is a key enzyme in arachidonic acid metabolism and prostaglandin production. It is stimulated by growth factors, cytokines, and promoters of cancer cells, leading to the production of PG at the site of inflammation, and is

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involved in cell proliferation (8-11). Expression of COX-2 enzymes, COX-2 mRNA, and protein in various cancers in humans, including lung and especially type NSCLC have been greatly enhanced and are thought to be potentially involved in the pathogenesis of lung cancer (12-18). Recent evidence also indicates that biosynthesis of prostaglandin is increased in cancer cells (19). Celecoxib decreases the intratumor prostaglandin E2 (PGE2) levels via enzymatic inhibition of COX-2 activity without affecting the COX-2 expression (20). Celecoxib, a highly selective COX-2 inhibitor, has shown antitumor effects in human tumor xenograft models of Lewis lung carcinoma (21). Celecoxib also induces apoptosis of cancer cells through inhibition of other mediators involved in the pathogenesis of the disease (22). Due to non-overlapping mechanisms, docetaxel combination with celecoxib for the treatment of NSCLC has shown considerable synergistic effects (2, 23-25). Similar to docetaxel, celecoxib is a lipophilic compound (27) and systemic administration of celecoxib stimulates heart attacks (28).

Lung carcinoma chemotherapy through pulmonary route is considered very efficient as this organ has a large absorptive surface of the alveolar region, delivers drugs directly to the tumor tissues, less systemic availability resulting to lower adverse side effects on normal cells, low metabolic activity in comparison with other routes of administration and avoidance of gastrointestinal and first-pass metabolic degradation (26-28). Therefore, novel and safe drug deliveries are needed in order to supply drug directly to the tumor cells in the lung thereby healthy organs would not be affected by the toxicities of the chemotherapeutic agents.

Porous microspheres with an aerodynamic mean size of 5–30 μm can escape from phagocytic clearance and can be deposited in the deep lung with an enhanced residence time have been proved desirable for pulmonary drug delivery (29,30). Therefore, we have recently designed and developed biodegradable porous PLGA microspheres loaded with docetaxel and celecoxib in order to improve therapeutic efficacy and reduce drug systemic toxicity (unpublished study). Therefore, development and validation of a rapid, simple, and yet sensitive high-performance liquid chromatography (HPLC) method for the quantitation of docetaxel and celecoxib for *in vitro* and *in vivo* characterization of developed docetaxel-celecoxib - loaded porous microspheres was of primary interest for us.

Reversed-phase HPLC and UV spectrophotometric methods for measurement of celecoxib (31,32) and docetaxel (33) in pure form and in liposomal or solid dosage forms have been reported. However, HPLC methods for concurrent measurements of docetaxel and celecoxib in delivery systems or in biological specimen have not yet been reported.

Sensitive methods for quantification of docetaxel in plasma using liquid chromatography-tandem mass spectrometry following liquid-liquid extraction (LLE), protein precipitation (PP) and/or solid phase extraction (SPE) (34-36), PP with UV detection (37), SPE or LLE with UV detection (38-43) have so far been reported. Measurement of celecoxib in biological fluids following LLE (44-48), PP (49, 50), LLE (51, 52) or SPE (53) after PP, and SPE (54-58) have been described.

Generally, PP deteriorates the chromatographic column and reduces the sensitivity of the assay due to sample dilution. SPE procedures is a high cost and time-consuming clean up procedure requiring a large volume of samples unsuitable for the processing of multiple samples in pharmacokinetic studies, and HPLC method based on MS or MS-MS are not affordable or readily available in most laboratories. Our described method does not utilize LC-MS, SPE, PP, or large volume of plasma and yet sensitive. The method is rapid, simple and appropriate for *in vitro* evaluation and pharmacokinetic studies in small animals and rodents following concurrent administration of docetaxel and celecoxib as with the PLGA porous microparticles developed in our laboratory. Paclitaxel was used as the internal standard (IS) in animal pharmacokinetic studies. This method was validated for its selectivity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines. The validated method was applied in *in vitro* studies of docetaxel- and celecoxib-loaded porous microparticles and following administration of formulations containing both drugs.

MATERIAL AND METHOD

Chemicals and reagents

Docetaxel, celecoxib, paclitaxel, diazepam, flutamide, budesonide, dexamethasone, and poloxamer (pluronic F-127) from Sigma Chemical Co. (St. Louis, MO); diethyl ether, acetonitrile, and methanol from Caledon (Ontario, Canada); n-hexane, isoamyl alcohol, and chloroform from Merck (Germany). All reagents and solutions were either HPLC or analytical grades.

docetaxel-and celecoxib-containing PLGA porous microparticles were developed and prepared in our laboratory.

Determination of UV wavelength

The suitable wavelength for simultaneous determination of docetaxel and celecoxib for *in vitro* study and docetaxel and celecoxib and internal standard (paclitaxel) for *in vivo* study was achieved by wavelength scanning over the range of 200 - 400 nm with a Shimadzu UV-160 (Shimadzu, Japan) double beam spectrophotometer.

Chromatography system and conditions

The apparatus used was a Waters HPLC system model 746 (USA), consisting of a model 515 intelligent solvent delivery pump, a 100-µL injection loop, a computerized system controller, and a Waters 2487 UV detector. Chromatographic separation was performed on a µ-Bondapak C18 (250 mm × 4.6 mm, Waters, Ireland) column. The mobile phase consisted of water/acetonitrile (55/45 v/v) for *in vitro* and *in vivo* studies and eluted at a flow rate of 1.2 mL/min. The column effluent was detected at 230 nm.

Internal standard and extracting solvent

To achieve a suitable internal standard for plasma analysis, five drug substances including flutamide, diazepam, paclitaxel, budesonide, and dexamethasone were examined. To test their interference with docetaxel and celecoxib retention times, each compound was injected into the HPLC system individually and in combination with docetaxel and celecoxib. Chromatograms of blank plasma extracted with extracting solvents were also recorded to assess the interference of the analytes with plasma endogenous substances.

Standard solutions of celecoxib, docetaxel and internal standard paclitaxel

One mg of celecoxib or docetaxel was separately weighed out and their mixture was dissolved in 1 mL methanol. Solution was further diluted with methanol to obtain 10 and 100 µg/mL standard stock solutions for *in vitro* and *in vivo* studies, respectively. Calibration curve standard solutions of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/mL were prepared for simultaneous measurement of celecoxib and docetaxel in dissolution medium by further dilution of *in vitro* standard stock solution in poloxamer 1.5%. For *in vivo* studies, working standard solutions at concentrations of 0.5, 1, 2.5, 5, 10, 15, 20, 30, and 40 µg/mL containing both celecoxib and docetaxel were achieved by further dilution of *in vivo* standard stock solutions in methanol. paclitaxel (IS) stock solution at concentration of 15 µg/mL was prepared in methanol.

Calibration procedure

For the preparation of calibration curves in dissolution medium, 30 µL of each *in vitro* working standard solutions were injected into the injection port and drug concentrations versus peak areas for each drug were plotted. To obtain the calibration curve in plasma, 10 µL of *in vivo* working standard solutions ranging from 0.5 - 40 µg/mL with paclitaxel (IS) at 15 µg/mL was added into 100 µL blank plasma. Drugs standard concentrations ranging from 0.05 - 4 µg/mL and IS at a fixed concentration of 1.5 µg/mL in plasma was achieved. Five mL n-hexane and isoamyl alcohol (97:3) were added to each tube, vortexed for 2 minutes, and centrifuged at 5,000 rpm for 5 min. The upper layer was then transferred to a clean test tube and evaporated to dryness under nitrogen gas. The residue was reconstituted in 100 µL of the mobile phase, mixed well and 50 µL of the final clear solution was injected into the HPLC system. By plotting peak area ratios of celecoxib or docetaxel to paclitaxel versus drug concentrations, the calibration curve for each drug was constructed.

Method validation

Selectivity and specificity

In order to test the interfering effect of components of porous microparticle matrix with docetaxel, celecoxib, and Paclitaxel peaks, blank microparticles devoid of drugs were prepared in poloxamer 1.5% w/v (used as the external phase in preparation of microspheres), centrifuged, and the upper liquid was removed and injected into the HPLC system. For *in vivo* tests, blank rat plasma samples without any analyte were pretreated with LLE as described before. The selectivity of the analytical method was confirmed by analysis of a concentration equal to LOQ of the assay method for docetaxel and celecoxib in dissolution medium and plasma. The ability of the method to separate all substances from docetaxel and celecoxib was assessed by a resolution between the peaks corresponding to various compounds. The peak factors were calculated using equations below:

$$Tailing\ f\ \text{actor} = \frac{W_{5\%}}{2\text{F}}$$

where, $W_{5\%}$ is the peak width at 5% of the peak height and F is the front peak half-width.

$$Resolution\ f\ \text{actor} = \frac{\mathrm{RT_2 - RT_1}}{0.5\times(W_1 + \mathrm{W_2})}$$

where, RT is peak retention time and W is peak width.

Linearity

Distribution of the residuals i.e. the percent differences of the back-calculated concentrations (measured) from the nominal (real) concentrations were determined to validate the correlation. For *in vitro* determinations, the calibration model will be accepted if the residuals are less than 2% and for *in vivo* measurements within 20% for the lower limit of quantifications and within 15% for all other calibration levels and at least 2/3 of the standards meet this criterion. The calibration curves were also evaluated by the correlation coefficient, slope, and intercept.

Precision, accuracy, and recovery

The intra- and inter-day variations of the assay in dissolution medium and plasma were determined by replicate analysis of samples at concentrations within the range of calibration curves in a single analytical run on the same day and at three successive days, respectively, using the same stock solutions. The percent coefficient of variance (% CV) or relative standard deviation (RSD) and accuracy were determined using the following equations.

$$\%\ Precision = (standard\ deviation/average\ concentration) \times 100$$
$$\%\ Accuracy = (measured\ concentration/nominal\ concentration) \times 100$$

The recoveries of docetaxel and celecoxib at three concentrations of 0.05, 1.5, and 3 µg/mL were evaluated by comparison of the peak areas obtained after extraction of a known amount of docetaxel and celecoxib from plasma with those obtained from the same amounts of drugs in methanol.

Limit of detection (LOD) and limit of quantitation (LOQ) determination

Limit of detection was determined using the signal-to-noise ratio by comparing the results of the test samples with known concentrations of the analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision, and variability.

Robustness

The robustness of the HPLC method for *in vivo* measurements was determined by analysis of samples under a variety of conditions such as small changes in the fraction of acetonitrile of the mobile phase, in the pH, and in the mobile phase flow rate. Standard strength of 15 µg/mL was used to prepare the samples. The final nominal concentration of docetaxel and celecoxib and paclitaxel (IS) was 1.5 µg/mL. Samples were prepared by the method described before. Percent changes in retention time, tailing factor, resolution factor, peak area ratio, and accuracy were calculated and compared with those obtained for the set-up method.

Application of the method

***in vitro* assessment of prepared formulation**

The proposed validated method was utilized for determination of docetaxel and celecoxib in dissolution medium of PLGA porous microparticles containing both drugs. Polymeric PLGA microparticles were prepared by simple W/O/W emulsion. One mL of docetaxel and celecoxib containing microparticle suspension were added into 24 mL of dissolution medium directly. The dissolution studies were performed at 37 °C and 300 rpm. In the scheduled time 100 µL of the medium were centrifuged at 12,000 rpm for 10 minutes. Residual discarded and 30 µL of the clear solution was injected into the HPLC system. docetaxel and celecoxib concentrations in the samples were then calculated using peak area responses and the previously plotted standard curve. This experiment was performed on three independent microparticle formulations.

Pharmacokinetic studies

Male Wistar rats (n = 3), weighing 200-230 g, were obtained from Laboratory Animal Resource Center of School of Pharmacy and Pharmaceutical Science of Isfahan University of Medical Science (Isfahan, Iran). Animals were maintained under normal conditions and allowed free access to water and food. Animal experiments were approved by Iran National Committee for Ethics in Biomedical Research (ethical approval ID: IR.MUI.RESEARCH.REC.1397.387) which was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals. Possible efforts were made to decrease animal numbers and distress. Two mg celecoxib and 2

mg docetaxel were accurately weighed out and dissolved in a mixture of 130 µL ethanol and 200 µL Tween 80 and then 670 µL normal saline was added and mixed vigorously. The injection volume of the clear solution was adjusted to deliver 5 mg/kg of either docetaxel or celecoxib through the animals' tail vein. Before and 0.25, 0.5, 0.75, 1, 2, 4, and 6 h after injection, 250 µL of blood was taken through retro-orbital sinus vein using heparinized capillary without anesthesia from each rat and centrifuged by heparinized microtube at 5,000 rpm for 5 minutes. One hundred µL of plasma was transferred into a clean glass test tube and thereafter 10 µL IS with a concentration of 15 µg/mL was added. Plasma was extracted using the LLE method described before. Fifty µL of the final reconstituted solution was injected into the injection port of the HPLC system. By comparing the ratio obtained from the standard curve and peak area ratio of docetaxel or celecoxib to internal standard from samples, docetaxel, and celecoxib concentrations were determined.

Pharmacokinetic analysis

Plasma concentration-time data were fitted in a two-compartment open model $C_t = Ae^{-\alpha t} + Be^{-\beta t}$, where, C_t is the drug concentration at time t; A is residual intercept and B is the intercept of an ordinate axis, and α and β are the corresponding first-order disposition rate constants. Finally, pharmacokinetic parameters including the area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$), the apparent volume of distribution (V_d), systemic plasma clearance (CL), distribution half-life ($T_{1/2\alpha}$), elimination half-life ($T_{1/2\beta}$) and mean residence time (MRT) were calculated by standard methods.

RESULTS

Determination of UV wavelength

In order to acquire appropriate UV wavelength for simultaneous verification, solutions of docetaxel, celecoxib, and paclitaxel in the mobile phase were scanned by UV spectroscopy in the range of 200-400 nm. Three tested compounds jointly have considerable absorbance at 230 nm.

Selection of appropriate extracting solvent and internal standard

In order to achieve suitable internal standard several compounds such as flutamide, diazepam, paclitaxel, budesonide, and dexamethasone with similar lipophilicity and UV absorptivity to docetaxel and celecoxib were tested. Flutamide (Fig. 1b) and diazepam traces appeared adjacent to the docetaxel peak showing a double peaks pattern. Budesonide (Fig. 1c), dexamethasone (Fig. 1d), and paclitaxel (Fig. 1e) were considered appropriate internal standards with no interferences with plasma endogenous materials once diethyl ether was used as the extracting solvent, however, a late eluting peak at 40 min was observed (Fig. 1f) resulting in unacceptable run time. Extraction of plasma, serum, pure water, and heparin with diethyl ether revealed that this late peak is most likely due to the plasma endogenous substances. PP prior to the extraction of plasma samples with diethyl ether not only could fully wipe off the late eluting peak but also dramatically reduced the drug extraction efficiency. Chloroform and n-hexane were examined separately to extract the analytes. With chloroform, the 40-min late peak was disappeared but another one appeared at 25 min. With the use of n-hexane no further late peak was detected but docetaxel was not extracted efficiently.

Although budesonide (Fig. 1c) and dexamethasone (Fig. 1d) were completely resolved from the docetaxel peak, they interfered with endogenous plasma peaks when n-hexane:isoamyl alcohol (97:3) was used as an extracting solvent. Ultimately, paclitaxel was considered the most appropriate IS once n-hexane:isoamyl alcohol (97:3) was used as extracting solvent. The mixture of n-hexane:isoamyl alcohol resulted in no late eluting peaks and significant recovery of analytes.

Selectivity and specificity

The HPLC chromatograms recorded for the blank dissolution (Fig. 2) revealed almost no peaks during the run time and all substances were eluted completely and appeared as separate resolved peaks. Representative chromatograms show a clear separation of docetaxel and celecoxib in a poloxamer 1.5% W/V solution, which was used as the external phase for the preparation of the microspheres. Figure 3 shows typical chromatograms of blank plasma and plasma spiked with docetaxel, celecoxib, and paclitaxel. As illustrated, three substances eluted with good resolution as three separate resolved peaks within 20 min. Initial peaks related to the plasma matrix appeared up to10 min, which did not interfere with the analyte peaks. Docetaxel, paclitaxel, and celecoxib eluted at 10.9, 12.4, and 16.8 min, respectively. Table 1 displays the peak factors of docetaxel and celecoxib with their adjacent peaks. Hence, the presented method is selective for the simultaneous determination of docetaxel and celecoxib.

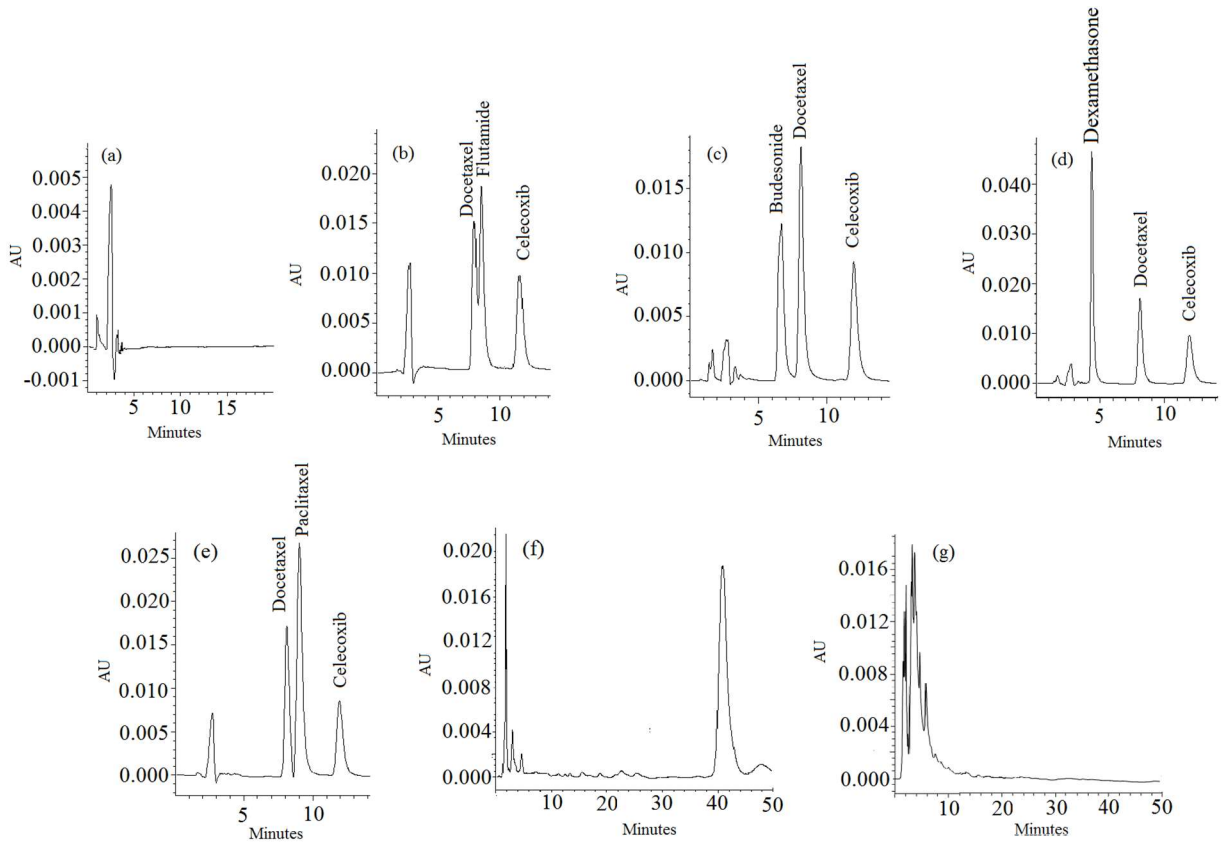

Figure 1. HPLC chromatograms of blank dissolution medium (a), docetaxel (10 µg/mL) and celecoxib (3 µg/mL) with different internal standards (10 µg/mL). Flutamide (b), budesonide (c), dexamethasone (d), paclitaxel (e), 45-min eluting peak (f), blank plasma samples extracted with diethyl ether (f), and blank plasma samples extracted with n-hexane:isoamyl alcohol (g) eluted with acetonitrile:water (50:50).

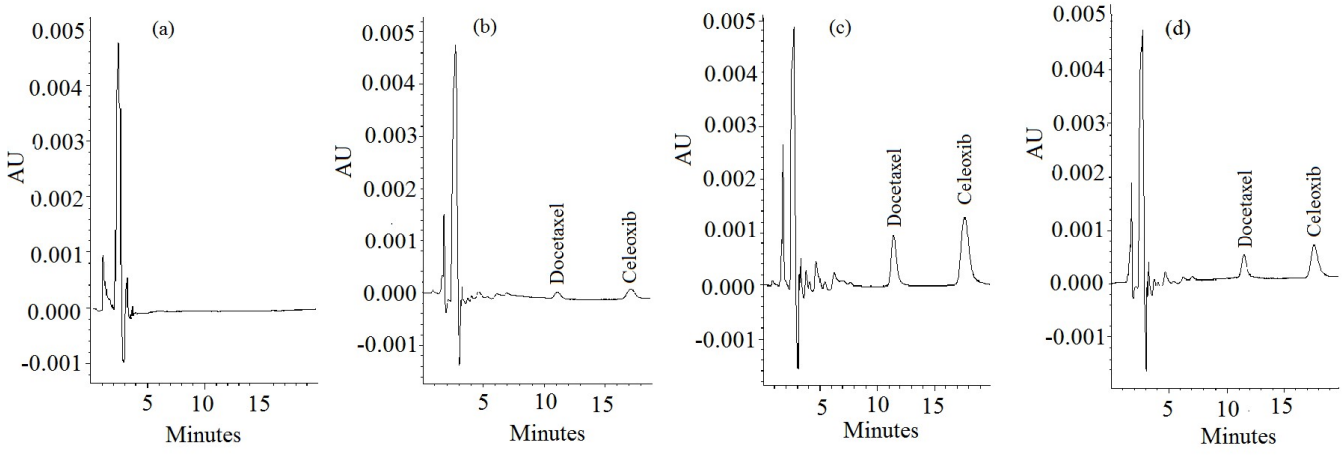

Figure 2. HPLC chromatograms of blank dissolution medium (a), dissolution medium containing 0.1 µg/mL docetaxel and celecoxib (b), dissolution medium containing 1 µg/mL docetaxel and celecoxib (c), and docetaxel and celecoxib released from porous particles in dissolution experiment after 36 h (d).

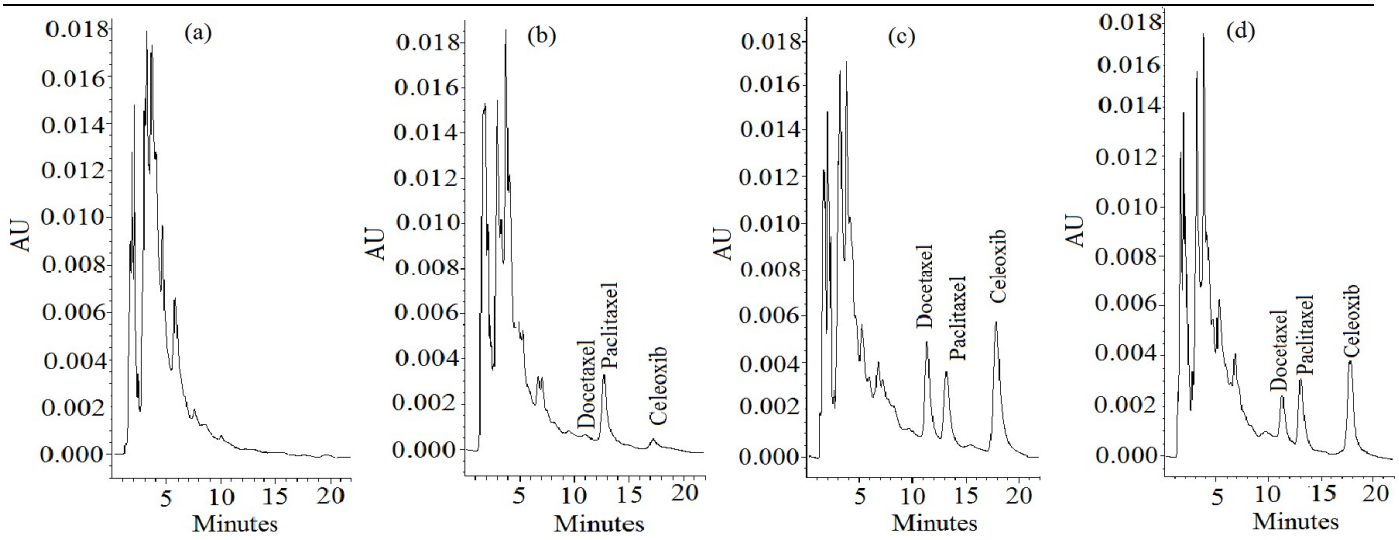

Figure 3. HPLC chromatograms of blank plasma extracted with n-hexane:isoamyl alcohol (97:3) (a), plasma contained 0.05 µg/mL docetaxel and celecoxib (b), plasma contained 3 µg/mL docetaxel and celecoxib (c), and plasma taken from rat 30 min after IV bolus injection of 5 mg/kg docetaxel along with 5 mg/kg celecoxib (d).

Table 1. docetaxel and celecoxib peaks related parameters

Tailing factor			Resolution factor				Retention time			Peak area ratio		Accuracy	
DTX	PTX	CXB	Plasma-DTX	DTX-PTX	PTX-CXB	DTX-CXB	DTX	PTX	CXB	DTX	CXB	DTX	CXB
1.07	1.16	1.20	1.22	1.80	6.59	4.65	10.9	12.4	16.81	0.61	1.31	1.05	0.39

Calibration curves and linearity

Calibration curves for docetaxel and celecoxib were plotted in dissolution medium and plasma as described before. As shown in Fig. 4, the developed method demonstrated excellent linearity for docetaxel and celecoxib for both dissolution and pharmacokinetic studies as indicated with mean correlation coefficients > 0.999. The regression equations are shown in Fig. 4 (a) and (b).

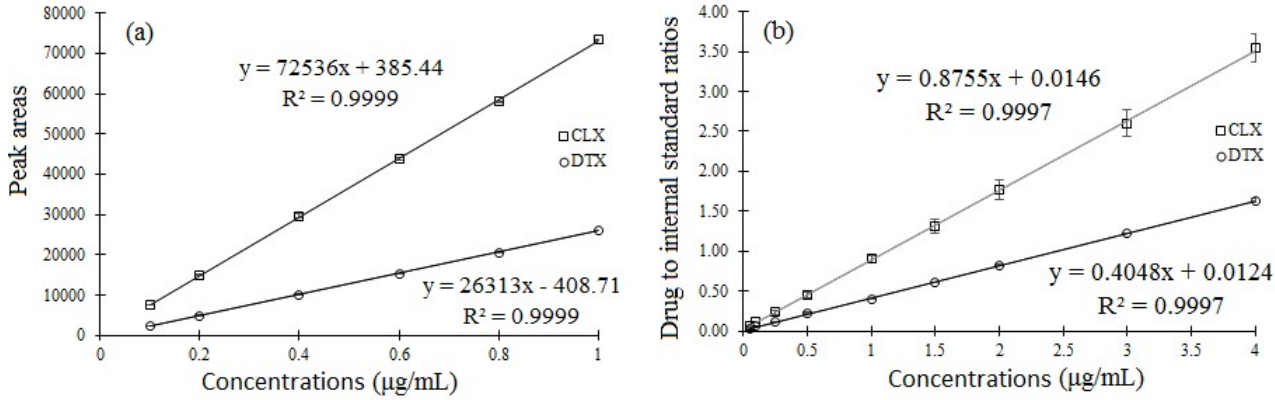

Figure 4. Calibration curves of docetaxel and celecoxib in: dissolution medium (a), plasma (b). Each point represents means ± SD (n = 3). Due to small variations amongst experiments, in many data points, particularly in dissolution medium test, SD bars are not visible.

Precision, accuracy and recovery

Results attained for precision and accuracy studies of the method in dissolution medium and plasma specimens are presented in Table 2 and Table 3, respectively. All results comply with the acceptance criteria defined in the International Conference on Harmonization (ICH) guidelines showing well precision and accuracy of the method. Table 4 shows the percent recoveries of docetaxel, celecoxib, and paclitaxel in plasma at three concentrations of quality control i.e. low, medium, and high.

Table 2. Intra- and inter-day precision and accuracy of the HPLC method developed for determination of docetaxel and celecoxib in dissolution medium

		Intra-day precision and accuracy				Inter-day precision and accuracy			
	Concentration (µg/mL)	Mean	S.D.	R.S.D. (Precision)	Accuracy (%)	Mean	S.D.	R.S.D. (Precision)	Accuracy (%)
Docetaxel	0.1	0.101	0.0015	1.43	101.3	0.101	0.0014	1.41	101.3
	0.2	0.201	0.0031	1.54	100.5	0.201	0.0026	1.28	100.4
	0.4	0.399	0.0033	0.82	99.84	0.400	0.0030	0.75	100.0
	0.6	0.596	0.0037	0.62	99.41	0.596	0.0034	0.58	99.33
	0.8	0.798	0.0052	0.66	99.76	0.798	0.0051	0.63	99.76
	1.0	1.003	0.0045	0.45	100.3	1.004	0.0039	0.39	100.3
Celecoxib	0.1	0.099	0.0007	0.72	99.44	0.100	0.0007	0.66	100.0
	0.2	0.200	0.0015	0.77	100.1	0.200	0.0017	0.85	100.1
	0.4	0.404	0.0070	1.73	100.8	0.403	0.0058	1.45	100.7
	0.6	0.598	0.0023	0.38	99.70	0.598	0.0038	0.63	99.58
	0.8	0.794	0.0017	0.22	99.22	0.794	0.0015	0.19	99.27
	1.0	1.005	0.0026	0.25	100.4	1.005	0.0031	0.30	100.4

S.D., Standard deviation; R.S.D., Relative standard deviation.

Table 3. Intra and inter-day precision and accuracy of the HPLC method for determination of docetaxel and celecoxib in plasma

		Intra-day precision and accuracy				Inter-day precision and accuracy			
	Concentration (µg/mL)	Mean	S.D.	R.S.D. (Precision)	Accuracy (%)	Mean	S.D.	R.S.D. (Precision)	Accuracy (%)
Docetaxel	0.05	0.049	0.0072	14.74	98.01	0.051	0.0079	15.40	102.4
	0.1	0.114	0.0110	9.67	113.8	0.113	0.0133	11.78	112.6
	0.25	0.258	0.0049	1.89	103.3	0.255	0.0128	5.02	102.2
	0.5	0.535	0.0277	5.19	106.9	0.530	0.0258	4.88	105.9
	1	0.940	0.0020	0.21	93.99	0.946	0.0065	0.69	94.61
	1.5	1.484	0.0040	0.27	98.95	1.481	0.0074	0.50	98.70
	2.0	1.999	0.0096	0.48	99.93	2.006	0.0167	0.83	100.3
	3.0	3.022	0.0054	0.18	100.7	3.018	0.0072	0.24	100.6
	4.0	4.034	0.0808	2.00	100.8	4.023	0.0654	1.63	100.6
Celecoxib	0.05	0.044	0.0074	16.86	87.90	0.045	0.0024	5.38	90.04
	0.1	0.108	0.0078	7.22	107.8	0.105	0.0119	11.39	104.7
	0.25	0.254	0.0030	1.18	101.4	0.245	0.0271	11.04	98.19
	0.5	0.483	0.0402	8.32	96.70	0.489	0.0415	8.49	97.72
	1	1.004	0.0347	3.46	100.4	1.015	0.0486	4.79	101.5
	1.5	1.490	0.0691	4.64	99.35	1.490	0.1052	7.07	99.31
	2.0	2.034	0.0691	3.40	101.7	2.027	0.1403	6.92	101.3
	3.0	2.983	0.1137	3.81	99.44	2.984	0.1929	6.47	99.47
	4.0	4.032	0.1600	3.97	100.8	4.086	0.1989	4.87	102.2

S.D., Standard deviation; R.S.D., Relative standard deviation.

Table 4. Percent recoveries of docetaxel, celecoxib, and paclitaxel extracted from plasma (n = 3) with n-hexane:isoamyl alcohol (97:3) at three different concentrations

	Recovery (%)		
Concentration (µg/mL)	Docetaxel	Celecoxib	Paclitaxel (IS)
0.05	87.98 ± 1.86	92.45 ± 1.89	-
1.5	84.20 ± 0.72	89.57 ± 1.55	78.36 ± 0.24
3	77.91 ± 0.95	85.20 ± 0.91	-

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ for docetaxel and celecoxib in dissolution medium were 0.05 µg/mL and 0.1 µg/mL, respectively. The LOD for docetaxel and celecoxib in plasma was approximately 0.01 µg/mL. The LOQ of docetaxel and celecoxib corresponding to a coefficient of variation of less than 15% was 0.05 µg/mL once 100 µL of the plasma sample was extracted, dried, reconstituted in 100 µL of the mobile phase of which 50 µL was injected into the HPLC system.

Robustness

Fig. 5 illustrates the robustness data of the assay method. It shows the percent changes in retention time, tailing factor, resolution factor, peak area ratio, and accuracy compared to the set-up method parameters listed in Table 1. Method robustness was checked after deliberate modifications in the mobile phase composition, flow rate, and pH.

***in vitro* and *in vivo* application of the method**

The method described here was successfully applied for simultaneous quantification of docetaxel and celecoxib in dissolution studies of docetaxel- and celecoxib-containing porous microparticles, and pharmacokinetic study using rat plasma. Fig. 6a illustrates the dissolution profiles of docetaxel and celecoxib released from microparticles and Fig. 6b shows the concentration-time profiles of docetaxel and celecoxib following IV administration of 5 mg/kg docetaxel and 5 mg/kg celecoxib. Table 5 includes pharmacokinetic parameters.

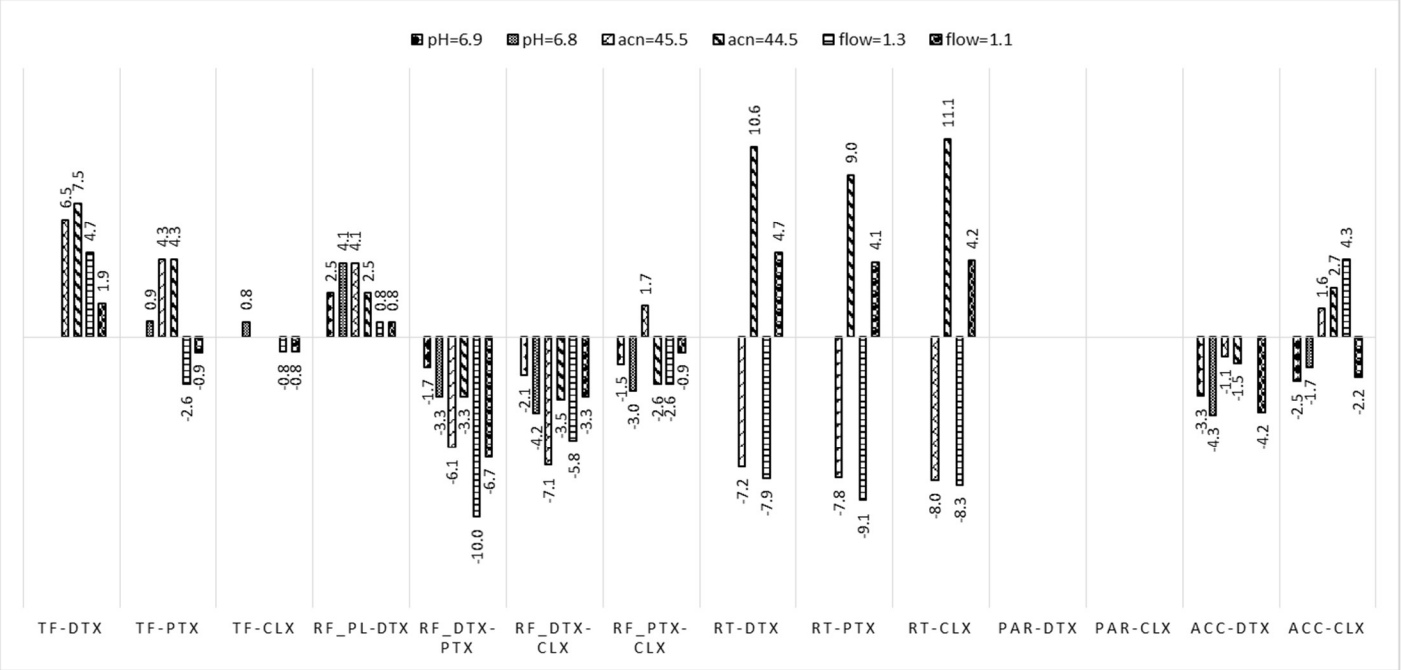

Figure 5. Robustness result of the assay method. It shows the percent changes in retention time (RT), tailing factor (TF), resolution factor (RF), peak area ratio (PAR), and accuracy (ACC) compared with set-up method parameters mentioned in Table 1. DTX, Docetaxel; CXB, Celecoxib; PL, Plasma.

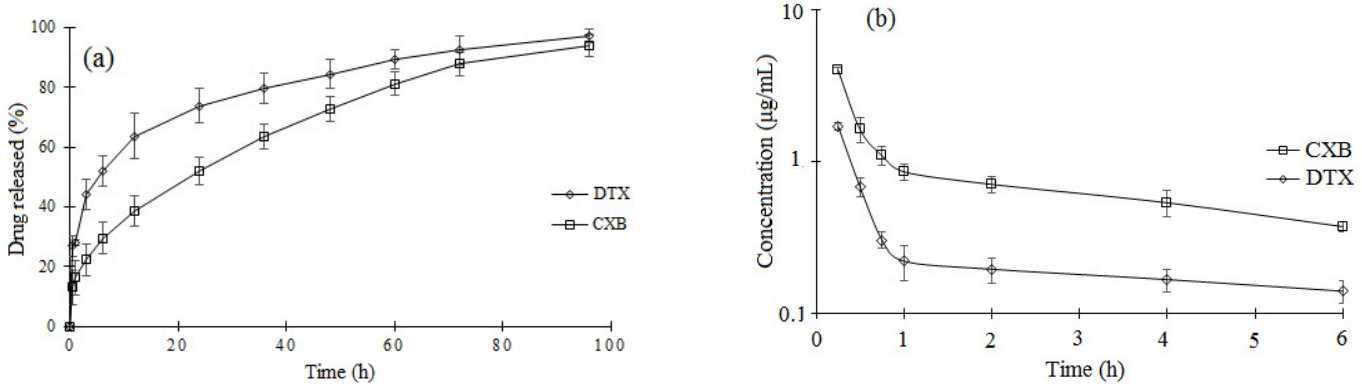

Figure 6. Release profiles of docetaxel and celecoxib liberated from porous microparticles (a), and concentration-time profiles of docetaxel and celecoxib fallowing IV administration of 5 mg/kg of docetaxel and celecoxib (b) (n = 3).

Table 5. The plasma pharmacokinetic parameters of docetaxel and celecoxib (n = 3)

	$AUC_{0-\infty}$ ($mg.h.L^{-1}$)	$AUMC_{0-\infty}$ ($mg.L^{-1}).h^2$	CL_{TB} $L.(h.kg)^{-1}$	MRT (h)	V_{dss} ($L.kg^{-1}$)	A ($mg.L^{-1}$)	α (h^{-1})	$T_{1/2\,\alpha}$ (h)	B ($mg.L^{-1}$)	β (h^{-1})	$T_{1/2\beta}$ (h)
Docetaxel	3.91 ± 0.17	32.39 ± 4.43	1.28 ± 0.06	8.29 ± 1.19	10.62 ± 1.75	6.79 ± 0.20	5.80 ± 0.27	0.12 ± 0.01	0.24 ± 0.06	0.09 ± 0.02	8.20 ± 1.46
Celecoxib	8.20 ± 0.74	38.11 ± 3.85	0.61 ± 0.06	4.65 ± 0.09	2.85 ± 0.23	11.06 ± 0.83	5.38 ± 0.39	0.13 ± 0.01	1.00 ± 0.14	0.16 ± 0.004	4.26 ± 0.09

AUC, Area under the zero-moment curve; AUMC, Area under the first moment curves; CL_{TB}, Total body clearance; MRT, Mean residence time; V_{dss}, Volume of distribution; $T_{1/2\,\alpha}$, Distribution half-life; $T_{1/2\beta}$, Elimination half-life.

DISCUSSION

In the current study, our purpose was to develop a simple, sensitive, and reliable HPLC method for simultaneous determination of docetaxel and celecoxib, which can be applied both in *in vitro* (dissolution characterization) and *in vivo* (pharmacokinetics studies) assessment of porous microparticles developed in our laboratory and loaded with these two drugs. Dissolution is a characterization test used to guide the formulation and is the only test that measures the rate of drug release *in vitro* as a function of time, which predicts drug release *in vivo*. As illustrated, three substances docetaxel, paclitaxel, and celecoxib eluted as sharp symmetrical peaks at 10.9, 12.4, and 16.8 min, respectively, and overall separation run time lasted 20 min and thereafter the system was ready for the next injection. The small peaks related to the plasma matrix appeared in the first 10 min and did not interfere with the analyte peaks. Three substances were eluted completely without peak tailing indicating that the assay method involves high specificity and selectivity from endogenous substances and other associated agents. The precision (repeatability) and accuracy of the calibration standard concentrations for both dissolution medium and plasma specimens were within the acceptable limits as defined in the ICH guidelines indicating that the developed method is precise, accurate, and reproducible for measuring docetaxel and celecoxib.

The method robustness referring to the ability of the assay to remain unaffected by small but deliberate changes to chromatographic conditions provides an indication of its reliability during routine usage (59). The robustness of the method checked after deliberate alterations of the mobile phase composition, mobile phase pH and flow rate, showed that the changes of the operational parameters did not lead to any essential changes in the performance of the chromatographic system. The tailing factor for docetaxel, celecoxib, and paclitaxel always ranged from 1.0 to 1.2, and drugs were well separated under all the changes carried out. The accuracy of docetaxel and celecoxib was acceptable under most conditions and did not show significant changes when the critical parameters were altered. Considering the result of alterations in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust. The percent recoveries of docetaxel and celecoxib were desirable under most

conditions and did not show a significant change when the critical parameters were altered.

To extract docetaxel and celecoxib from plasma samples, most of analytical methods have made the use of multiple steps LLE, SPE or PP followed by evaporation to prepare and concentrate samples prior to injection into the HPLC column. UV-HPLC assay based on PP with acetonitrile for quantification of docetaxel and celecoxib in plasma have been reported. PP has resulted in higher LOQ for DTX (3.1 μg/mL) (60) which is much greater than the value accomplished in our study (0.05 μg/mL) making the assay not applicable to a very low volume of plasma taken from small animals. Sample preparation involved PP for celecoxib used 1:1 ratio of acetonitrile to plasma to avoid dilution of the sample and to prevent the reduction in the sensitivity of the assay but this may lead to inadequate precipitation of the protein contents in the samples and deterioration of the chromatographic column in the long run (49,61). In our study, PP with acetonitrile was tested and percent recovery of docetaxel and celecoxib decreased dramatically.

Solid phase extraction technique for drug extraction and sample cleanup has been considered as a more efficient approach for increasing method sensitivity. PP followed by SPE also used to quantify docetaxel in rabbit plasma, which exhibited a LOQ 10 ng/mL (62), removed the interfering substances, and offered good extraction recoveries of docetaxel. However, most HPLC assays for docetaxel determination in biological fluids using the SPE cleanup method (63-66) required a large volume of samples (1-4 mL) and long eluting run times. In the method of Stormer (67) for determination of celecoxib, PP with acetonitrile, evaporation of the organic phase to dryness, and dissolution of the residue in water prior to SPE make the extraction procedure difficult. Rose *et al* (68) used more costly normal phase HPLC in which PP was used prior to SPE.

To circumvent the aforementioned limitations, we developed and validated a sensitive and reliable HPLC method for the simultaneous determination of docetaxel and celecoxib following single-step LLE and UV detection. In this context, the isocratic RP-HPLC method with LLE and UV detection for analysis of docetaxel in rat plasma and tissues (42) and celecoxib in human plasma (48) we have already proposed were also considered.

One of the specific features of the present HPLC assay is one-step efficient LLE of docetaxel, celecoxib, and paclitaxel from plasma making the method more convenient for pharmacokinetic studies of these drugs. Amongst different extracting solvents examined in the preliminary evaluation as outlined in "selection of appropriate extracting solvent and internal standard" section, a mixture of n-hexane and isoamyl alcohol (97:3) employed, and HPLC was performed isocratically on a reversed-phase column with UV detection. The extraction procedure of this assay is economical and convenient with good extraction efficiency as determined by comparison of the peak areas of the spiked plasma samples with those of un-extracted docetaxel or celecoxib solution. The recoveries were consistent for the samples as demonstrated by very low standard deviations. Recoveries were not dependent on the plasma concentrations, which consequently resulted in good linearity of the calibration curve. Good linear relationships were found when the peak area ratios of docetaxel or celecoxib to the internal standard were plotted versus the plasma concentrations. In the present work, paclitaxel met all typical requirements as an appropriate IS. This agent is stable during the analysis, readily available, and well-resolved from docetaxel and celecoxib. The use of IS increases the accuracy and precision of the assay.

To achieve the highest resolution, sensitivity, and elution under an isocratic condition, the mixtures of acetonitrile with various proportions of water were assessed as the mobile phase. A binary mixture of water/acetonitrile at 55:45 (v/v) proved to be the most effective combination as evidenced by more efficient resolution, lack of tailing, noiseless baseline, and satisfactory retention and run times. The gradient mode has already been used to resolve docetaxel peak from that of IS; where the composition of the mobile phase should be continuously changed throughout the run (69) making the method more complex and not sufficiently reproducible compared to isocratic methods. The LOQ of the present assay for docetaxel and celecoxib in *in vitro* and *in vivo* studies is adequate for pharmacokinetic characterization of tested drugs in small animals from whom limited volumes of plasma could be taken at each time point.

The docetaxel and celecoxib plasma concentration versus time exhibited a biexponential plot demonstrating a two-compartment open model with a distribution phase followed by an elimination phase. The calculated pharmacokinetic values given in Table 5 are in line with the results of previous studies for celecoxib (45); however, some pharmacokinetic parameters including $AUC_{0-\infty}$, systemic clearance, and

distribution volume for docetaxel are similar to those reported by Luo *et al* (70).

CONCLUSION

The present investigation describes an HPLC method using UV detection for the simultaneous analysis of docetaxel and celecoxib in plasma with LLE of drugs and IS from plasma specimens and for *in vitro* characterization of formulations. The method was validated and met the requirements of selectivity, linearity, recovery, accuracy, precision, and robustness. This validated method was successfully used to quantify docetaxel and celecoxib simultaneously in the release of the drugs from microparticles and pharmacokinetic studies. n-hexane:isoamyl alcohol (97:3) mixture as the extracting solvent resulted in a highly efficient recovery of the drugs from plasma samples. Since docetaxel and celecoxib will be co-administered for the treatment of a wide range of cancers such as NSCLC, the developed method has a great potential for routine therapeutic drug monitoring of these two drugs.

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