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Validated HPLC method for the simultaneous determination of taxol and ellagic acid in a *Punica granatum* fruit extract containing combination formulation

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A simple, rapid, precise and accurate isocratic reversed-phase high performance liquid chromatography (HPLC) method was developed and validated for the simultaneous determination of paclitaxel and ellagic acid in a combination nanoformulation. Separation was achieved using a 25 × 4.6 mm column, particle size 5 μm C18 reverse phase column (Luna), with a mobile phase consisting of methanol and 0.05% H_3PO_4, in gradient elution mode with a mobile phase flow rate of 1 mL/min, using UV visible detection at 230 nm. Sharp and well defined peaks were obtained at retention times of 13.75 min. and 11.6 min. for paclitaxel and ellagic acid, respectively. Regression analysis showed a good linear relationship ($r^2 = 0.996 \pm 0.0011$) and ($r^2 = 0.993 \pm 0.0011$) over wide ranges of 5–500 μg/ml and 1–500 μg/ml for paclitaxel and ellagic acid, respectively. LOD and LOQ of paclitaxel were 30 ng/ml and 100 ng/ml, respectively, while for ellagic acid LOD and LOQ were 300 ng/ml and 1 μg/ml, respectively. The accuracy of the method was determined by recovery studies using the standard addition method and was found to be in the range of 99.61–101.21% and 98.70–102.22% for paclitaxel and ellagic acid, respectively. The relative standard deviation (% RSD) for precision, repeatability and robustness was less than 2%. The ellagic acid content in fruits of *Punica granatum* and combination formulation with paclitaxel was analyzed and found to be 0.04% w/w and 0.0012% w/w, respectively. The proposed, developed and validated HPLC method for the simultaneous quantification of ellagic acid and paclitaxel can be used for the quality control and standardization of several crude drugs and different combination formulations, in which ellagic acid is present.

1. Introduction

Punica granatum is commonly known as pomegranate. It is now placed under the family Lythraceae in the Angiosperm phylogeny group, after recent phylogenetic studies, it having been formerly placed under the family Puniceae (http://www.mobot.org/mobot/research/apweb/). The fruit has several potential medicinal uses in its juice (Faria et al. 2007), peel and seeds (Tran et al. 2010). The primary component found in its juice is ellagic acid and therefore most commercially available pomegranate extracts are standardized to contain 40% or more of ellagic acid (Lansky 2006). Ellagic acid is 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione. Although there are a number of other extracts with antioxidant potential it has been shown that pomegranate juice has an antioxidant capacity (Gil et al. 2000) 2–3 times higher than that of red wine or green tea. In particular, pomegranate extract reduces lipid peroxidation (Chidambara et al. 2002) and oxidative stress on macrophages (Rosenblat et al. 2006) in animal models. It thus has a wide range of therapeutic benefits but most research corroborates its antioxidant, anticarcinogenic (Hassoun et al. 2004) and anti-inflammatory properties. The importance of the various constituents in the juice can be inferred from a study in which a 19% reduction in oxidative stress in mouse peritoneal macrophages, a 42% decrease in cellular lipid content and a 53% increase in reduced glutathione levels (Rosenblat et al. 2006) was observed from the residue after extraction of the juice from the fruit. A number of studies have been performed showing its anticarcinogenic potential. This pertains to the polyphenolic content of the juice. After the rind, which has 1.892 + 0.1070 mg/g dry weight of polyphenols, the juice has 0.063 ± 0.0003 mg/g dry weight (Dikmen et al. 2011). The mechanisms of action of *Punica granatum* mainly comprise antiproliferative, antiaromatase and NFKb modulatory pathways, and antioxidation (Jurenka 2008). The anticancer activity of *Punica granatum* has been extensively studied for prostate cancer and breast cancer, paclitaxel being the first-line drug for breast cancer, including advanced stages, or recurrent prostate cancer. The mechanism by which it acts is by stabilizing the microtubule mesh which plays an important part in cell division (Yonemoto et al. 2007). Food drug interaction studies show that neither has a negative effect on the activity of the other (Hemalswarya et al. 2006).

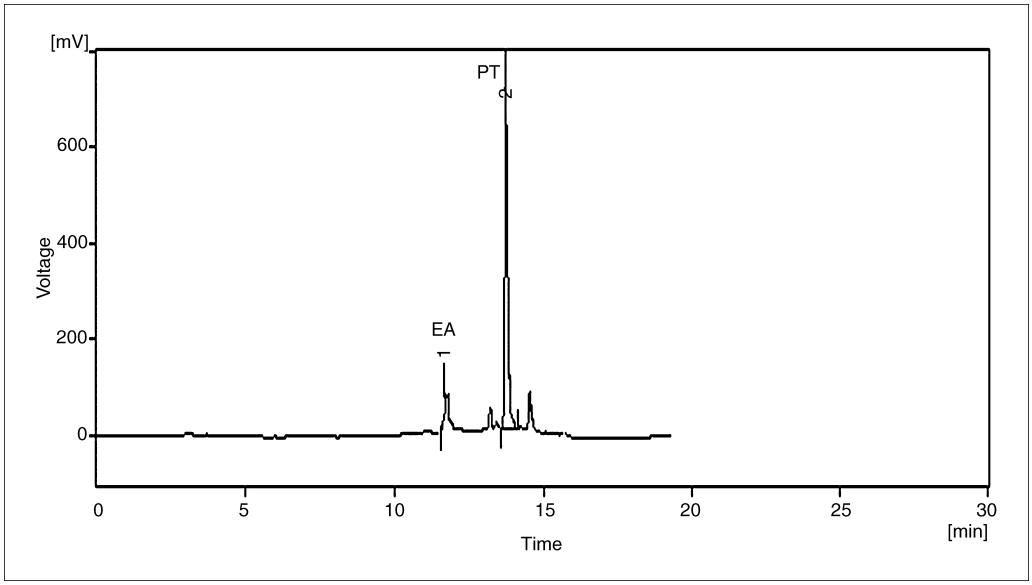

Fig.: Reversed phase HPLC separation of paclitaxel and phenolic compounds, mainly ellagic acid extracted from nanoparticulate formulation using 60% methanol mixture. λ=280 nm. Peak: (EA) ellagic acid; (PT) paclitaxel; sensitivity 0.01

In the study reported here we developed a HPLC method with UV detection for routine analysis of paclitaxel and ellagic acid simultaneously in combined formulations containing pomegranate extract.

2. Investigations, results and discussions

2.1. HPLC analysis

Eluting mixtures of methanol-acetic acid-orthophosphoric acid were tested at various proportions in an attempt to resolve the extracted ellagic acid and paclitaxel by HPLC. The composition of the eluent was repeatedly adjusted until satisfactory resolution was achieved. The Fig. shows the separation of ellagic acid and paclitaxel extracted from a nanoparticulate solution using 60% methanol-acetic acid-orthophosphoric acid mixture, which was quite similar to the the original nanoparticulate formulation.

Ellagic acid was eluted first followed by some other compounds. Peak PT, i.e. paclitaxel, was eluted later with a mobile phase mixture of methanol-acetic acid. Retention time for ellagic acid was found to be 11.6 min. Both simple and complex phenols varied between 11.6 min and 12.1 min. Secondly, the less polar paclitaxel was eluted using a less polar methanol/acetic acid mixture with an RT of about 13.75 min.

In all the samples analysed, the most abundant compound was paclitaxel, peak PT (RT 13.75 min). A solvent mixture consisting of methanol-acetonitrile-phosphoric acid 3:1:3 (v/v/v) was used for preparing samples prior to injection. Solvent efficiency was the deciding factor for preparing the mixture. A wavelength of 230 nm was used for both phenols and paclitaxel.

Detection of phenols is usually achieved at $\lambda = 280$ nm, even though this is not the maximum wavelength, and for paclitaxel the wavelength is 227 nm. At 230 nm there is good detection of both paclitaxel and ellagic acid simultaneously.

2.2. Calibration of paclitaxel and ellagic acid

Plots of peak area against concentration were linear in the range 1 to 500 μg mL^{-1} and 5 to 500 μg mL^{-1} with good correlation coefficients ($r^2 = 0.993 \pm 0.0011$) and ($r^2 = 0.996 \pm 0.0010$) for ellagic acid and paclitaxel respectively. Results from regression analysis are listed in Table 1, they indicate a good linear relationship between peak area and concentration over a wide range.

2.3. Validation

The accuracy of the method was determined by measurement of recovery using the standard addition method. Pre-analyzed samples were spiked with paclitaxel and ellagic acid standards at three different concentrations, 50, 100, and 150% of the amount present, and the mixtures were reanalyzed. Recovery was in the range 98.69–103.21% for paclitaxel and 98.70–102.22% for ellagic acid (Table 2).

The precision of the method was assessed by measurement of repeatability and intermediate precision. For repeatability, six different standard solutions of three different concentrations were analyzed. For intermediate precision, intra-day, inter-day, and inter-system precision was evaluated. Intra-day and inter-day precisions were determined by making measurements in triplicate six times a day on six different days. Inter-system precision was done by repeating the same procedure with a different HPLC system. The RSD of peak area and retention time were calculated and are reported in Table 3. The low RSD values are indicative of the good reproducibility of the method, which can be adopted by any laboratory for routine analysis of ellagic acid in crude drug and combination nanoformulations.

The robustness of the method was assessed by introducing small changes in the conditions (intentional changes in the flow rate and the detection wavelength) for analysis of a standard solution of concentration 100 μg mL^{-1}. The standard deviation and RSD of the peak area and retention time were calculated and are listed in Table 6. The low values of the RSD show the method to be robust.

The limits of detection and limits of quantification were determined on the basis of signal-to-noise ratio. The concentration of sample for which the signal-to-noise ratio was 3 was fixed as the LOD whereas the concentration for which the signal-to-noise ratio was 10 was fixed as the LOQ; the values being 30 ng/mL and 100 ng/mL respectively for paclitaxel and 300 ng/mL and 1 μg/mL respectively for ellagic acid.

2.4. Analysis of paclitaxel and ellagic acid in Punica granatum extract containing combination formulation

The validated method was used for analysis of a combination formulation. The peak areas obtained from triplicate sample analysis and the regression equation obtained from the calibra-

Table 1: Linear regression data for calibration plot of ellagic acid and paclitaxel (n = 3)

Drug	Ellagic acid	Paclitaxel
Linear range	1–500 μg mL^{-1}	5–500 μg mL^{-1}
Correlation coefficient	0.993 ± 0.0011	0.996 ± 0.0010
Intercept ± SD	–230.176 ± 19.10222	–23.9176 ± 138.2191
Regression equation	$y^a = 27.19x^b – 228.9$	$y^a = 34.55x^b – 16.57$
Slope ± SD	27.18692 ± 0.008579	35.48565 ± 3.633353a

[a] Peak area.
[b] Concentration of standard (μg mL−1).

Table 2: Accuracy of the method (n = 3)

		Paclitaxel		
Amount (%) of standard added to sample	Theoretical content (μg mL^{-1})	Amount of drug recovered (μg ± SD)	Amount of drug recovered (%)	RSD (%)
0	78	77.7 ± 0.87178	99.61538	1.121982
50	117	120.7667 ± 2.042874	103.2194	1.691587
100	156	153.9667 ± 0.763763	98.69658	0.496057
150	195	197.3667 ± 702468	101.2137	1.369263
		Ellagic acid		
0	2.4	2.433333 ± 0.055076	101.3889	2.263385
50	3.6	3.553333 ± 0.025166	98.7037	0.70824
100	4.8	4.83 ± 0.14	100.625	2.898551
150	6	6.133333 ± 0.047258	102.2222	0.770513

tion plot were used to calculate the ellagic acid and paclitaxel contents of the sample. The contents of paclitaxel and ellagic acid were found to be 0.046% and 0.00102% (*w/w*), respectively, before extraction from nanoparticles, and 0.048% and 0.00485% (w/w) after extraction from nanoparticles.

2.5. Conclusion

A simple, economic, accurate, precise, and reproducible gradient HPLC–UV method has been developed and validated for analysis of paclitaxel and ellagic acid simultaneously in the

Table 3: Repeatability of method (*n* = 6)

	Peak area			
Conc. (μg/ml)	Paclitaxel		Ellagic acid	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
50	1824.667 ± 20.599	1.1	1263 ± 7.810	0.618389
100	3656 ± 14.422	0.3	2415.667 ± 23.692	0.980784
300	9623.667 ± 7.637	0.07	7207 ± 87.068	1.208116

Table 4: Precision of method for ellagic acid (*n* = 6)

Concn (μg mL^{-1})	Inter-day precision		Intra-day precision		Inter-system precision	
	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)
50	1239.667 ± 13.20353	1.065088	1250.333 ± 22.59056	1.806763	1249.667 ± 21.38535	1.711285
100	2378 ± 48.21825	2.027681	2398 ± 68.73864	2.866499	2383.333 ± 49.50084	2.076958
300	7200.333 ± 81.45142	1.131217	7210.333 ± 62.88349	0.87213	7171.667 ± 117.0741	1.632452

Table 5: Precision of method for paclitaxel (n = 6)

Conc. (μg mL−1)	Inter-day precision		Intra-day precision		Inter-system precision	
	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)
50	1863.333 ± 20.50203	1.100288	1841.333 ± 8.736895	0.474487	1866.333 ± 17.50238	0.937795
100	3619.667 ± 19.21805	0.530934	3641.667 ± 31.78574	0.872835	3649.667 ± 36.85557	1.009834
300	9643.667 ± 23.62908	0.245022	9623.667 ± 37.52777	0.389953	9626 ± 33.28663	0.345799

Table 6: Robustness of method to change of flow rate (concentration 100 μg mL^{-1})

Ellagic acid				
Flow rate (mL min–1)			Mean peak area ± SD	RSD (%) of peak area
Actual	Used	Level		
1	0.8	−2	1268 ± 7	0.55205
	1	0	1263 ± 13.0767	1.035368
	1.2	+2	1255.667 ± 14.57166	1.160472
Wavelength				
230	228	–2	1248.667 ± 1.154701	0.092475
	230	0	1257.333 ± 0.57735	0.045919
	232	+2	1252 ± 5.196152	0.415028
Paclitaxel				
Flow rate (mL min−1)			Mean peak area ± SD	RSD (%) of peak area
Actual	Used	Level		
1	0.8	–2	1858.667 ± 18.87679	1.015609
	1	0	1878.333 ± 1.527525	0.081323
	1.2	+2	1843.667 ± 1.154701	0.062631
Wavelength				
230	228	–2	1838.333 ± 17.61628	0.958275
	230	0	1854.667 ± 1.154701	0.062259
	232	+2	1869.667 ± 12.70171	0.679357

crude drug and combination formulations. The method is simple and sufficiently sensitive over a fairly wide linear range. It can be used, under general laboratory conditions, for routine quality control of the crude drug *Punica granatum* and for different herbal formulations containing these plant ingredients.

3. Experimental

3.1. Materials

Paclitaxel was a sample donated by Fresenious Kabi. Pomegranates were purchased from a local vendor and were extracted/ground for juice.

3.2. Reagents and standards

Acetonitrile, methanol, hexane, isopropanol (2-propanol) filtered through a 1.5-mm filter, acetic acid, and water were all of HPLC grade and were purchased from Merck (Darmstadt, Germany).
The standard ellagic acid was purchased from Sigma Aldrich (Steinheim, Germany).

3.3. Extraction of polyphenols from Punica granatum

Extraction was carried out by method proposed by Kotamballi et al. (2002). Pomegranates (*P. granatum* var. Ganesha) were purchased from a local market. The peels were manually removed, sundried, and powdered. The powdered peel (100 g) was extracted by stirring with MeOH at 30 °C for 4 h. followed by filtration for removal of peel particles. The residue was then re-extracted with pyridine and filtered. The extracts were pooled and concentrated under vacuum at 40 °C.

3.4. Co-extraction of polyphenols and paclitaxel from combination formulation

Ten grams of powdered nanoformulation was extracted at room temperature with two 25-ml portions of absolute methanol. The residue was extracted again under the same conditions with two 25-ml portions of methanol/acetic acid (80:20, v/v). The extracts were combined and brought to dryness in a vacuum rotary evaporator at 40 °C. The residue was dissolved in 5 ml of a methanol/acetic acid/orthophosphoric acid (3:1:2, v/v/v) mixture and analyzed for ellagic acid and paclitaxel content by HPLC.

3.5. HPLC procedure

A Shimadzu (Shimadzu Corporation, Kyoto, Japan) HPLC system consisting of an LC-10AD delivery module with a double plunger reciprocating pump, UV-VIS SPA 10A detector, column oven (CTO-10A) and 20-ml injection loop was used. The column used was an Apex octadecyl 104 C18 with 5-mm packing (Jones ChromatographyLlimited, Colorado, USA).
Detection was performed at 230 nm for both ellagic acid and paclitaxel. The elution solvents used were A (0.05% orthophosphoric acid in water), B (100% methanol). The samples were eluted according to the following gradient: 80% A/20% B; 60% A/40% B in 5 min; 0% A/100% B in 10 min, 60% A/40%B in 15 min, and 80% A and finally 20% B in 20 min. Flow rate was 1 ml/min and run time was 30 min. The run was performed at 32 °C. The sample injection volume was 20 μl. Identification of compounds was achieved by comparing their retention time values with those of standards. Data were collected and processed using Class-VP Chromatography Laboratory Automated software (Shimadzu Corporation).

3.6. Quantitation and recovery of paclitaxel and ellagic acid

The initial paclitaxel content of samples was determined by diluting approximately 200 mg of nanoparticles in 1 ml of methanol/acetic acid/orthphosphoric acid mixture (3:1:2, v/v/v) and analyzing the sample solution by HPLC. Initial paclitaxel contents were then calculated from the integrated peak areas of the samples and the calibration curve of the paclitaxel standard. Good linearity was achieved in the range 5–500 ppm for paclitaxel ($r^2 = 0.996$) and 1–500 ppm for ellagic acid ($r^2 = 0.993$). Recovery of paclitaxel was determined by subjecting nanoparticulate samples to extraction and by comparing the amounts of paclitaxel extracted to the initial amounts determined above (without extraction). Six nanoparticulate samples with different initial paclitaxel contents were used. Extraction was performed as described in Section 3.4, and analysis was performed by HPLC. A recovery study was also carried out for phenolic compounds. Ellagic acid, being the most abundant phenol present in all samples, could be detected easily and was therefore quantified in the nanoparticulate samples after extraction. Recovery of the above compounds was determined by comparing the amounts of phenols extracted from the nanoparticulate samples, to the amounts found prior to extraction.

Three replicates were carried out for each sample. Means with standard deviations are reported.

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