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Research Article

Simultaneous quantification of eurycomanone, epimedin C and icariin in film coated tablets containing *Radix Eurycomae longifoliae* extract and *Herba Epimedii* extract using HPLC – DAD

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Abstract

Introduction. This study aimed at developing an HPLC-DAD method for simultaneous determination of eurycomanone (EU), epimedin C (EC) and icariin (IC) in EE tablets containing *Radix Eurycomae longifoliae* extract and *Herba Epimedii* extract (EE tablets).

Methods. Several HPLC conditions (detection wavelength, mobile phase) and sample preparation conditions (type of solvent, ratios of tablet powder and solvent) were investigated.

Results. The optimized HPLC condition employed a Shim-Pack RP18 column and a detection wavelength of 254 nm. The mobile phase consisted of acetonitrile and water containing 0.1% H_3PO_4 in gradient elution. The method was selective, linear, precise (RSD < 2%) and accurate with recoveries in the range of 98.51–104.58% for EU, 96.22–104.80% for EC and 97.50–104.96% for IC.

Conclusion. The method for simultaneous quantification of EU, EC and IC in EE tablets by HPLC – DAD was developed and validated for the first time and could be applied for quality control of EE tablets.

Keywords

Eurycomanone, Epimedin C, HPLC-DAD, Icariin, Simultaneous quantification

Introduction

Tongkat Ali (*Radix Eurycomae longifoliae*) and Horney Goat weed (*Herba Epimedii*) are two popular traditional medicine for supporting and treating sexual dysfunction. These medicinal herbs have been reported their abilities to increase the testosterone contents and improve the quantity and quality of sperm (Susumu et al. 1985; Oshima et al. 1987; Morita et al. 1992; Mitsunaga et al. 1994; Khari et al. 2014; Naseer et al. 2015). Consequently, the EE tablet

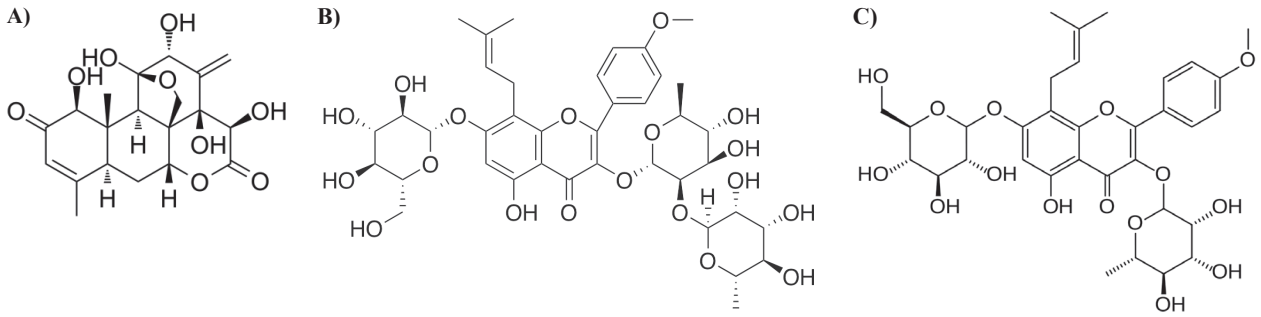

Figure 1. Chemical structures of (a) eurycoumanone, (b) epimedin C and (c) icarriin.

containing 25 mg of *Radix Eurycomae longifoliae* extract and 200 mg of *Herba Epimedii* extract (EE tablet) had been developed for these treatments.

Epimedin C (EC) and icariin (IC), the two active ingredients of Horney Goat weed, had been reported their remarkable effects such as neuroprotection, improving testosterone, and osteoporosis treatment (Chan et al. 2009; Meng et al. 2005; Susumu at al. 1985). According to Chinese Pharmacopoeia 2010, EC and IC were used as markers for Horney Goat weed. Eurycomanone (EU), a bioactive compound of Tongkat Ali, could increase the synthesis of testosterone in Leydig cells by inhibiting the conversion of testosterone into estrogen (Low et al. 2013). EU was also used as a marker of Tongkat Ali freeze dried extract according to Malaysian standard 2011.

Standardization is one of the difficult tasks for quality control of herbal medicine due to the complex nature of constituents of plant-based drug. In recent years, several analytical technologies have been developed to overcome these problems. Currently, high performance liquid chromatography equipped with a diode-array detector (HPLC-DAD) has been employing in quality control of several herbal medicines for its convenient, sensitive, accurate and powerful technique. The HPLC-DAD method has been employed to simultaneously quantify 15 flavonoids (including EC and IC) in *Epimedium* spp. (Chen et al. 2007). The HPLC-DAD quantification method of EU in *Herba Epimedii* extract and the related products has also been reported (Norhidayah et al. 2015; Mohamad et al. 2013). However, there has been no report on the simultaneous quantification of these 3 markers (EU, EC and IC) in one product using the HPLC-DAD method. Therefore, the present study aimed at developing and validating a HPLC-DAD method for EU, EC and IC simultaneous determination from EE tablets.

Materials and methods

Materials and reagents

The EE tablets were supplied by Traditional Medicine Hospital of Ho Chi Minh city (Vietnam). The EU, EC and IC reference standards (gifts from University of Medicine and Pharmacy at Ho Chi Minh city, Vietnam) were used at the contents of 96.9%, 90.3% and 97.2%, respectively. Methanol (MeOH), ethanol (EtOH), chloroform ($CHCl_3$), formic acid (HCOOH), phosphoric acid (H_3PO_4) and ethyl acetate (EtOAc) were of analytical grade and purchased from Xilong Chemical Co., Ltd. (China). Acetonitrile (ACN) was of HPLC grade, purchased from Scharlau (Spain). EE tablets were obtained from Traditional Medicine Hospital of Ho Chi Minh city (Vietnam).

Optimization of HPLC conditions

High performance liquid chromatography (HPLC) analysis was conducted with a Shimadzu LC 2030C 3D plus system (Shimadzu, Japan) equipped with a quaternary pump and a photodiode array detector. Chromatographic separation was performed on a Shim-pack GIST C18 (4.6 × 250 mm, 5 µm). The flow rate and the injection volume were 1 ml/min and 10 µl, respectively. The column temperature was kept at 30 °C. The detection was carried out at the optimized wavelength of the three targeted markers. The peaks of markers were confirmed by comparing their retention times and UV spectra with those of the standards. The mobile phases included water containing 0.1% (v/v) H_3PO_4 (A) and acetonitrile (B). Various gradient elution programs of the mobile phase were tested to achieve the required separation of three targeted markers (Table 1). The mobile phase program was chosen based on the peak purity, asymmetry, resolution of the targeted peaks.

Table 1. Mobile phase composition and gradient elution conditions.

Condition	Run time (min)	Water containing 0.1% H_3PO_4 (%)	Acetonitrile (%)
Condition I	0–15	89.5	10.5
	15–17	89.5 → 69	10.5 → 31
	17–20	69 → 72	31 → 28
	20–35	71	29
	35.01–45	30	70
	45.01–55	89.5	10.5
Condition II	0–16	90.5	9.5
	16–18	90.5 à 71	9.5 → 29
	18–21	71 → 73	29 → 27
	21–35	72	28
	35.01–45	30	70
	45.01–55	90.5	9.5
Condition III	0–16	91.5	8.5
	16–18	91.5 → 71	8.5 → 29
	18–21	71 → 73	29 → 27
	21–35	73	27
	35.01–45	30	70
	45.01–55	91.5	8.5

Optimization of preparation method for test solution

Screening extraction solvent for test solution preparation by thin layer chromatography

Test solutions: 20 EE tablets were randomly sampled, grinded and well mixed to obtain EE tablet powder. A same amount of EE tablet powder was extracted with the same volume of different solvents (methanol, acetonitrile, water and ethanol) to obtain 4 different test solutions. For test solution prepared by methanol, 750 mg EE tablet powder (equivalent to one EE tablet) were exactly weighed into a 25 ml volumetric flask. 15 ml methanol was then added and the sample was sonicated for 10 min. The volumetric flask was then filled up to 25 ml by methanol and the sample was filtered through a 0.22 µm membrane filter to obtain test solution. Similarly, the other three solvents (acetonitrile, water and ethanol) were also employed to prepared acetonitrile, water or ethanol test solutions, respectively.

Standard solutions: EU, EC and IC were separately weighed and dissolved in methanol to obtain the standard solutions with the concentrations of 15 µg/ml, 90 µg/ml and 9 µg/ml, respectively.

The thin layer chromatography (TLC) was employed to analyze the composition of 4 test solutions using two different TLC conditions as follows:

Condition A: 10 µl of each solution (EU standard solution and 4 test solutions) was applied on the same TLC plate. The mobile phase included $CHCl_3$ and MeOH at the volume ratio of 9:1. Detection was carried out by using H_2SO_4 10% in EtOH, followed by heating at 105 °C.

Condition B: 40 µl of each solution (EC and IC standard solutions and 4 test solutions) was applied on the same TLC plate. The mobile phase included EtOAC, HCOOH and H_2O at the volume ratio of 8:1:1. Detection was carried out by using H_2SO_4 10% in EtOH, followed by heating at 105 °C.

The best solvent was selected based on its capability of simultaneous extraction of EU, EC and IC from EE tablet powder with the lowest interference of the other components in EE tablets.

Screening the ratio of EE tablet powder and extraction solvent for preparation of test solution

Two different ratios were screening as follows:

- The ratio A: 750 mg of EE tablet powder was extracted by 25 ml of the selected solvent.
- The ratio B: 750 mg of EE tablet powder was extracted by 50 ml of the selected solvent.

The EE tablet powder was exactly weighed into the volumetric flask. The extraction solvent was added and the mixture was sonicated for 10 min. The volumetric flask was filled up to the requirement volume by the selected solvent. The samples were then filtered through a 0.22 µm membrane filter. Each investigated ratio was performed in three separate replicates. The extraction efficiency of 2 ratios was analyzed by the selected HPLC method. The best ratio was selected based on the capability to give the maximum simultaneous extraction efficiency of the three markers with the lowest volume of extraction solvent.

Method validation

The analysis method was validated according to International Conference on Harmonisation (ICH) guidelines in terms of system suitability, specificity, linearity, precision, accuracy, LOD and LOQ.

Specificity

Specificity was evaluated using the optimized HPLC condition to analyte the blank sample, test solution, standard solution and test solution spiked with three markers. The test solution was prepared from EE tablet powder using the optimized method for test solution preparation. Extraction solvent was used as blank sample. The mixture of three markers in methanol was used as the standard solution.

System suitability

System suitability was carried out by injecting six replicates of the test solution. The theoretical plate number (N), resolution (Rs), asymmetric factor (As) and repeatability (RSD) of retention time and peak area of the three targeted markers were investigated.

Linearity

The mixed standard stock solution was prepared by exactly weighing and simultaneously dissolving EU, EC and IC in methanol to obtain the concentrations of 0.145 mg/ml, 0.723 mg/ml, 0.136 mg/ml, respectively. The stock solution was diluted to prepare the standard solutions in the concentration range of 7.3–58 µg/mL for EU, 72.3–578.4 µg/mL for EC and 6.8–54.4 µg/mL for IC. The calibration curves were generated by plotting peak areas of the standards versus the concentrations. Linearity was assessed by the value of correlation coefficient (r^2).

Lower limit of detection and lower limit of quantification

The sensitivity of the analysis method was determined by lower limit of quantification (LLOQ) and lower limit of detection (LLOD). The LLOD was determined at the lowest concentration providing a signal-to-noise (S/N) ratio of 3:1 while the LLOQ was set at the lowest concentration giving the S/N ratio of 10:1.

Precision and accuracy

The intra-day precision was evaluated by analyzing six replicates of standard solutions within one day. The inter-day precision was determined on three consecutive days. The intra- and inter-day precision were evaluated by the relative standard deviation (RSD).

The accuracy of the method was determined by adding standard markers into the test solution. The samples were spiked at three different levels (80%, 100%, and 120%) of the three targeted markers. The contents of each marker were calculated using the calibration curve and the recovery percentages were determined.

Results and discussion

Optimization of HPLC conditions

Selection of UV wavelength for simultaneous detection of three targeted markers

The maximum absorption wavelength of EU, EC and IC were 245 nm, 270 nm and 270 nm, respectively (Fig. 2). At the wavelength of 245 nm, EU gave the maximum absorption while EC and IC gave the minimum absorption. In contrast, at the wavelength of 270 nm, EC and IC gave the maximum absorption while EU gave the minimum absorption. At the wavelength of 254 nm, all three markers could be simultaneously detected with acceptable sensitivity. Therefore, the wavelength for simultaneous detection of three targeted markers was selected at 254 nm.

Screening of mobile phase

The EE tablet which contained *Radix Eurycomae longifoliae* extract and *Herba Epimedii* extract included several complex ingredients. In order to improve the separation capacity, acidified water (0.1% v/v, H_3PO_4) was used in mobile phases to reduce the peak tailing and obtain the acceptable shapes of peaks. To achieve optimum separation, different eluting mobile phase programs were screened. Fig. 3 reveals that EU peak overlapped with the adjacent peaks and the EC peak overlapped with IC peak in condition I. In condition II, the two peaks of EC and IC were completely separated, however, EU peak still overlapped with the adjacent peaks. In condition III, the peaks of the three markers were well separated with the resolution above 1.5 and the peak purity of the three markers met the requirement of an HPLC analysis method (Fig. 4). Therefore, condition III was chosen as the optimized mobile phase program for HPLC condition.

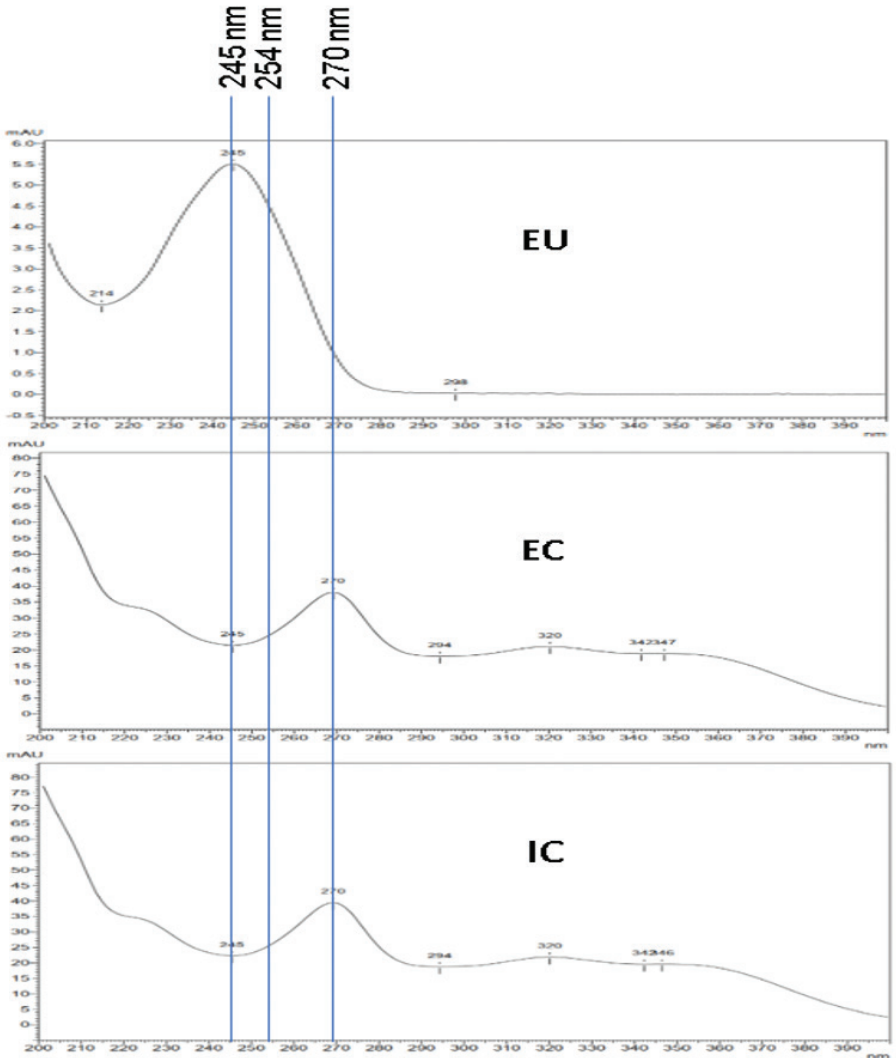

Figure 2. UV spectra (200–400 nm) of EU, EC and IC.

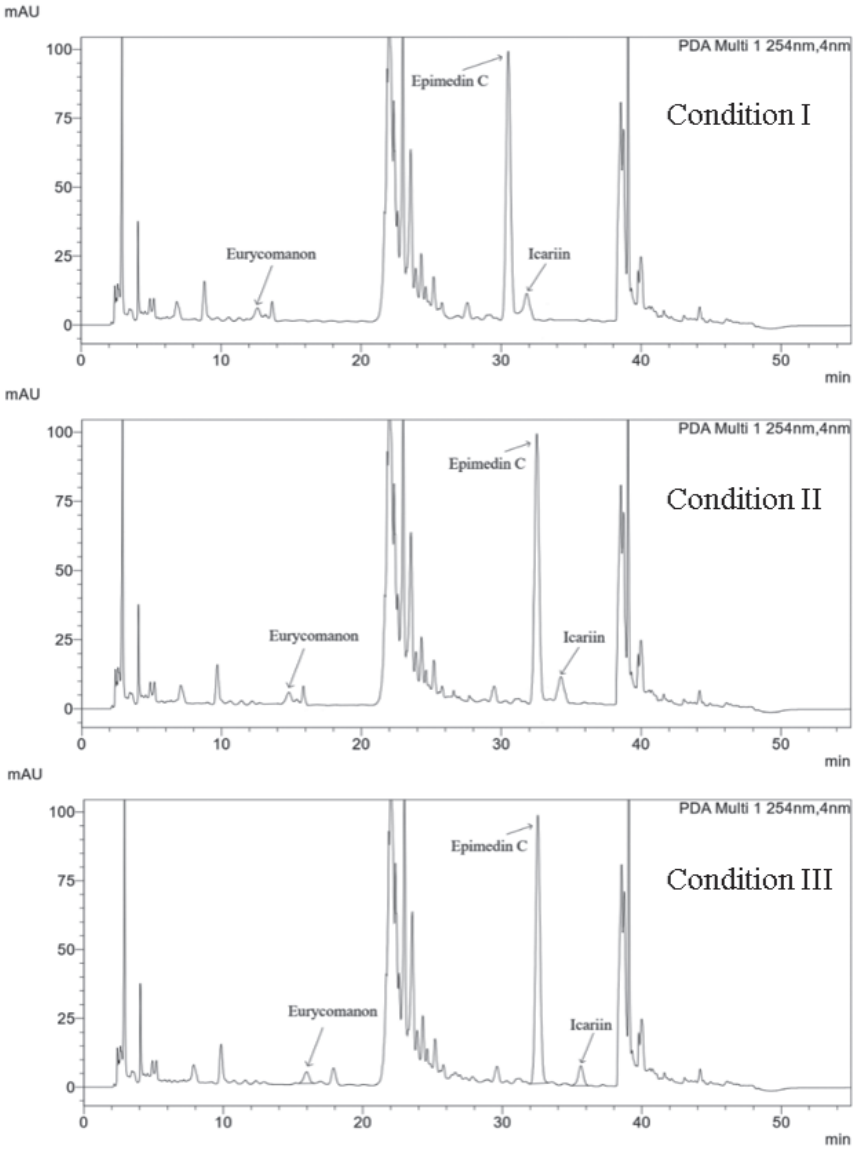

Figure 3. HPLC chromatograms of the test solution eluting by different mobile phase programs.

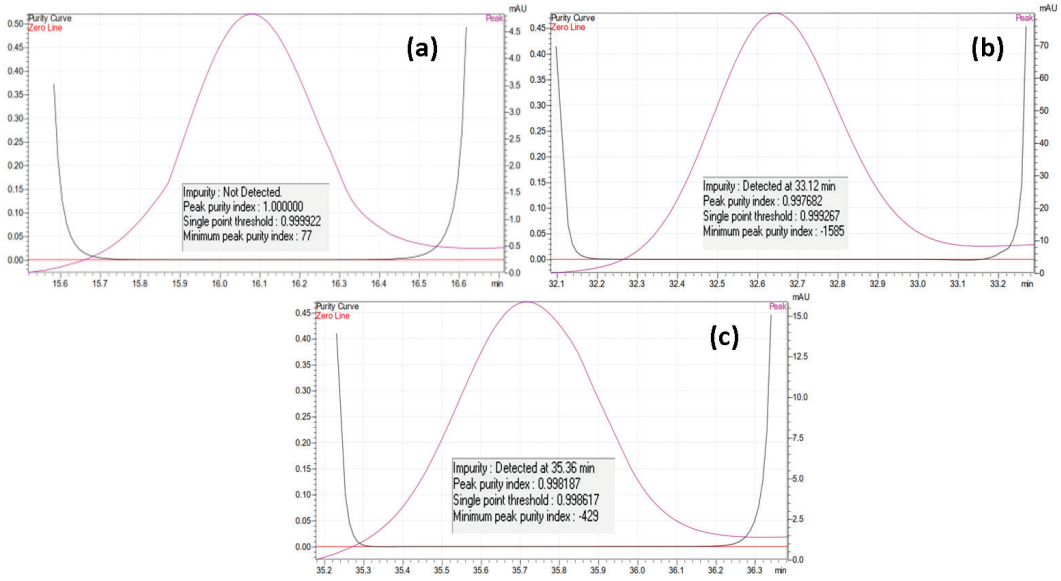

Figure 4. The purities of (a) EU, (b) EC and (c) IC peaks obtained from condition III.

Optimization of preparation method for test solution

Screening extraction solvent for test solution preparation by TLC

In order to optimize the extraction solvent for preparation of test solution, the extraction efficiency of various solvents (MeOH, H_2O, CH_3CN and EtOH) were screened. TLC results show that three markers could not be extracted by acetonitrile (Fig. 4). Ethanol, methanol and water gave the better extraction efficiency when compared with acetonitrile. Among the three extraction solvents, methanol exhibited the best solvent for test solution preparation due to the efficiency to simultaneously extract the three markers with low interference of the other components from EE tablet powder. Therefore, methanol was selected as the optimized extraction solvent.

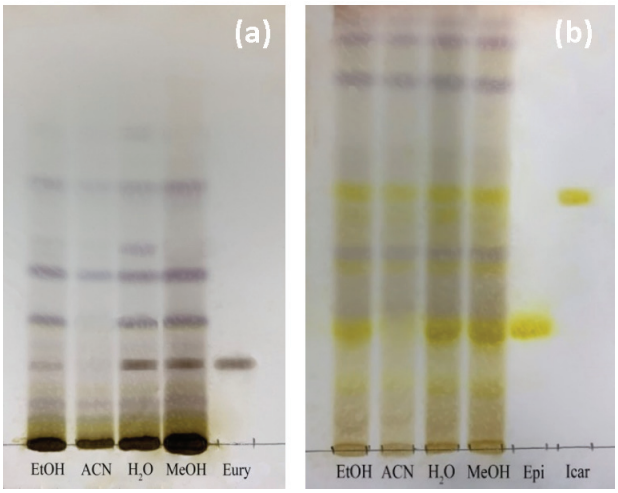

Figure 5. TLC chromatograms of 4 test solutions analyzed by (a) condition A and (b) condition B. EtOH, ACN, H_2O and MeOH were test solutions prepared by ethanol 96%, acetonitrile, water and methanol, respectively. Eury, Epi and Icar were standard solutions of EU, EC and IC, respectively).

Screening the ratio of EE tablet powder and extraction solvent for test solution preparation

HPLC results showed that the contents of three markers prepared by using two investigated ratios were not significantly different ($p > 0.05$). With the same amount of EE tablet powder, when the volume of methanol increased two times, the contents of three targeted markers did not increase. Therefore, the ratio A was selected to prepare the test solution.

Table 2. Contents of three markers in the EE tablet powder determined by two investigated ratios of EE tablet powder and extraction solvent.

Contents of three markers in the EE tablet powder (mg/g)			
Ratio	**EU**	**EC**	**IC**
A	0.3686	6.0408	0.5758
	0.3656	5.8947	0.5702
	0.3574	5.9577	0.5650
B	0.3657	5.9657	0.5729
	0.3641	6.0417	0.5717
	0.3658	5.9880	0.5720

Method validation

System suitability

The developed method resulted in the elution of EU at 15.92 min, EC at 32.59 min, and IC at 35.68 min (Table 3). The total run time was 55 min. The % RSD values of peak area and retention time for three targeted peaks were less than 2%. The resolution of the EU, EC and IC peaks in the test solution were greater than 1.5 and the peak asymmetry of the three markers was between 0.8–1.5. All the results showed that the proposed method met requirements of system suitability.

Table 3. The results of system suitability.

		t_R (min)	S (mAU.s)	Rs_1	Rs_2	As	N
EU	Mean	15.92	106338	1.62	1.52	0.99	8251
	%RSD	0.61	1.37				
EC	Mean	32.59	2278305	1.52	1.52	1.06	44367
	%RSD	0.10	0.83				
IC	Mean	35.68	183768	1.64	1.54	0.91	44568
	%RSD	0.11	1.18				

Specificity

There was no peak in the blank sample at the retention times of the EU, EC and IC peaks in the standard solution. The chromatogram of the test solution gave three peaks with retention times in coincident with that of the EU (15.92 min), EC (32.59 min) and IC (35.68 min) peaks in the standard solution (Fig. 6). When the EU, EC and IC standards were added to the test solution, the EU, EC and IC peak areas increased. UV spectra of EU, EC and IC in the test solution were found to be coincident with the UV spectra of the corresponding standard. No significant interference was detected at the retention times of the three targeted markers in the test solution. Therefore, the analytical method was specific for simultaneous determination of the targeted markers.

Table 4. Linearity and sensitivity of three investigated markers in EE tablets.

Components	**Linear range (μg/ml)**	**Regression equation**	**Correlation coefficient (r^2)**	**LLOD (μg/ml)**	**LLOQ (μg/ml)**
EU	7.3–58	y = 7673.9x	r^2 = 0.9987	2.2	6.5
EC	72.3–578.4	y = 9300.1x	r^2 = 0.9997	10.8	32.5
IC	6.8–54.4	y = 7951.7x	r^2=0.9987	2.0	6.1

y: peak areas (mAU), x: concentrations of markers (μg/ml)

Linearity, lower limit of detection and lower limit of quantification

Table 4 shows that all standard curves exhibited good linearity with high values of correlation coefficients (r^2>0.998).

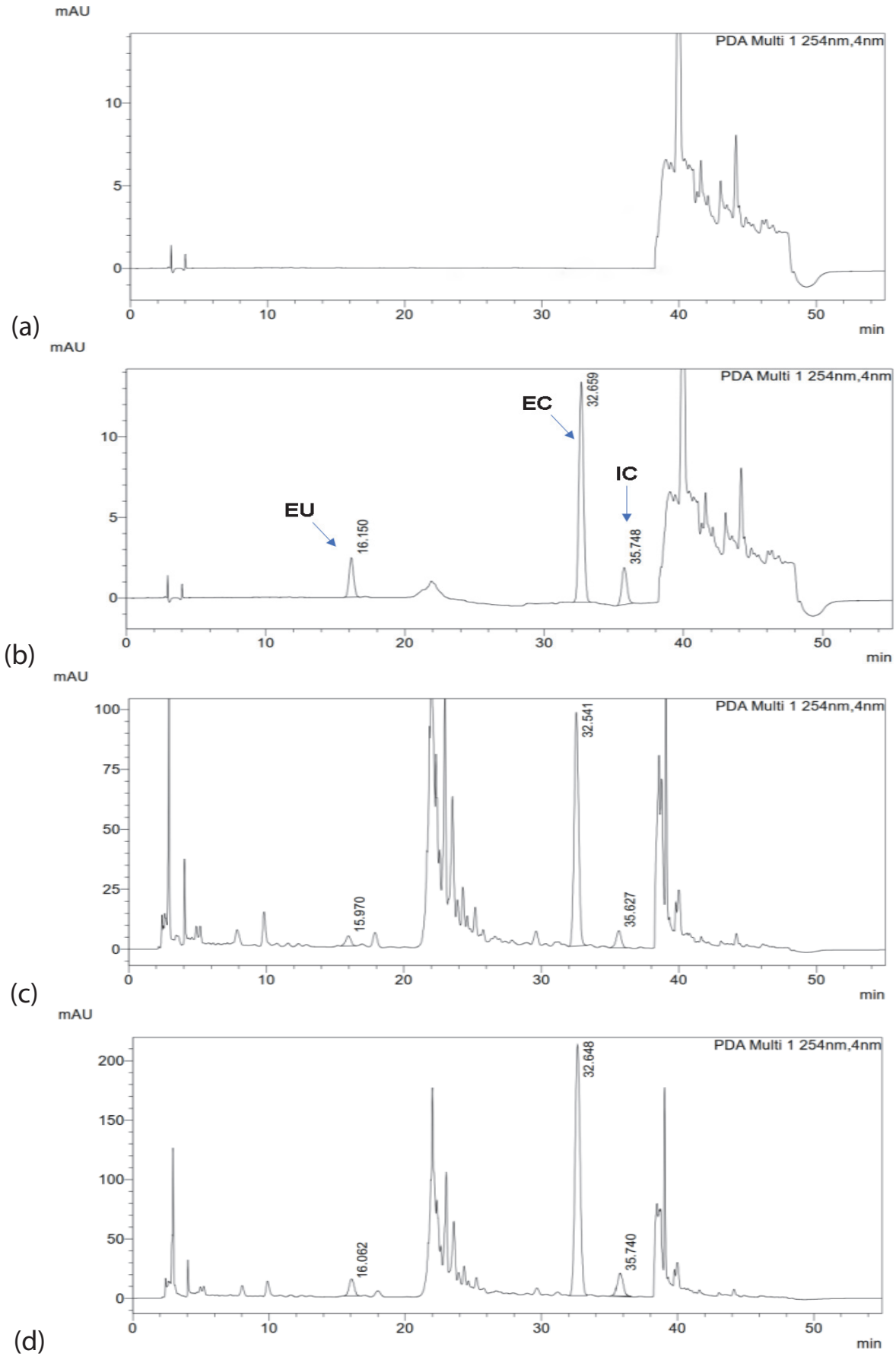

Figure 6. HPLC chromatograms of (a) blank sample; (b) mixture of standard solution; (c) test solution; (d) test solution spiked with three markers.

The values of LLOD were 2.2 µg/ml, 10.8 µg/ ml, 2.0 µg/ml and the values of LLOQ were 6.5 µg/ml, 32.5 µg/ml, 6.1 µg/ml for EU, EC and IC, respectively.

Precision and accuracy

The inter- and intra-day precision data at working concentrations are summarized in Table 5.

The RSD values (%) for all markers were within 2%. The inter- and intra-day results showed no significant variation in the analysis of three targeted markers.

The developed method had good accuracy with the recovery ranged from 98.51 to 104.58% for EU, 96.22–104.80% for EC and 97.50–104.96% for IC with RSD of less than 3% (Table 6). All data of accuracy study were found to be within the required range (Huber et al. 2007). Therefore, the analytical method possessed good reproducibility with acceptable precision and accuracy.

The analytical method for simultaneous determination of EU, EC, IC in EE tablet met the requirements of systematic suitability, selectivity, linearity, precision and accuracy according to ICH criteria. Therefore, this method could be used to simultaneously analyse EU, EC, IC in EE tablet.

Analysis of EE tablet samples

The developed HPLC analysis method was applied for simultaneous determination of three markers in three batches of EE tablets. The average contents of EU, EC and IC in one EE tablet were 0.313 mg, 5.867 mg and 0.546 mg, respectively (Table 7).

The contents of three markers in 3 batches of EE film coated tablets have good repeatability. This good results could be achieved in a simultaneous combination of the good repeatability of both manufacturing procedure and quantitative process. Thus, the quantitative method was confirmed its good repeatability.

Conclusion

The HPLC – DAD method for simultaneous quantification of EU, EC and IC in EE tablets was successfully developed and reported for the first time. The quantification method was selected based on the screening of several HPLC conditions and the sample preparation conditions. The developed method showed good sensitivity, precision, accuracy and could be used to simultaneously quantify EU, EC and IC in EE tablets for their quality assurance.

Table 5. Analytical results of inter- and intra-day test.

Precision		Tablet content (mg/tablet)		
		EU	EC	IC
Intra-day (n = 6)	Mean	0.3287	5.9641	0.5677
	RSD%	0.48	1.60	1.59
Inter-day (n = 6)	Mean	0.3345	5.9897	0.5620
	RSD%	1.88	1.41	1.67

Table 6. Analytical results of accuracy test.

Compound	Spiked amount (mg)	Measured amount (mg)	Recovery (%)	%RSD
EU	0.2610	0.2671	102.32	2.00
		0.2593	99.33	
		0.2571	98.51	
	0.3197	0.3331	104.19	2.35
		0.3241	101.37	
		0.3180	99.45	
	0.4000	0.4139	103.47	1.84
		0.4035	100.89	
		0.4183	104.58	
EC	4.85	4.6668	96.22	0.62
		4.7232	97.39	
		4.7085	97.08	
	6.05	6.3107	104.31	2.60
		6.3407	104.80	
		6.0460	99.93	
	7.21	7.1990	99.85	2.42
		7.3151	101.46	
		6.9747	96.74	
IC	0.45	0.4667	103.71	2.62
		0.4556	101.25	
		0.4429	98.42	
	0.54	0.5265	97.50	1.55
		0.5365	99.35	
		0.5430	100.56	
	0.68	0.6878	101.14	2.16
		0.6475	101.11	
		0.7137	104.96	

Table 7. The contents of three markers in three batches of EE tablets.

Compound	Contents (mg/tablet)			
	Batch No. 01NMDDH	Batch No. 02NMDDH	Batch No. 03NMDDH	Mean ± SD
EU	0.338	0.304	0.296	0.313 ± 0.022
EC	5.966	5.800	5.834	5.867 ± 0.088
IC	0.568	0.525	0.546	0.546 ± 0.022

Acknowledgements

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