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Research Article

STABILITY-INDICATING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR ANALYZING INJECTION DOSAGE FORMULATION CONTAINING MEDROXYPROGESTERONE ACETATE AND ESTRADIOL CYPIONATE

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ABSTRACT

Objective: Stability-indicating reversed-phase high-performance liquid chromatography method with photodiode array detection is described for the assay of medroxyprogesterone acetate (MDA) and estradiol cypionate (ECA) in bulk and injection dosage form.

Methods: MDA and ECA were determined on a Cosmicsil (250 mm × 4 mm) C18, 5 µm analytical column using mobile phase of 0.1 M KH_2PO_4 and acetonitrile (65:35 v/v) supplied isocratically by a flow rate of 1 ml/min. During stress testing, the sample was subjected to stress with 0.1 N HCl, 0.1 N NaOH, 30% hydrogen peroxide, water, and 105°C in oven and sunlight. Method validation was done in accordance with international conference on harmonization.

Results: The linear response was obtained over the concentration range from 2.5 to 7.5 µg/ml for ECA and 12.5 to 37.50 µg/ml for MDA. The recoveries of MDA and ECA were 99.31%–99.45% and 99.59%–99.79%, with relative standard deviation ranging from 0.021% to 0.217% and 0.027% to 0.187%, respectively. The limits of detection for MDA and ECA were 0.097 µg/ml and 0.042 µg/ml, respectively. The method was able to selectively quantitate MDA and ECA in the presence of the degradation products and, hence, can be considered as stability-indicating one. Proposed method was applied to the quantification of MDA and ECA in injection dosage form with good precision and accuracy.

Conclusion: The method can be employed for routine and quality control analysis of MDA and ECA simultaneously.

Keywords: Medroxyprogesterone acetate, Estradiol cypionate, Contraceptive, Quantitation, Stress test.

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INTRODUCTION

Medroxyprogesterone acetate (MDA) belongs to a class of drugs called progestogens. MDA prevents pregnancy [1]. MDA shows its action by preventing the complete development of woman's egg. MDA through thickening the mucus surrounding the cervix makes tough for sperm to get to the egg [2]. Furthermore, MDA is used to treat endometriosis, uneven or absence of menstrual periods, unusual uterine bleeding, reduce the possibility of endometrial hyperplasia (while the person is on estrogen therapy), and prevent overgrowth in the lining of uterus in postmenopausal women having estrogen replacement therapy [3,4]. [(6S,8R,9S,10R,13S,14S,17R)-17-acetyl-6,10,13-trimethyl-3-oxo-2,6,7,8,9,11,12,14,15,16-decahydro-1H-cyclopenta[a] phena nthren-17-yl] acetate is the IUPAC name for MPA and the chemical structure is given in Fig. 1.

Estradiol cypionate (ECA) is a prodrug of estradiol, which is a major estrogenic steroidal female hormone circulating endogenously in the women body [5,6]. ECA shows its effects in the body by binding to the estrogen receptor α and β subtypes [7]. These receptors are present in ovaries, breasts, skin, prostate, bone, uterus, and brain. For women, ECA is prescribed to reduce menopause symptoms such as vaginal dryness and hot flashes [8]. ECA is also prescribed to women who cannot secrete adequate estrogen due to conditions such as hypogonadism and primary ovarian failure. [(8*R*,9*S*,13*S*,14*S*,17*S*)-3-hydroxy-13-methyl-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[*a*]phenanthren-17-yl] 3-cyclopentylpropanoate is the IUPAC name for ECA and the chemical structure is given in Fig. 1.

In women, MDA and ECA combination is employed as monthly once injectable suspension contraceptive to avoid pregnancy [9-12]. When given to women, MDA and ECA combination blocks the gonadotropins secretion that further stops maturation of follicles and ovulation. This combination reduces the sperm penetration by thickening of cervix and decreasing the volume of cervical mucus. MDA and ECA combination decreases the chances of implantation by thinning of endometrium. To the best of our survey on literature, there is only one paper in literature on using RP-HPLC as an analytical technique for the determination of MDA and ECA [13].

Stress degradation testing plays a significant role in the development of a stability-indicating analytical method [14,15]. This testing assists to detect and determine the degradation products of drug substance that might form at some stage during storage, formulation development, manufacturing, and packaging. Therefore, it is recommended to carry out stress degradation test to evaluate the effect of acid, alkaline, oxidation, temperature, and sunlight on the drug [16]. There is no any analytical method reported in literature that analyzes MDA and ECA simultaneously using stability-indicating RP-HPLC method. Hence, the purpose of this investigation was to develop and validate a stability-indicating RP-HPLC method that analyzes MDA and ECA in bulk and injection suspension.

METHODS

Instrumentation

A Waters Alliance HPLC 2695 system with a PDA 2998 detector was used for detection and analysis of MDA and ECA. Data acquisition was carried out using Empower 2 software.

Materials

Chemicals and solvents used in this investigation are of analytical grade (AR) and HPLC grade, respectively. Acetonitrile (HPLC grade) was

obtained from Merck India Ltd. (Mumbai, India). Potassium dihydrogen phosphate (KH_2PO_4), hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), and sodium hydroxide (NaOH) used in this study are of AR grade and were from SD Fine Chemicals Ltd. (Mumbai, India). MDA and ECA standard reference substances were given as gift samples by Rainbow Pharma Training Labs (Hyderabad, India). Lunelle™ injectable suspension with MDA and ECA at 25 mg and 5 mg labeled concentration, respectively, was used in this investigation. Water used was purified using Millipore purification system (Millipore, USA)

Standard solutions

MDA (250 µg/ml) and ECA (50 µg/ml) stock solution were prepared by dissolving appropriate quantities of drugs in the mobile phase. Sequential dilutions of stock solution were done with mobile phase to reach working standard solutions at five different concentrations for calibration curve:

- 2.5, 3.75, 5.0, 6.25, and 7.5 µg/ml of ECA
- 12.5, 18.75, 25, 31.25, and 37.50 µg/ml of MDA.

For testing validation parameters, working standard solution with a concentration of 5 µg/ml and 25 µg/ml of ECA and MDA, respectively, was prepared from stock solution by dilution with mobile phase.

Optimized chromatographic conditions

Separation and analysis of MDA and ECA were achieved on Cosmicsil C18 column (4.6 mm × 250 mm; 5 µm particle size) with an isocratic flow rate of 1.0 ml/min. Isocratic mobile phase consisted of 0.1 M KH_2PO_4 and acetonitrile (65:35, v/v), with pH adjusted to 4.0. The mobile phase and sample solutions were filtered through 0.45 µm membrane filter and degassed earlier to use. Injection volume was 10 µl for samples and standards. Analyses were performed at 25°C temperature. Samples were analyzed with PDA detector at 236 nm.

Calibration curves of MDA and ECA

To establish calibration curve, series of the dilutions of stock solution with mobile phase were made to achieve the calibration standards of MDA and ECA ranging from 12.5 to 37.50 and 2.5 to 7.5 µg/ml, respectively. The solutions are filtered using 0.45 µm membrane filter. The solutions were into HPLC system, keeping the injection volume at 10 µl volume. Calibration curves of MDA and ECA were plotted between the drugs peak area and their relevant concentration. Regression equation was also derived using the concentration and peak area data.

Assay of MDA and ECA in injection suspension

Powder in the injection bottle, equal to 25 mg of MDA and 5 mg of ECA was dissolved in 100 ml of mobile phase in a volumetric flask (100 ml), sonicated for 20 min, and filtered through 0.45 µm membrane filter. The stock solution was diluted properly with mobile phase to achieve a test sample concentration of 5 µg/ml and 25 µg/ml of ECA and MDA, respectively. The test sample was analyzed as stated above. The amount of MDA and ECA in injection dosage form was computed from calibration curves or regression equations of MDA and ECA.

Forced degradation test

A stability-indicating method is one which is defined as a method that acceptably enumerates the drug without interference from process impurities and degradation products. The stability-indicating ability of the proposed RP-HPLC method was assessed by subjecting 10 ml of stock injection sample solution (MDA – 250 µg/ml and ECA – 50 µg/ml) to accelerated degradation by acid, alkaline, water, thermal, hydrogen peroxide, and photolytic conditions. Following degradation, the stock injection sample solution was diluted to get test sample (concentration: 5 µg/ml – ECA and 25 µg/ml – MDA) and analyzed by the proposed method. The acidic degradation was performed by preparing the sample solution in 0.1 N HCl, sonicated for 30 min at room temperature and neutralizing with 0.1 N NaOH. Sample solution prepared in 0.1 N NaOH was used for alkaline hydrolysis valuation, where the solution was sonicated at room temperature for 30 min and neutralized with 0.1 N HCl. For the study under neutral situation, the stock injection sample is mixed with water and sonicated at room temperature for 30 min. Oxidative degradation was induced by mixing the stock injection sample solution with 30% H_2O_2 followed by sonication at room temperature for 30 min. Thermal and photodegradation were induced by exposing the injection powder in a Petri dish to 105°C for 30 min in oven and to sunlight for 24 h, respectively. The degraded samples were injected into the HPLC system and analyzed. The stability-indicating ability of the proposed method was established by the satisfactory separation of degradant peaks from the peaks of MDA and ECA.

RESULTS

Method development

The objective of the current investigation was to develop a stability-indicating RP-HPLC method for the estimation of MDA and ECA simultaneously in bulk and in injection dosage form. Several stationary phases (Waters C18, Hibar C18, Inertsil C18, and Cosmicsil C18) and mobile phases (orthophosphoric acid:methanol, 0.1 M KH_2PO_4:methanol, and 0.1 M KH_2PO_4:acetonitrile) with different ratio, flow rate, and pH were tested. Finally, a combination of a Cosmicsil C18 column (250 mm × 4.6 mm, 5 µm) and a mobile phase of 0.1 M KH_2PO_4:acetonitrile (ratio – 65:35 v/v; flow rate – 1 ml/min; and pH 4.0) gave the optimum separation, good peak shape, and sensitivity toward MDA and ECA. 236 nm was selected for detection and analysis since good sensitivity was found for MDA and ECA at this wavelength. A typical chromatogram acquired with the proposed method is represented in Fig. 2.

Method validation

As indicated by the International Conference on Harmonization (ICH) guidelines Q2 (R1), the developed RP-HPLC method was validated [17].

System suitability test is usually performed to confirm repeatability of the chromatographic system, resolution, and column efficiency to make sure its capability for the analysis of MDA and ECA. For this test, standard drug solution (5 µg/ml – ECA and 25 µg/ml – MDA) was injected into the HPLC system 6 times and system suitability parameters were analyzed. The values are in limits (Table 1) and confirmed the suitability of the method. The column efficiency was >2000 plate count, tailing factors are lesser than 2.0, percent relative standard deviation of peak area and retention were lesser than 2.0, and resolution is >1.5.

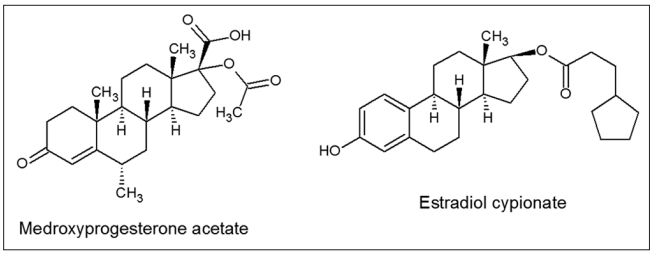

Fig. 1: Structures of drugs selected in this investigation

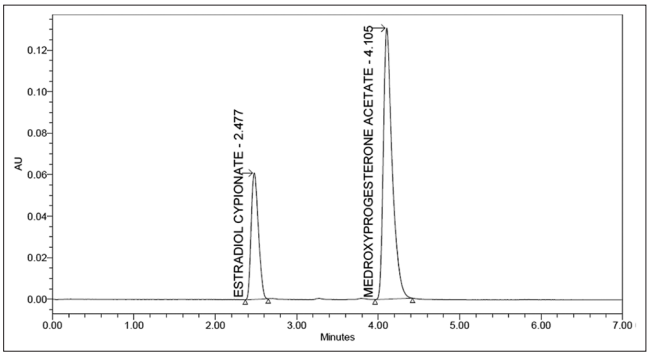

Fig. 2: Chromatogram of medroxyprogesterone acetate and estradiol cypionate after method optimization

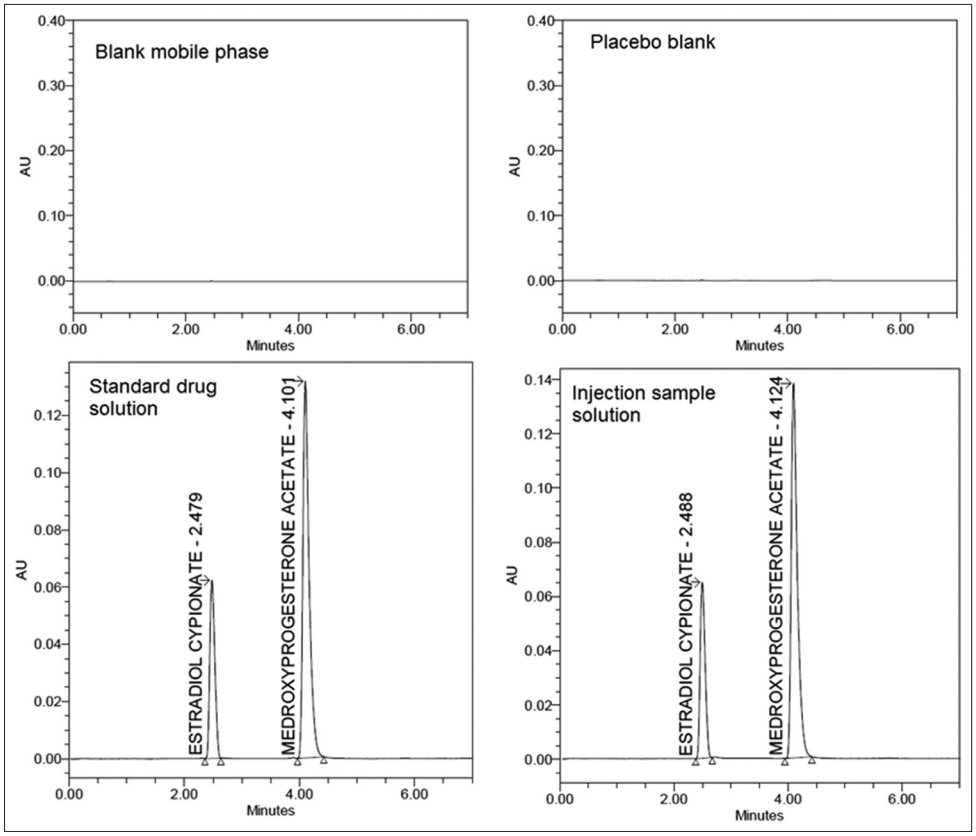

Fig. 3: Chromatograms demonstrating the method selectivity

Table 1: System suitability values and recommendations

Drug	Repeatability of system		Column efficiency		Resolution and percentage RSD (n=6)
	Mean peak area and percentage RSD (n=6)	Mean retention time and percentage RSD (n=6)	Plate count and percentage RSD (n=6)	Peak tailing and percentage RSD (n=6)	
MDA	378,144 and 0.106%	2.478 and 0.046%	3752 and 1.172%	1.256 and 1.207%	
ECA	990,436 and 0.183%	4.101 and 0.076%	7126 and 1.264%	1.562 and 0.536%	1.562 and 0.339%
Recommended limits	RSD≤2	RSD≤2	>2000	≤2	≤1.5

MDA: Medroxyprogesterone acetate, ECA: Estradiol cypionate, RSD: Relative standard deviation

For method selectivity establishment, chromatogram of standard drug solution (5 µg/ml – ECA and 25 µg/ml – MDA) was compared with chromatograms of placebo blank, blank mobile phase, and injection sample solution (5 µg/ml – ECA and 25 µg/ml – MDA) (Fig. 3). The method is selective for MDA and ECA analysis because any detectable interfering peaks at the retention time of MDA and ECA were not found in chromatograms of placebo blank, blank mobile phase, and injection sample solution [18].

The method confirmed linearity in the range of 12.5–37.50 µg/ml (MDA) and 2.5–7.5 µg/ml (ECA). The regression line equation was y = 75671x + 113.7 for MDA and y = 39586x + 480 for ECA. The goodness of fit, regression coefficient (R^2), was found to be 0.9996 and 0.9998 for MDA and ECA, respectively. The results indicated a linear association between the drug concentration and area of peak.

The limit of detection (LOD) and limit of quantitation (LOQ) were evaluated on the basis of signal-to-noise ratio (SNR). An SNR of 3:1 was considered for LOD and 10:1 for LOQ. For this, different dilute solutions of MDA and ECA were analyzed by the proposed method. The calculated LOD values are 0.042 µg/ml and 0.097 µg/ml for ECA and MDA, respectively. While LOQ values are 0.139 µg/ml and 0.324 µg/ml for ECA and MDA, respectively. The values proved method sensitivity.

Table 2: Precision and accuracy for the quantification of medroxyprogesterone acetate and estradiol cypionate

Precision			
Drug	**Concentration taken (µg/ml)**	**Peak area* (mAU)**	**RSD (%)**
MDA	25	990616	0.017
ECA	5	378343	0.049
Accuracy			
Drug	**Concentration taken (µg/ml)**	**Concentration found* (µg/ml)**	**Recovery (%)**
MDA	25	24.85	99.42
ECA	5	4.98	99.55

*Mean of six values. MDA: Medroxyprogesterone acetate, ECA: Estradiol cypionate, RSD: Relative standard deviation

To study proposed method precision, standard drug solution (5 µg/ml – ECA and 25 µg/ml – MDA) was injected 6 times and peak area of MDA and ECA was recorded. Information shown in Table 2 indicated an adequate level of method precision (%RSD value is <2.0%).

For studying method accuracy, six standard drug solutions (5 µg/ml – ECA and 25 µg/ml – MDA) were analyzed by the proposed method. Percent recovery calculated is shown in Table 2. The percent recovery value indicates an acceptable level (≈ 100%) of method accuracy.

The blank placebo spiked at three concentrations of MDA and ECA was used to evaluate the recovery ability of the method. The recovery of MDA and ECA by the proposed method was determined from the analysis of three replicate samples at each concentration level. The mean recoveries of MDA and ECA are represented in Table 3. No significant common excipients effect was observed. Hence, the method was selective and accurate.

The method robustness was established by executing the analysis of standard drug solution using different detection wavelengths, column temperatures, ratio of mobile phase, flow rates, and pH of mobile phase [19]. The effect of change on the system suitability parameters was studied. In all changed conditions, the system suitability values are in limits (Table 4), hence, proved the method robustness.

Stress test was performed, to demonstrate the innate stability of MDA and ECA, and to prove specificity and stability-indicating nature of the proposed method, as indicated by the ICH guidelines Q1A (*R2*) [16]. After degradation period, the stressed samples were analyzed. The presence of related peaks, peak interference, percentage degradation, and peak purity for MDA and ECA was checked (Table 5). All the conditions assessed in the stress tests resulted in degradation products formation (Fig. 4). The stability-indicating ability of the method was demonstrated by the adequate separation of degradant peaks from MDA and ECA peaks. Peak purity for MDA and ECA peaks was checked (peak purity angle < peak threshold) and indicated that they are pure from excipients/degradants. As a result, the method is reliable to detect any probable change in the drug product analyzes during stability tests.

Application

The proposed method was productively applied to the quantification of MDA and ECA simultaneously in injection dosage form. Procedure explained in section "Assay of MDA and ECA in injection suspension" was followed. Mean percent recovery and percent relative standard deviation for MDA and ECA of three determinations were recorded. The results are shown in Table 6. The determined content of MDA and ECA was equivalent to the respective labeled amounts.

DISCUSSION

The described stability-indicating RP-HPLC technique is sensitive, selective, reliable, and reproducible assay method which is successfully applied to separate and analyze MDA and ECA in the presence of their degradation products, to determine MDA and ECA content in injection dosage form. The use of photodiode array detector was a supportive tool for peak identity and purity of MDA and ECA under different conditions of degradation. The parameters of system suitability are within the acceptability limits. The linearity curve acquired shows a close relationship between concentration of drugs (MDA and ECA) and peak areas (MDA and ECA). The method was accurate, as the percentage of recovery was close to 100%. The RSD values below 1% signify the method's precision. The method has been demonstrated as specific since there is no interference in placebo peak and blank mobile phase

Table 3: Recovery of medroxyprogesterone acetate and estradiol cypionate using proposed method

Drug	Concentration added	Concentration found* (µg/ml)	Recovery* (%)	RSD (%)
MDA	12.5	12.430	99.45	0.067
	25.0	24.857	99.43	0.021
	37.50	37.243	99.31	0.217
ECA	2.5	2.497	99.79	0.186
	5.0	4.977	99.60	0.110
	7.5	7.470	99.59	0.027

*Mean of three values. MDA: Medroxyprogesterone acetate, ECA: Estradiol cypionate, RSD: Relative standard deviation

Table 4: Effect of slight changes on the system suitability data of medroxyprogesterone acetate and estradiol cypionate

HPLC condition	Value	MDA			ECA	
		Theoretical plates	Tailing	Resolution	Theoretical plates	Tailing
Flow rate (ml/min)	0.9	6925	1.57	8.45	3524	1.25
	1.1	7608	1.58	8.67	3722	1.26
Column temperature (°C)	23	8064	1.57	8.79	3808	1.22
	27	8142	1.58	9.01	3937	1.22
Mobile phase ratio (0.1 M KH_2PO_4:acetonitrile)	60:40	7608	1.58	8.67	3722	1.26
	70:30	8064	1.57	8.79	3808	1.22
Mobile phase pH	3.9	7121	1.57	8.68	3546	1.24
	4.1	7287	1.56	8.76	3661	1.24
Detection wavelength (nm)	234	7062	1.55	8.71	3570	1.29
	238	7130	1.56	8.70	3590	1.23

MDA: Medroxyprogesterone acetate, ECA: Estradiol cypionate, HPLC: High-performance liquid chromatography

Table 5: Results of medroxyprogesterone acetate and estradiol cypionate stress test

Stress applied	MDA			ECA			Rt of Deg
	%Deg	PPA	PT	%Deg	PPA	PT	
0.1 N HCl	13.37	0.389	0.589	12.41	0.271	0.754	3.267, 3.785
0.1 N NaOH	10.07	0.286	0.487	10.77	0.295	0.652	3.688
30% H_2O_2	10.81	0.190	0.486	9.72	0.358	0.657	1.587, 3.777
105°C	13.34	0.209	0.584	12.76	0.464	0.853	3.254, 3.610, 5.747
Sunlight	11.61	0.241	0.586	11.54	0.357	0.862	0.162, 3.265, 3.615, 7.180, 7.594
H_2O	7.9	0.381	0.584	8.77	0.214	0.756	3.279, 3.756

%Deg: Percentage degradation, PPA: Peak purity angle, PT: Purity threshold, Rt of Deg: Retention of degradants

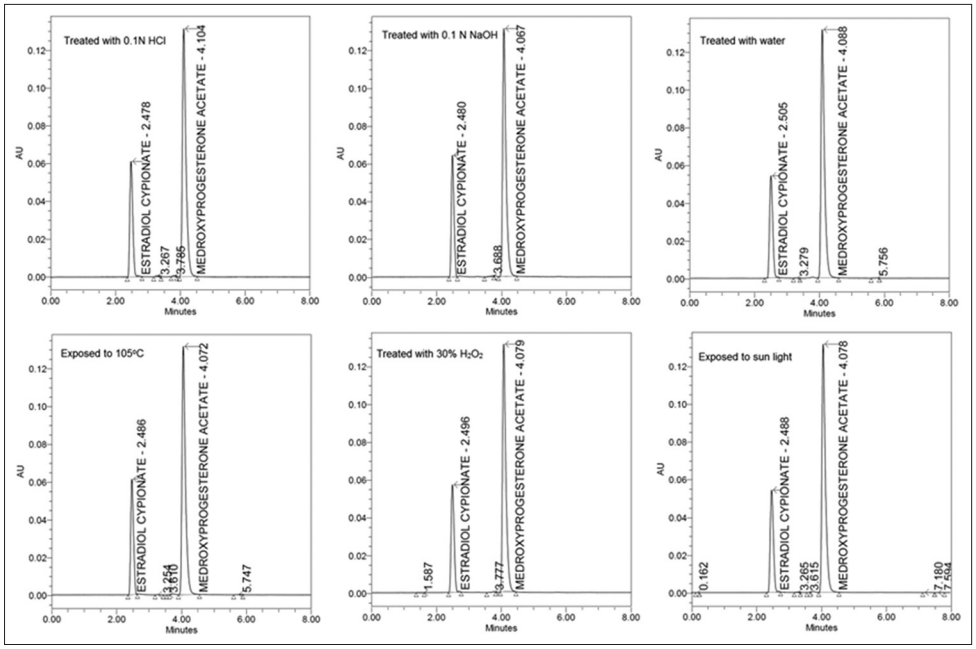

Fig. 4: Chromatograms of the medroxyprogesterone acetate, estradiol cypionate, and degradants

Table 6: Analysis of formulations having medroxyprogesterone acetate and estradiol cypionate

Dosage form	Drug	Content (mg)	Determined (mg)	Percentage recovery and percentage RSD
Lunelle™	MDA	25	24.85	99.39 and 0.70
	ECA	5	4.98	99.67 and 0.182

*Mean of three values. MDA: Medroxyprogesterone acetate, ECA: Estradiol cypionate, RSD: Relative standard deviation

as to the retention time of MDA and ECA. Forced degradation studies indicated stability-indicating ability of the method proposed.

CONCLUSION

The stability-indicating RP-HPLC method for the simultaneous analysis of MDA and ECA is described using Cosmicsil (250 mm × 4 mm) C18, 5 µm analytical column, and mobile phase of 0.1 M KH_2PO_4 and acetonitrile (65:35 v/v). The method has been found to be reliable and suitable for the assay of MDA and ECA in the presence of stress degradants. This method was simple, sensitive, rapid, accurate, precise, and free from interference by excipients and degradants. Due to these features, this method can be employed for routine and quality control analysis.

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COMPETING INTEREST

Authors declare that no conflicts of interest exist in this investigation.

AUTHORS' CONTRIBUTIONS

SR and SRXR designed the study. SR performed the experiments and AK analyzed the data and reviewed. SRXR supervised the experiments and reviewed the data and supported to the writing of the manuscript.

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