

Original Article

## A RAPID STABILITY INDICATING HPLC-METHOD FOR DETERMINATION OF NORETHISTERONE ACETATE IN PLASMA, TABLETS AND IN A MIXTURE WITH OTHER STEROIDS

HISHAM HASHEM<sup>1,2</sup>, SOAD S. ABD EL-HAY<sup>1</sup>, THOMAS JIRA<sup>3</sup>

<sup>1</sup>Faculty of Pharmacy, Pharmaceutical Analytical Chemistry Department, Zagazig University, Egypt, <sup>2</sup>Faculty of Pharmacy, Pharmaceutical Chemistry Department, Jazan University, Saudi Arabia, <sup>3</sup>Institute of Pharmacy, Pharmaceutical/Medicinal Chemistry, Ernst-Moritz-Arndt-University Greifswald, F.-L.-Jahn-Str. 17, D-17487 Greifswald, Germany  
Email: hisham413@yahoo.com

Received: 21 Nov 2014 Revised and Accepted: 15 Dec 2014

### ABSTRACT

**Objectives:** This study reports about a simple, rapid and accurate, routine-HPLC method for quantification of Norethisterone acetate (NA) in bulk powder, tablets and plasma.

**Methods:** The chromatographic separation is carried out using isocratic binary mobile phase consisting of acetonitrile (ACN) and water (H<sub>2</sub>O) in the ratio of 55: 45 at the flow rate of 1 mLmin<sup>-1</sup> and 40 °C. A diode array detector is used at 240 nm for detection.

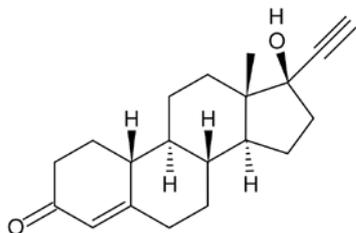
**Results:** The elution time of NA is found to be 2.946±0.01 minutes. The method is validated for system suitability, linearity, precision, limits of detection and quantitation, specificity, stability and robustness. The robustness study is done for small changes in temperature, flow rate, the wavelength of detection, mobile phase content of ACN and injection volume. Stability tests are done through exposure of the analyte solution for four different stress conditions: Reflux with 1N hydrochloric acid (HCl), reflux with 1N sodium hydroxide (NaOH), reflux with 30% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and exposure to ultraviolet radiation (UV) radiation. The limits of detection and quantitation are 0.0625 and 0.125 µg/mL<sup>-1</sup>, respectively. The recovery value of this method is 101.90% and the reproducibility is within 2.16.

**Conclusion:** This is a rapid stability indicating HPLC method for determination of NA and can be applied for determination of NA in presence of other steroids as Levonorgestrel, Estradiol and Norethisterone.

**Keywords:** Norethisterone acetate, Stability indicating method, Quantitation, Plasma.

### INTRODUCTION

Norethisterone or norethindrone (19-nor-17 $\alpha$ -ethynyltestosterone) (Scheme 1) is a synthetic progestagenic compound and widely used for hormone replacement therapy and contraception. It works through inhibition of the secretion of pituitary gonadotrophins to prevent follicular maturation and ovulation [1].



Scheme 1: Structure of Norethisterone

Various analytical techniques were described for an assay of NA in bulk form, pharmaceuticals and biological samples. They mainly include HPLC [2-8], TLC [7,9], gas chromatography [10-17], radio immunological [10], argentometry [18] and electrochemical methods as differential-pulse polarography and voltammetry [19-22] and spectrophotometry [23].

Literatures reveal that there is only one previous method dealing with degradation study during determination of norethisterone [8] but this method includes some drawbacks such as using of gradient mobile phase, too long separation time (about 40 minutes) and incomplete validation of the method. Therefore the aim of this study is to find an inexpensive, simple and rapid stability indicating fully validated chromatographic method applying isocratic mode for determination of NA in bulk powder, tablets and plasma and to

overcome the problems in all previously reported chromatographic methods as long time of analysis and expensive detectors [2-8].

### MATERIALS AND METHODS

#### Chemicals

HPLC analytical grade ACN and methanol (MeOH) were purchased from Mallinckrodt Baker B. V. (Deventer, Netherland). Water was deionized and doubly distilled. Mobile phase components were degassed before use. Authentic NA was kindly supplied from Schering AG (Berlin, Germany).

#### Instrumentation

**HPLC:** HP1090 II model equipped with a diode array detector (DAD) system (Hewlett-Packard, Waldbronn, Germany).

**pH-meter:** pH values were measured using pH meter Metrohm 713<sup>®</sup> (Switzerland).

**UV-lamp:** Modell FMR 10 UV-Blutbestrahlungsgerät, 220 Volts, 50 Hz (VEK Präcitronek, Dresden, Germany)

#### Chromatography

The separations were achieved with an analytical reversed phase c-18 column 150 x 3.0 mm I. D. (Bridge column XBridge™, Waters, Ireland) with an isocratic mobile binary phase of ACN/H<sub>2</sub>O (55.0/45.0, v/v) at a flow rate of 1 mL min<sup>-1</sup> at 40 deg C oven temperature. Detection was achieved at 240 nm. The hold-up times (t<sub>0</sub>) were determined for each mobile phase composition via uracil peak.

#### Preparation of stock and standard working solutions

The stock solution of NA (1 mg mL<sup>-1</sup>) was prepared by dissolving 50 mg NA in methanol to make 50 mL of solution. The working standard solutions were prepared by diluting aliquots of the stock solution to obtain concentrations ranging from 0.0156 to 100 µg mL<sup>-1</sup>.

The calibration graph was constructed by plotting the peak areas obtained at the wavelength of 240 nm versus the corresponding injected concentrations.

#### Sample preparation (for tablets)

Twenty tablets of Steronate® (labeled as containing 5mg NA per tablet, HI Pharm., Cairo, Egypt) was weighed, and then the average mass per tablet was determined. The tablets were grounded to a fine homogeneous powder. A quantity of the homogeneous powder of NA equivalent to the weight of one tablet was accurately transferred into a 100 mL volume calibrated flask containing 70 mL methanol (Deventer, Netherland). The content of the flask was sonicated for about 10 minutes and then made up to the volume with methanol. The solution was then filtered through a 0.45 µm milli-pore filter (Gelman, Germany) and the first 10 mL was rejected. The desired concentrations of the NA were obtained by accurate dilution with methanol. The solution was directly analyzed according to the general analytical procedure.

#### Sample preparation (for spiked plasma)

9 mL of plasma is spiked with 1 mL of NA solution in MeOH (0.5 mg mL<sup>-1</sup>). Then the solution is put in the centrifuge for 10 minutes. After that 1 mL of the supernatant is taken, mixed with 1 mL of ACN and left in sonicator for 5 minutes. Then the solution is put in centrifuge for 10 minutes and filtered. Finally a certain amount of filtrate is injected into chromatograph.

#### Intentional degradations

Intentional degradation was attempted using acid, base, hydrogen peroxide and UV-radiation. Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the method. Then 10 mL of 1 mg mL<sup>-1</sup> NA/methanol was transferred in three 50 mL rounds bottomed flasks to perform the first three degradation tests. 10 mL of each of 1N HCl, 1N NaOH and 30% H<sub>2</sub>O<sub>2</sub> were added to the first 3 flasks for acidic, basic and oxidative degradations, respectively. Each of the three flasks was refluxed for about 1 hour. After the completing of the degradation treatment, samples were allowed to cool to room temperature and treated as follows: The pH values of the first and second flasks were neutralized with dilute NaOH and dilute HCl, respectively. To the third flask 1N sodium bisulphite solution was added to destroy H<sub>2</sub>O<sub>2</sub>. The volume of all the three flasks was adjusted to 50 mL with methanol. Samples were injected and analyzed against control samples (lacking of degradation treatment). For degradation through UV-radiation 2 mL of the sample was left in UV radiation from 1 to 10 minutes and then the radiated solution

diluted with methanol to 10 mL, then finally injected into chromatograph and compared with the control sample.

## RESULTS AND DISCUSSION

#### System suitability

The results of six runs indicate high system suitability (Table 1). The retention time (*t<sub>R</sub>*-value) of NA is 2.946 ± 0.01 minutes. The RSD of peak area is 0.017%.

Table 1: System suitability and regression data

Parameters	Results
<b>System suitability</b>	
<i>t<sub>R</sub></i> ± SD (min)	2.946 ± 0.01
N	17078 (pro meter)
k'	1.964
<b>Linearity and regression data</b>	
Linearity range (µg mL <sup>-1</sup> )	0.125-50
Detection limit (µg mL <sup>-1</sup> )	0.0625
Quantitation limit (µg mL <sup>-1</sup> )	0.125
Slope (RSD)	36.726 (0.220)
Intercept (RSD)	4.522 (2.661)
Coefficient of determination (R <sup>2</sup> )	0.9998
<b>Comparison with a reported method [8]</b>	
t-test*	1.57(2.26)
F-ratio*	3.88(5.19)

\* Values in parentheses are the tabulated *F*- and *t*-values at P = 0.05.

#### Selectivity and specificity of the method

The resulted peak after tablet analysis is found to be homogeneous and there are no co-eluting peaks indicating specificity of the method. Comparison between the chromatogram of the raw NA and that of extracted NA from tablets indicates that the excipients in the formulation did not interfere with the determination of NA (Fig. 1). Each of twenty two pharmaceutical substances (mainly steroids) was simultaneously injected with NA (Table 2) for examination of specificity of the method. Only Chlormadinone acetate and Flumethasone pivalate were found to interfere with the method. Fig. 2 shows that the method can be applied for separation of NA in combination with Norethisterone and Levonorgestrel or with Estradiol and Levonorgestrel. Fig. 2 reflects that increase in the amount of ACN% to greater than 55% led. to co-elution of the steroids in combinations.

Table 2: Specificity of the proposed method

Not interfered			Interfered	
Levonorgestrel	Testosterone	Triamcinolone	Nifedipine	Chlormadinone acetate
Norethisterone	Propionate	Prednisolone	Diclofenac Na	Flumethasone pivalate
Estradiol	Hydrocortisone	Chlorpromazine	Atenolol	
Methylprednisilone	Fluocortilone	Salicylic acid	Chlorthalidone	
Betamethasone	Difluocortilone	Ketoprofen	Phenobarbital	
	Dexamethasone			

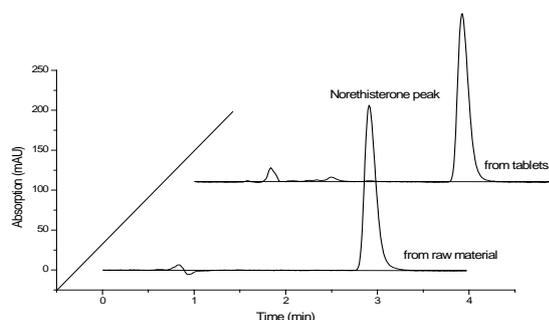
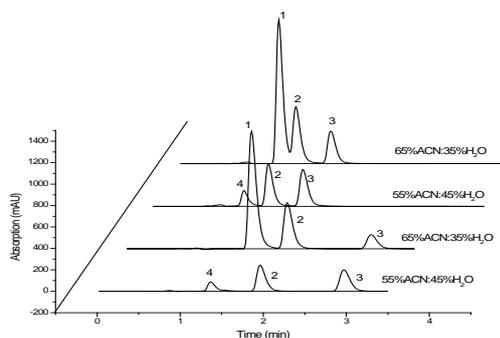


Fig. 1: NA from bulk powder and from tablets

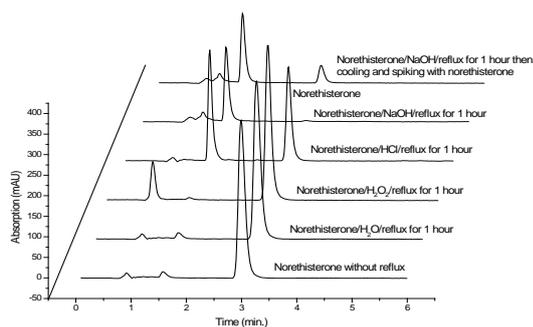
#### Stability of the analytical solution and stability tests

The results (Fig. 3 and 4) of stress degradation indicate that NA is strongly affected with reflux with NaOH or exposure to UV radiation. Reflux with H<sub>2</sub>O<sub>2</sub> or HCl leads to nearly the same degradation of NA but the effect here is weaker than in cases of NaOH and UV. There is no interference with the peak of the intact drug indicating that the method is stability indicating. The full run time for separation of the intact NA from its degradants is about 5 minutes which is very short comparing with the previously published work [8].

Stability of the standard solution was studied by injection of the prepared solution at periodic intervals into the chromatographic system up to about 7 days. The results indicate that the RSD of the peak area was within 1.02%.



**Fig. 2: Separation of NA in combination with other steroids**  
1- Norethisterone 2- Levonorgestrel 3- NA 4- Estradiol.



**Fig. 3: Stability tests on NA**

#### Linearity and range

Twelve concentrations of NA solution ranging from 0.0156 to 100  $\mu\text{g mL}^{-1}$  were analyzed. The graph of peak area against concentration proved linear in the range of 0.25-50  $\mu\text{g mL}^{-1}$  and the linearity equation is:  $Y = 36.726 X + 4.522$  and regression coefficient = 0.9998.

**Table 3: Precision of the proposed method**

	Intraday (n=5)			Interday (n=5)		
	2 $\mu\text{g mL}^{-1}$	10 $\mu\text{g mL}^{-1}$	20 $\mu\text{g mL}^{-1}$	2 $\mu\text{g mL}^{-1}$	10 $\mu\text{g mL}^{-1}$	20 $\mu\text{g mL}^{-1}$
Found	2.003	10.482	19.232	1.973	10.369	19.672
Recovery %**	100.15	104.82	96.16	98.65	103.69	98.36
RSD*	1.00	2.00	0.24	2.54	1.17	1.11

\*\*Recovery (%) = an observed concentration x 100/used concentration, \*RSD = S. D. x 100/mean

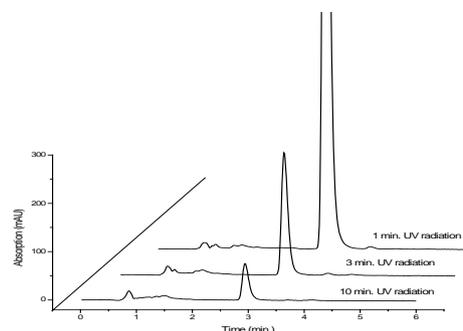
**Table 4: Applications**

	Steronate® tablets			Spiked plasma		
	50 $\mu\text{g}$	10 $\mu\text{g}$	5 $\mu\text{g}$	10 $\mu\text{g}$	5 $\mu\text{g}$	5 $\mu\text{g}$
Taken	47.595	10.109	4.889	10.331	5.088	5.088
Found	95.19	101.09	97.78	103.31	101.76	101.76
Recovery %	98.02			102.535		
Mean	3.02			2.64		
RSD						

**Table 5: Robustness of the method**

Changed factors	Deg C	Flow rate (mLmin <sup>-1</sup> )		ACN (%)		Wavelength of detection (nm)		Injection Volume ( $\mu\text{L}$ )		
		peak	t <sub>R</sub>	peak	t <sub>R</sub>	peak	t <sub>R</sub>	peak	t <sub>R</sub>	
Changes	38,	0.99,	54.9,	239,	19.9,					
	39	1.00	55.0	240	20.0					
	and	and	and	and	and					
	40	1.01	55.1	241	20.1					
Tested parameter	peak	t <sub>R</sub>	peak	t <sub>R</sub>	peak	t <sub>R</sub>	peak	t <sub>R</sub>	t <sub>R</sub>	
C. V. (%)	1.58	0.04	2.38	0.86	1.58	0.61	2.05	0.02	0.68	0.21

The limit of detection (LOD) giving S/N of 3 (signal to noise ratio in terms of peak height), was found to be 0.0625  $\mu\text{g mL}^{-1}$ . The limit of quantification (LOQ) giving S/N of 10 (signal to noise ratio in terms of peak height), was found to be 0.125  $\mu\text{g mL}^{-1}$  (Table 1).



**Fig. 4: Effect of UV-radiation on NA**

#### Reproducibility and precision of the method

The % RSD observed on the replicates (Table 3) indicates the reproducibility and hence the precision of the system.

Results (Table 3) show that there were high intra- and inter-day precisions (both within 2.54 %). Intra-day precision was assessed injecting of the standard solution at two concentrations five times during a day. Inter-day precision was assessed injecting of the standard solution at two concentrations every day for five days.

#### Accuracy and application

Analysis of NA in tablets formulation and spiked plasma showed high accuracy with recovery of  $98.020 \pm 3.02\%$  and  $102.535 \pm 2.640$ , respectively (Table 4). The results were compared with a reported method [8]. The values of *f* and *t* indicate that there is no significant difference between both methods (Table 1). Fig. 5 shows separations of NA in the plasma sample.

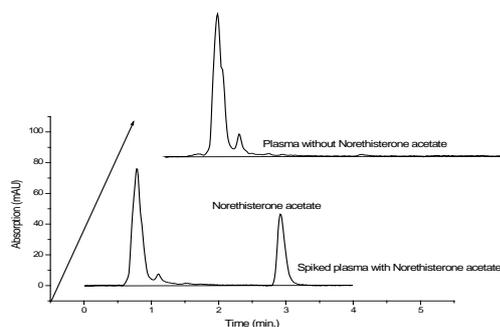


Fig. 5: Chromatogram of plasma with and without NA

### Robustness of the method

The robustness of the present method was evaluated in the terms of temperature, flow rate, content of ACN in mobile phase, wavelength of detection and injection volume (Table 5). The slight variations in the examined factors had no significant effect on the shape of the peak. The results co-efficient of variation (C. V.%) indicate that the method is more sensitive to changes in wavelength and in the flow rate greater than to changes in the other factors. Compared with retention times ( $t_R$ -values), peak areas were more affected with the slight changes in the chromatographic conditions.

### CONCLUSION

This study reports about a stability indicating HPLC method for determination of Norethisterone acetate (NA) in bulk powder, tablets and plasma. The proposed method is suitable also for separation of NA from some other steroids which can be used with it in combination for contraception. Compared with the published HPLC-methods, this method represents a strong reduction of analysis time and it is considered as a stability indicating method. The full run time for separation of the intact NA from its degradants is about 5 minutes which is very short comparing with the previously published work. With the proposed method a satisfactory separation of NA from the degradation products, an extended linear range and a rapid analysis time was obtained. A high recovery of NA in tablets and plasma was achieved. No interference from the excipients was noticed.

### CONFLICT OF INTEREST

Declared none

### ACKNOWLEDGEMENT

Authors thank the above-mentioned companies for the friendly supply of analyte and column.

### REFERENCES

- Hammond GL, Rabe T, Wagner JD. Preclinical profiles of progestins used in formulations of oral contraceptives and hormone replacement therapy. *Am J Obstet Gynecol* 2001;185:S24-S31.
- Swynnerton NF, Fischer JB. Determination of ethynylestradiol and norethindrone in synthetic intestinal fluid and in timed-release oral formulations. *J Liq Chromatog* 1980;3:1195-204.
- Gluck JA, Shek E. Determination of ethynylestradiol and norethisterone in an oral contraceptive capsule by reversed-phase high performance liquid chromatography. *J Chromatogr Sci* 1980;18:631-6.
- Loo JC, Brien R. Analysis of norethindrone in plasma by high-performance liquid chromatography. *J Liq Chromatogr* 1981;4(5):871-7.
- Fayad PB, Prevost M, Sauve S. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry optimized for the analysis of steroid hormones in urban wastewaters. *Talanta* 2013;115:349-60.
- Gonzalo-Lumbreras R, Izquierdo-Hornillos R. High-performance liquid chromatographic optimization study for the separation of natural and synthetic anabolic steroids: Application to urine and pharmaceutical samples. *J Chromatogr B* 2000;742:1-11.
- Boglárika B, Géza R, Mária M, Zoltán V, Katalin FF. OPLC, a method between TLC and HPLC, for purity testing of norethisterone bulk drug substance and tablet. *Planar Chromatogr* 2003;16:359-62.
- Krishna PM, Rao BT, Kumar RK, Venkateswarlu P. Development and validation of method for the determination of related substances of norethindrone in norethindrone tablets and degradation studies. *Int J Chem Tech Res* 2011;3:143-8.
- Ali I, Hussain I, Saleem K, Aboul-Enein HY. Development of efficient SPE-TLC method and evaluation of biological interactions of contraceptives with progesterone receptors. *Arabian J Chem* 2012;5:235-40.
- Fotherby K, Warren RJ, Shrimanker K, Siekmann L, Siekmann A, Breuer H. Plasma levels of norethisterone measured by radioimmunoassay and gas chromatography-mass fragmentography. *J Steroid Biochem* 1979;10:121-2.
- Braselton WE, Lin TJ, Mills TM, Ellegood JO, Mahesh VB. Identification and measurement by gas chromatography-mass spectrometry of norethindrone and metabolites in human urine and blood. *J Steroid Biochem* 1977;1:9-18.
- Braselton WE, Lin TJ, Ellegood JO, Mills TM, Mahesh VB. Accumulation of norethisterone and individual metabolites in human plasma during short-and long-term administration of contraceptive dosage. *Am J Obstet Gynecol* 1979;133:154-60.
- Sahlberg BL, Landgren BM, Axelson M. Metabolic profiles of endogenous and ethynyl steroids in plasma and urine from women during administration of oral contraceptives. *J Steroid Biochem* 1987;26:609-17.
- Stanczy FZ, Roy S. Metabolism of levonorgestrel, norethindrone, and structurally related contraceptive steroids. *Contracept* 1990;42:67-96.
- Rizk MS, Zakhari NA, Walash MI, Toubar SS, Brooks CJ, Anderson R. Gas chromatography and mass spectrometry of dimethylethylsilyl ether derivatives of norethisterone metabolites in plasma. *Acta Pharm Nord* 1991;4:205-10.
- Pommier F, Sioufi A, Godbillon J. Simultaneous determination of norethisterone and six metabolites in human plasma by capillary gas chromatography with mass-selective detection. *J Chromatogr B* 1995;674:155-65.
- Pommier F, Sioufi A, Godbillon J. Quantitative determination of norethisterone acetate in human plasma by capillary gas chromatography with mass-selective detection. *J Chromatogr A* 1996;750:75-81.
- Khattab FI, Ashour FM, Amer MM. Argentometric methods for the determination of acetylenic steroids, ethinyloestradiol and norethisterone. *J Pharm Belgique* 1983;38:147-55.
- Opheim LN. Determination of noretisterone in tablets by differential pulse polarography. *Anal Chim Acta* 1977;89:225-9.
- Chatten LG, Yadav RN, Binnington S, Moskalyk RE. Polarographic study of certain progestogens and their determination in oral contraceptive tablets by differential pulse polarography. *Analyst* 1977;102:323-7.
- Yadav RN, Teare FW. Determination of fluoxymesterone, norethandrolone, prednisolone, and prednisone in tablets by differential pulse polarography. *J Pharm Sci* 1978;67:436-8.
- Ghoneim MM, Abushoffa AM, Moharram YI, El-Desoky HS. Voltammetry and quantification of the contraceptive drug norethisterone in bulk form and pharmaceutical formulation. *J Pharm Biomed Anal* 2007;43:499-505.
- Ayad MM, EL-Henawee MM, Abdellatef HE, EL-Sayed HM. Spectrophotometric determination of levonorgestrel, norethisterone acetate, mometasone furoate and fluticasone propionate using 4-amino-antipyrine. *Bull Fac Pharm (Cairo Univ)* 2005;43:24-8.