Polarographic Investigation of Dienogest

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Dienogest (DGN), an orally administered synthetic progestin that has shown to have selective progesterone receptor (PR) agonistic activity and oral progestational potency on endometrium. In this paper, the investigation of electrochemical reduction of Dienogest on dropping mercury electrode (DME) is achieved and resulted in a reduction scheme that involves a two electron reduction of 3-keto-delta-4 group in the A-ring of the molecule. This observation is supported by comparison of the compound with model compounds of testosterone and norethisterone. In this study, electrochemical determination of the compound and its validation is also studied by differential pulse polarography (DPP). The results yielded good precision, accuracy and reproducibility. Calibration range was found as $2.00 \times 10^{-6}$ M to $1.00 \times 10^{-4}$ M, with LOD and LOQ values as $0.58 \times 10^{-6}$ M and $1.90 \times 10^{-6}$ M, respectively.

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Steroids are organic compounds that contain a characteristic arrangement of four cycloalkane rings that are joined to each other. They can be found in animals, plants, fungi and microorganisms. Dienogest (DGN, (17α)-17-hydroxy-3-oxo-19-norpregna-4,9-diene-21-nitrile, Qlairista), is an orally active semisynthetic, steroidal progestogen, which is reported to display no androgenic properties.1,2

Owing to its activity, and potentially favorable side effect profile, DGN has received approval as a monotherapeutic and it was the first agent approved by the European Union in 2010 for the treatment of endometriosis.

In order to elucidate its activity several modern analytical methods including radioimmunoassay and separation methods with excellent sensitivity and selectivity such as gas or liquid chromatography combined with mass spectrometry (LC/GC-MS), have been developed and used to study DGN and its metabolites in plasma.3,4 urine, salivain,5 animal tissue6 and pharmaceutical formulations.7 Although these methods are extremely powerful, most of them are time consuming, expensive and need complicated sample pretreatment, involving various types of derivatization, extraction, and purification steps.

On the other hand, electroanalytical methods have proven to be very sensitive, selective, cheap and rapid for the determination of active pharmaceutical ingredients (APIs) in their dosage forms and are an available alternative for the analysis of electroactive compounds. The advantage of experimental electrochemical techniques in the field of quantitative analysis of drugs arise from their simplicity, low cost and relatively short analysis time as compared with other techniques. Voltammetric techniques are most suitable for investigation of the redox properties of APIs, and these techniques can give insight into the metabolic fate, in-vivo redox process or pharmaceutical activity.8-11

To our best knowledge, there has been no published research on the electrochemical reduction mechanism and detection of DGN in the official pharmacopoeias as of today. In the present work, the reduction mechanism of DGN in aqueous buffered solutions has been studied by comparison with model compounds, namely testosterone and norethisterone, whose electrochemical studies could be found in the literature.12,13 The comparison of the compound under investigation with these model compounds led to some conclusions about the potential electroactive centers under working conditions, and as a result, a reduction mechanism for DGN has been proposed.

In this study, also, a new, simple, rapid, selective, sensitive and fully validated electrochemical method is developed for determination of DGN. The proposed method involves Differential Pulse Polarography (DPP) achieved by a dropping mercury electrode (DME) and it offers good precision, accuracy and reproducibility. This method is inexpensive, time-saving and easy to handle experimentally when compared with already published methods, requiring neither any pre-treatment of the formulation nor determination of specificity and selectivity since the used electrodes are not affected by the impurities in the studied solution. Such basic information on solution chemistry of DGN is useful in understanding the conversion of the compound to its active form, and this understanding is especially valuable since this process also occurs in an aqueous medium in the human body.

Materials and Methods

Chemicals and solutions.—Dienogest (DGN) and its pharmaceutical dosage form Qlairista was obtained by Roche Pharm. Ind. (Istanbul, Turkey). All chemicals and buffers used were of reagent or spectral grade purity. NaH2PO4, NH4Cl, H3PO4, NaOH and HCl were supplied by Fluka and Riedel-de Haen while CH3COOH, Na2HPO4.2H2O, NH3, NaHCl, H2PO4, NaOH and HCl were supplied by Merck. H2SO4 was supplied by Tekkim and CH3COONa was supplied by Riedel-de Haen. DMSO was obtained from Sigma-Aldrich. Distilled water was used for preparing buffer solutions.

Instrumentation.—For polarography experiments, current-voltage curves were recorded by using a Polargraphic Analyser Model ENTEK 2017 as well as capillary electrodes with characteristics of $m = 2.0$ mgs$^{-1}$, $t_1 = 2.5$ s at $h = 64$ cm. Electrochemical experiments were carried out in a Kalousek cell in combination with a saturated calomel reference electrode (SCE). The pH measurements of the buffer solutions were achieved using a Denver Instrument Model UB-10 pH-meter in combination with a glass electrode.

Operating conditions for DC polarography experiments were: damping, 1s; current range, 0.05 mA; scan range, 5 mV$^{-1}$ or 2mV$^{-1}$; scan range, 0 mV to $-2000$ mV and for DPP were: dropping time, 2s; pulse amplitude, 50 mV; pulse width, 40ms; scan rate, 2 mVs$^{-1}$; current range, 0.05 mA; damping, 1s.

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1 or 2mVs$^{-1}$;
Preparation of the solutions.—Mechanistic studies to understand the reduction behavior of DGN were carried out by DC polarography in which a series of simple buffer solutions as well as acid and base solutions were prepared with pH values varying between 1.0 and 13.0. The buffer solutions were prepared such that the concentration of either the acidic or basic component was held constant throughout the series to eliminate the effect of varying buffer concentrations on the measured currents. CH₃COOH/CH₃COONa buffers were prepared by varying the composition of the acid component from 0.01 M to 0.30 M in order to achieve supporting solutions of pH varying from 3.7 to 5.7. Similarly, ammonia buffers were prepared by using NH₄ and NH₄Cl with concentration of the basic component varying from 0.01 M to 0.30 M, hence, by varying the pH from 8.3 to 10.3. HCl solutions of concentrations varying from 0.01 M to 0.1 M were used to achieve acidic solutions whereas NaOH solutions of the same concentrations were used to achieve an alkaline media. Phosphate buffers were used in the remaining pH range. Solutions with pH varying from 2.0 to 3.2 by using H₃PO₄ and NaH₂PO₄, pH from 5.7 to 7.7 by using NaH₂PO₄ and Na₂HPO₄ and pH from 10.8 to 12.0 by using Na₂HPO₄ and Na₃PO₄ were prepared in which the concentration of the acidic component was varied from 0.01 M to 0.30 M. Constant ionic strength of 0.30 M was maintained by addition of a neutral salt such as sodium chloride when needed.

Studies on tablets and validation were carried out by DPP in which the size of the mercury drop was kept constant by controlling the dropping time at 2s. The best response for studying the tablet dosage forms and validation parameters was obtained in pH 10.8 phosphate buffer solution with a composition of 0.10 M Na₂HPO₄ and 0.01 M NaH₂PO₄.

Stock solution of DGN for DC experiments was prepared in DMSO at 0.025 M concentration and stored in the dark at room temperature. No extra addition of DMSO was necessary and the required amount of the stock solution was added into the polarographic cell after 5 minutes of purging of the buffer solution by nitrogen. For DPP studies, dilutions were carried out by adding appropriate volumes of pH 10.8 phosphate buffer solution into the cell so that solutions of concentrations varying from 1 × 10⁻⁴ to 2 × 10⁻⁸ M of DGN were achieved.

Tablet assay procedure.—Ten tablets of Qlairista were finely powdered and weighed. The mass of a single tablet was calculated. Each tablet was claimed to contain 3 mg of DGN, thus the amount needed to prepare a stock solution containing 0.001 M pure DGN was weighed and transferred into a volumetric flask with a 50 mL volume where it was dissolved in DMSO to the volume. This solution was stirred in an ultrasonic bath for 30 min and left to settle. After the insoluble impurities have precipitated, necessary amounts from the clear supernatant liquor were taken into another volumetric flask for further dilution with the selected buffer. This solution was used to receive polarograms. The content amount of DGN was calculated from the corresponding regression equations.

Validation of the analytical procedure.—For the validation of the studied method, the precision and accuracy were checked by assaying seven replicate samples on the same day (within day) and different days (between days) over a week period for three different concentrations of DGN.

To avoid decomposition, all solutions were protected from light and used within one week. All measurements were carried out at ambient temperature of the laboratory (23–26°C). Polarograms of the sample solutions recorded a week after preparation did not show any appreciable change in assay values. The calibration equations were constructed by plotting the peak height against DGN concentration.

Recovery studies.—In order to demonstrate the applicability and show the accuracy of the proposed method, recovery experiments were performed. For this purpose a known amount of the pure DGN was added into the pre-analyzed tablet formulation and the mixtures were analyzed. The percent recovery was calculated by comparing the concentration obtained from spiked samples with the actual added concentration. Thus, the effects of commonly used excipients (e.g., shifting of peak potential or changing peak shape) in tablet dosage form were investigated. The recovery studies were obtained by using the related calibration equations for five or six repeated measurements. Hence, recovery experiments from tablets proved the reliability and suitability of both methods.

Results and Discussion

Reduction mechanism of DGN.—The nature of chemical and physical processes involved in any procedure used for the understanding of the behavior of a compound should be well understood. Such understanding of reaction schemes or mechanisms is of particular importance in electroanalytical procedures developed for investigation of drug active materials in order to relate the structure of the compound to its biological activity. Hence, every quantitative determination study should be accompanied by a mechanistic study to give an insight into its in vivo behavior in biological systems.

Investigation of electrochemical reduction mechanism of DGN has been achieved by carrying out a pH dependence of the compound by DC Polarography in common buffer solutions with pH values in the range of 1.0 to 13.0. Sample polarograms at three pH values; namely an acidic pH value (Figure 1A), a medium pH value (Figure 1B) and a basic pH value (Figure 1C) were chosen and shown together with the polarograms of the supporting electrolyte at each individual pH. As can be seen from this figure, a reduction wave with a half-wave potential that becomes more negative with increasing pH was observed up to pH 12.

The dependence of the half-wave potentials on pH of this reduction wave for DGN is given in Figure 2. This figure shows the linear dependence of half-wave potentials at pH values lower than 12, with a dE₁/₂/dpH value of 0.058 V between pH 1 and 6, and a dE₁/₂/dpH value of 0.055 V between pH 6.3 and 12. The shift of half-wave potentials to more negative values with increasing pH occurs due to the additional energy need for the proton transfer and the values of the slopes usually indicate the number of protons transferred before the first electron uptake. These calculated values of slopes indicate involvement of the same number of protons and electrons in this reduction process and this proton is involved in the rate-determining step preceding the electron transfer. Hence, the process occurs by addition of a proton in which an O–H bond is instantaneously formed, and then electrons are transferred. At pH values higher than 12, the E₁/₂ values were pH independent; indicating reduction of the unprotonated form.

The dependence of the limiting currents of the reduction wave for DGN is given in Figure 3. As can be seen in this figure, the measured limiting currents were pH independent throughout the pH range studied with slightly lower values at acidic pH values (pH between 1 and 6) than at higher pH values. The pH range in which lower current values were observed agrees with the two-segment linear dependence of the half-wave potentials. This may be due to a chemical reaction accompanying the reduction process, or due to the fact that the reduction wave was poorly developed at acidic medium. However, no conclusion about the faith of a possible side reaction could be driven out.

Information about the polarographic reduction of two related compounds, Testosterone and Norethisterone, were found in the literature. These published results were used for comparison with our experimental results on the reduction of DGN on DME in order to get evidence for our possible reduction mechanism. There is also another structurally related compound called Ethisterone, which could be used for comparison. However, no electrochemical study could be found about this compound in the literature. The chemical structures of these model compounds are given in the experimental part. As can be seen from these structures, all four compounds are derivatives of delta-3-ketosteroids. The only possible reduction site on this class of compounds is the keto group in the A-ring of the molecule, as given in the following Scheme 1.
The comparison of the half-wave potentials for the more positive wave of three related compounds; Testosterone, Norethisterone, Dienogest are given in Figure 4. As can be seen, all the plots are pH dependent having the same slope, indicating transfer of same number of protons before the electron uptake. However, for DGN the measured half-wave potentials are much more positive than the model compounds. This can be explained by the concept named conjugation. As can be seen from the chemical structures of these compounds, there are two differences in the DGN molecule when compared to the model compounds. One is that there is a cyano group on the five membered ring instead of the hydrogen at the same position for Testosterone and C-C triple bond for Norethisterone. Second difference is the presence of a double bond in the B-ring of DGN which is absent in the model compounds. This double bond causes delocalization of the electrons of the A and B rings and this conjugation makes the reduction of DGN easier on DME, leading to more positive reduction potential values. Since the cyano grouping on DGN is distant to the reduction center of the molecule, the inductive effect of this grouping is not considered as a cause in the shift of reduction potentials to more positive values.

As the results indicate, it is proved by comparison that DGN is reduced by a mechanism similar to that of Testosterone and Norethisterone on DME. In this reduction, only one single wave could be observed throughout the pH range studied. The half-wave potential of this wave is shifted to more negative potentials with increasing pH up to pH 12. The slope of this shift indicates transfer of same number of protons and electrons during the reduction process. Therefore, a reduction mechanism may be proposed based on our experimental results and the above mentioned discussions implying the reduced group on DGN to be the C=O bond of the compound (Scheme 1). This reduction occurs via two electron transfer, accompanied by two protons uptake, following H+ + e− + e− + H+ sequence. At pH values higher than 12, the half-wave potential of the observed wave was pH independent, indicating e− + e− + H+ + H+ sequence.

**DPP method developed for DGN.—**Validation.—DPP using a DME was used for quantitative determination of DGN and is based on the linear correlation between the peak current and concentration. For analytical purposes, best response and the best single peak shapes, peak current sensitivity, and reproducibility were obtained in phosphate buffer at pH 10.8. Two peaks were observed for DGN at this pH value; one at −1.45 V and the other at −1.55 V. The latter one was investigated (see Figure 5) since the more positive peak was proven to be a pre-adsorption peak from the mercury pressure and placebo (Estradiol Valerate tablets) experiments. The plot of DGN concentration vs. the peak current gave a linear calibration curve, indicating a diffusion-controlled process. In phosphate buffer at pH 10.8, the plot of the calibration curve for the reduction peak was linear between 2 × 10−4 and 1 × 10−4M (Fig. 6).

Characteristics of this plot and the related validation parameters are given in detail in Table 1. The low values of standard error of the slope and intercept and the greater correlation coefficient than 0.99 confirmed the precision of the proposed methods.

Several approaches are given in the ICH guideline (The International Council for Harmonisation of Technical Requirements to Pharmaceuticals for Human Use) to determine the limits of detection (LOD) and limits of quantification (LOQ) values. LOD and LOQ, repeatability (within day), reproducibility (between days), pre-cision, recovery, bias%, and selectivity were evaluated.9,14–17 The LOD and LOQ were calculated on the peak current using the equations LOD = 3(s/m) and LOQ = 10(s/m) where s is the standard deviation of the peak currents (five runs) and m is the slope of the related calibration equation. LOD and LOQ give the sensitivity of the proposed methods.

The precision of the proposed method was calculated by repeating seven experiments for the same solutions within the same day.
(repeatability) and seven experiments for 3 consecutive days from different solutions (reproducibility). The DGN concentrations were selected as $8 \times 10^{-5}$, $2 \times 10^{-5}$ and $6 \times 10^{-6}$ M for the precision of the experiments. The within day and between day precision, accuracy, and reproducibility were determined and described as RSD\% in Table I. The obtained results demonstrated good precision, accuracy, and reproducibility.

Standard sample solutions were stored at room temperature ($22^\circ$ C) in the dark and recorded every week. The solutions did not show any appreciable change in assay values even after a month. However, all solutions used for the validation experiments were freshly prepared to ensure the stability of analyte in the solutions.

**Tablet assay procedure.**—The proposed DPP technique was applied for the determination of DGN in pharmaceutical dosage forms in phosphate buffer at pH 10.8 (labeled DGN amount is 3 mg per tablet). The obtained results are given in Table II in detail. The validity was assessed by applying calibration curves and the standard addition methods. The results showed that the proposed method could be applied with a great success to DGN assay in tablet dosage form without any interference (Table II). The mean results for the determinations are very close to the amount of 3 mg.

On the basis of above results, DPP was applied for the direct determination of DGN in tablet dosage form, using the related calibration straight lines without any sample extraction, evaporation or filtration and after adequate dilutions.

In order to detect the interaction between the excipients and active ingredients, recovery studies were carried out after addition of known amounts of the pure drug to pre-analyzed formulations of DGN. These results indicated the absence of interference from commonly encountered pharmaceutical excipients used in the tablet formulations. The mean percentage recoveries based on the average of seven replicate measurements have showed no significant interference from excipients in the analysis of DGN (Table II).
The reduction mechanism proposed for the compound was based on the comparison with model compounds studied in the literature which involves a similar chemical structure to DGN. The similarity in the electrochemical behavior of the model compounds and the compound under investigation at the same conditions led to the conclusion that it is the keto group on the A ring of the molecule that is reduced to yield an alcohol.

The importance of DGN as a drug in the treatment of hormone replacement therapy and lack of a simple, inexpensive, reliable and reproducible method for its determination in tablet dosage forms resulted in an effort to establish a validated electrochemical method on the reduction behavior of the compound.

The DPP method offered in this study yields good precision and reproducibility (less than 2.0 of RSD % values), low LOD and LOQ values and good accuracy results. The method was also applied to tablets and the results of the direct measurements as well as the recovery studies showed that the proposed method could be applied with a great success to DGN assay in tablet dosage forms without any interference from the commonly encountered pharmaceutical excipients used in the tablet formulations.

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