Analytical Methods

Cite this: *Anal. Methods*, 2011, **3**, 1198

www.rsc.org/methods

TECHNICAL NOTE

Use of nitroanilines for spectrophotometric determination of ethinylestradiol in pharmaceutical formulations

Leonardo S. G. Teixeira,*[a] Maiana A. Teixeira,[a] Admar J. M. Bueno,[b] Cristiane F. Brito,[c] Alailson F. Dantas[a] and Antonio C. S. Costa[a]

Received 31st December 2010, Accepted 25th February 2011
DOI: 10.1039/c0ay00783h

Three nitroanilines (2-methoxy-4-nitroaniline, 2,4-dinitroaniline and 2-methyl-4-nitroaniline) were tested as spectrophotometric reagents for determination of ethinylestradiol (ETE). The determinations were based on absorbance measurments of the reaction product obtained from the coupling of each diazotized nitroaniline with ETE. Optimization of temperature, pH and diazotization coupling reaction time was realized. 2-Methyl-4-nitroaniline provided the more sensitive system for ETE spectrophotometric determination at 531 nm and the analytical response was linear in the concentration range of 0.65–10.0 μg ml^{-1}. The limit of detection and coefficient of variation were estimated as 197 μg l^{-1} and 4.5%, respectively. The results showed that the proposed method is simple and rapid, allowing selective determination of analyte. The method successfully determined ETE in oral contraceptive formulations.

1. Introduction

Oral contraceptives have had a positive impact on public health for the past four decades. Although a remarkably low incidence of side effects has been reported, there is still a need for reliable methods of routine analysis.[1] Ethinylestradiol (ETE), semi-synthetic estrogen, is a female sex hormone that is widely used in oral contraceptives. The formulations of this steroid in tablets of low dosage had presented a challenging analytical problem. Most oral contraceptive formulations, in current use, contain 10 μg or less of ETE in combination with an orally active synthetic progestin (norethindrone or levonorgestrel), whose concentration is 5–30 times that of the estrogen.[2] Thus, a rapid, sensitive, accurate and low-cost analytical procedure, unaffected by excess progestin, is required for the analysis of ETE in pharmaceutical preparations.

Many analytical methods have been established for ETE determination in a variety of sample matrices, including micellar electrokinetic chromatography,[3] gas chromatography,[4] chemiluminescence,[5] voltammetry,[6] spectrophotometry[2] and high-performance liquid chromatography (HPLC).[7] The technique predominantly used by the United States Pharmacopoeia is HPLC with UV detection, and that by European Pharmacopoeia for estrogen determination is UV spectrophotometry.[8] Although sensitive and selective, the methods involving HPLC are time-consuming and expensive, requiring skilled personnel for operating.

Because of its high sensitivity, operational facility and low cost, spectrophotometry is one of the most useful analytical tools and has been used in the analysis of pharmaceutical preparations,[9–11] including ETE determination.[2,12,13] ETE has an absorption maximum at 230 nm and can be directly determined by UV spectrophotometry. However, this method has low sensitivity and is naturally non-specific: the overwhelming majority of other compounds present in the sample are measured together with the ETE.[14]

Sensitivity and selectivity of spectrophotometric methods can be improved by reaction of the analyte that gives a new compound, whose absorbance can be monitored.[15] Thus, diazotization followed by coupling reaction of the analyte has been used for spectrophotometric determination of drugs in pharmaceutical preparations such as nimesulide,[16] sulfadiazine,[17] metoclopramide[18] and flutamide.[19] Formation of products with stable colours by diazotized nitroanilines coupled with phenolic compounds is the basis for a rapid and sensitive method for determination of pharmaceuticals containing a phenolic hydroxyl group such as ETE.[20]

In this work, three nitroanilines (2-methoxy-4-nitroaniline, 2,4-dinitroaniline and 2-methyl-4-nitroaniline) were tested as spectrophotometric reagents for determination of ethinylestradiol (ETE) and a procedure for determination of ETE in pharmaceutical formulations is proposed. The method is based on

[a]*Instituto de Química—Universidade Federal da Bahia, Campus Universitário de Ondina, 40.170-280 Salvador, Bahia, Brazil. E-mail: lsgt@ufba.br; Fax: +55 71 32355166; Tel: +55 71 32836829*
[b]*Departamento de Engenharia e Arquitetura—Universidade Salvador—UNIFACS, Av. Cardeal da Silva 132, 40.220-141 Salvador, Bahia, Brazil*
[c]*Serviço Nacional de Aprendizagem Industrial, Centro de Tecnologia Industrial Pedro Ribeiro—SENAI/CETIND, Av. Luiz Tarquínio Pontes, 938, 42700-000 Lauro de Freitas, Bahia, Brazil*

absorbance measurments of the reaction product of the coupling reaction between diazotized 2-methyl-4-nitroaniline and ETE, which is a convenient and stable compound for spectrophotometric determination.

2. Experimental

2.1. Apparatus

Spectrophotometric measurements were taken using a Cary 50 UV-visible spectrophotometer (Varian, Australia) with 1.00 cm glass cells. The pH measurements were carried out with a DM 20 pH meter (Digimed, Brasil). Chromatographic determinations were made using a Varian 9010 HPLC equipped with a Prostar autosampler (model 410), a Bondesil C18 column (250 × 4.6 mm; 5 μm) and a UV-VIS detector.

2.2. Reagents and solutions

All solutions were prepared using analytical-grade reagents and high-purity demineralized water obtained using a Milli-Q Water System (Millipore, France).

ETE was obtained from Sigma, and stock solutions (100.0 mg l^{-1}) were prepared in methanol (Merck). Working standard solutions were daily prepared by adequate dilution in methanol.

2-Methyl-4-nitroaniline solution (0.05%, m/v) was prepared by dissolving 0.100 g of this reagent (Aldrich) in 200 ml of 0.5 mol l^{-1} HCl.

2-Methoxy-4-nitroaniline (0.05%, m/v) was prepared by dissolving 0.100 g of this reagent (Aldrich) in 200 ml of 0.5 mol l^{-1} HCl.

2,4-Dinitroaniline (0.05%, m/v) was prepared by dissolving 0.100 g of this reagent (Aldrich) in 200 ml of 0.5 mol l^{-1} HCl.

Sodium nitrite solution (3.0%, m/v) was prepared by dissolving 3.0 g of $NaNO_2$ (Merck) in 100 ml of water.

Sodium acetate solution (2.0 mol l^{-1}) and sodium hydroxide solutions (0.1 mol l^{-1} and 1.0 mol l^{-1}) were prepared by dissolving appropriate amounts of the respective reagents in 200 ml of water.

Acetate buffer solution (pH 5.5) was prepared by mixing 1.0 mol l^{-1} trihydrate sodium acetate solution and 1.0 mol l^{-1} glacial acetic acid until the required pH.

2.3. Sample preparations

The pharmaceutical preparations and their labelled contents (amount present in one tablet) were *Neovlar* (Schering) containing 50 μg of ETE and 0.25 mg of levonorgestrel; *Microvlar* (Schering) containing 50 μg of ETE and 0.15 mg of levonorgestrel; and *Primosiston* (Schering) containing 50 μg of ETE and 2.0 mg of norethisterone acetate. All the contraceptives have excipients, such as magnesium stearate, starch, glycerol, polyethylene glycol, polyvinylpyrolidine, sucrose, lactose, calcium carbonate, titanium dioxide and iron oxide, which make up the total weight of the tablets.

Twenty-one tablets of each commercial pharmaceutical preparation were crushed to a fine powder. An appropriate amount of each powder sample was dissolved in 15 ml of methanol by sonication for 15 min. Insoluble excipient was removed by filtration through a 0.45 μm membrane filter. The residue was washed with methanol, and the filtered solution was diluted to appropriate volume with the same solvent.

2.4. Procedure

One millilitre of the nitroaniline and 1 ml of sodium nitrite solutions were taken in a 10 ml volumetric flask, and solutions were allowed to stand for 2 min at ambient temperature (25 °C). Then, the work solution containing 5.00 to 100 μg of ETE, 2 ml of sodium acetate solution and 2 ml of sodium hydroxide (0.05 mol l^{-1}) was added and allowed to stand for a further 15 min at ambient temperature. The solution in the flask was diluted with sodium hydroxide (1.0 mol l^{-1}), and the absorbance signal was obtained at a wavelength of absorption maximum in a 1 cm cell, against a blank prepared in the same way but without ETE.

3. Results and discussion

The maximum absorbance of ETE methanolic solution occurs at 230 nm. However, determination of ETE directly in contraceptive formulations was not possible because of the interference of other compounds in the UV spectral region, as previously observed by Berzas-Nevada *et al.*[2]

In a previous work, a colorimetric procedure, based on the formation of an azo dye by the condensation of diazotized 5-chloro-2,4-dinitroaniline with ethinylestradiol, was described.[20] In this work, three nitroanilines (2-methoxy-4-nitroaniline, 2,4-dinitroaniline and 2-methyl-4-nitroaniline), available in our laboratory, were tested as spectrophotometric reagents for determination of ethinylestradiol (ETE). The determinations were based on absorbance measurments of the reaction product obtained from the coupling of each diazotized nitroaniline with ETE. The products obtained by the coupling reactions of ETE with 2-methoxy-4-nitroaniline, 2,4-dinitroaniline and 2-methyl-4-nitroaniline showed absorption maximum at 487, 522 and 531 nm, respectively.

Nitrite ion reacts with diazotizable aromatic amines, such as the tested nitroanilines, in hydrochloric acid medium to form diazonium ion. This ion is responsible for the coloured azo dyes formed after coupling reactions with ETE. Optimization of temperature, pH and reaction time was realized using solutions containing 5 μg ml^{-1} of ETE and sufficient excess of each nitroaline and sodium nitrite, according to the experimental procedure described by Eldawy *et al.*[20]

The effect of temperature on the reactions was studied by varying the temperatures (0, 8, 25 and 32 °C) at which the diazotization and coupling reactions were carried out. The experiments were realized using controlled temperature bath, and at 32 °C the extent of colour development was less. However, at 25 °C and below, consistent and maximum absorbance signals were obtained for all tested nitroanilines. Therefore, the ambient temperature was chosen for the procedure, avoiding temperature control.

Acidic solutions of the nitroanilines were used in this study to facilitate the reaction with the nitrite to form diazonium ions, as in previous studies.[20,21] However, the coupling reaction can occur in alkaline or acidic medium, depending on the compounds

Table 1 Analytical characteristics of the spectrophotometric determination of ethinylestradiol using nitroanilines

	Reagent		
Analytical characteristic	2-Methyl-4-nitroaniline	2-Methoxy-4-nitroaniline	2,4-Dinitroaniline
λ_{max}/nm	487	531	522
Calibration equations[a]	$A = 0.010C + 0.005$	$A = 0.038C + 0.003$	$A = 0.025C + 0.004$
Correlation coefficient	0.998	0.999	0.999
Linear dynamic range/μg ml^{-1}	3.0–10.0	0.65–10.0	1.0–10.0
ε/l mol^{-1} cm^{-1}	2.47×10^3	1.11×10^4	7.30×10^3
LD/μg l^{-1} (3σ)	870	197	300
Coefficient of variation (% RSD)	4.8	4.5	4.0

[a] A: absorbance and C: concentration (μg ml^{-1}).

involved in the reaction. The absorbance signal of the product of the coupling reaction was studied at various pH values (3.5–12.0), and no significant variation in the absorbance occurred in pH range of 5.5–7.0, 5.0–6.5, and 5.0–7.0 using 2-methyl-4-nitroaniline, 2-methoxy-4-nitroaniline and 2,4-dinitroaniline. In subsequent studies, pH 5.5 was maintained for coupling reactions and pH was controlled using sodium acetate buffer.

The effect of the reaction time on diazotization and coupling was studied at room temperature according to the proposed procedure. Best absorbance signals were obtained when the diazotization and coupling reactions lasted for 2 and 15 min, respectively for the three nitroanilines.

Analytical characteristics of the proposed procedure and application

Calibration curves were constructed as described in the experimental procedure, and good correlation coefficients were found. The precisions (repeatability), calculated from 15 consecutive measurements and defined as the coefficient of variation of solutions containing 5.0 μg ml^{-1} of ETE, as well as the limits of detection (LOD), defined as the analyte concentration that gives a response equivalent to three times the standard deviation of the blank ($n = 10$), were calculated. The analytical characteristics of the procedure using the three anilines are summarized in Table 1. As can be seen, the procedure using the 2-methyl-4-nitroaniline provided the more sensitivity system for spectrophotometric determination of ETE. In this way, the proposed procedure using the 2-methyl-4-nitroaniline was then applied to ETE determination in oral contraceptive formulations. The diazotization-coupling reaction employing the 2-methyl-4-nitroaniline is shown in Fig. 1.

Previously, a solution containing ETE, norethindrone and levonorgestrel was treated to study the effect of norethindrone and levonorgestrel on ETE determination by the proposed method. The reaction selectivity was studied by determining 5 μg ml^{-1} of ETE in the presence of 200 μg ml^{-1} of levonorgestrel and 1000 μg ml^{-1} of norethindrone. The tolerance limit was taken as ±3% change in absorbance, which was not interfered by norethindrone and levonorgestrel.

The proposed method was used to determine ETE in oral contraceptive formulations. Results shown in Table 2 demonstrate that the diazotization-coupling reaction allowed determination of the analyte at VIS region, avoiding interferences of other compounds, including excipients present in the samples. Additionally this reaction increased the absorbance signal and thus the determination sensitivity when compared with direct determination of ETE at UV region. The molar absorptivity was estimated as 1.11×10^4 l mol^{-1} cm^{-1} which is higher than that

Table 2 Determination of ethinylestradiol in pharmaceutical preparations ($n = 3$)[a]

Sample	Labelled contents	Reference method[22]	Proposed method
NEOVLAR®	50	48.7 ± 0.4	49.7 ± 0.5
MICROVLAR®	30	29.4 ± 0.5	29.6 ± 0.5
PRIMOSISTON®	10	10.2 ± 0.3	10.4 ± 0.2

[a] Milligrams per tablet of ETE.

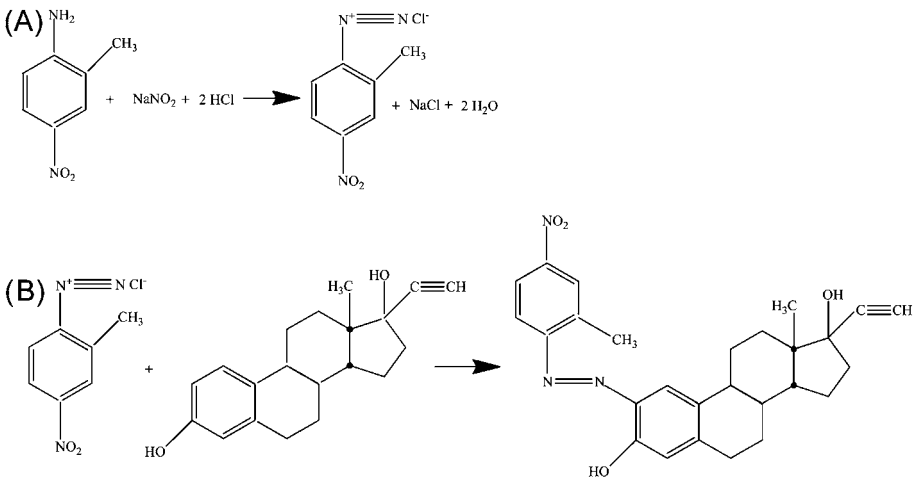

Fig. 1 (A) Diazotization reaction of the 2-methyl-4-nitroaniline. (B) Formation of an azo dye by the coupling reaction between the diazotized 2-methoxy-4-nitroaniline with ethinylestradiol.

obtained by direct measurements of ETE methanolic solution at 230 nm (7.4×10^3 l mol^{-1} cm^{-1}). The results were compared with those labelled for them and the results obtained by the reference method using HPLC.[22] Paired *t*-test (95% confidence level) did not show significant differences.

Conclusions

The proposed spectrophotometric method for determination of ETE is selective, simple, rapid, practical, reliable and inexpensive. The method may be used for routine analysis of oral contraceptive formulations without a time-consuming sample pretreatment prior to the analysis. Furthermore, the procedure has adequate accuracy, and the instrumentation required is not expensive and is easily available.

Acknowledgements

The authors acknowledge the financial support from CNPq and FAPESB.

References

1 E. M. Ghoneim, H. S. El-Desoky and M. M. Ghoneim, *J. Pharm. Biomed. Anal.*, 2006, **40**, 255–261.
2 J. J. Berzas-Nevada, J. Rodríguez-Flores and G. Castañeda-Peñalvo, *Anal. Chim. Acta*, 1997, **340**, 257–265.
3 J. J. Berzas, J. Rodríguez, G. Castañeda and M. J. Pinilla, *Chromatographia*, 1999, **49**, 65–70.
4 T. A. Ternes, H. Andersen, D. Gilberg and M. Bonerz, *Anal. Chem.*, 2002, **74**, 3498–3504.
5 C. Schneider, H. F. Scholer and R. J. Schneider, *Anal. Chim. Acta*, 2005, **551**, 92–97.
6 C. Li, *Bioelectrochemistry*, 2007, **70**, 263–268.
7 L. Mao, C. Sun, H. Zhang, Y. Li and D. Wu, *Anal. Chim. Acta*, 2004, **522**, 241–246.
8 S. Gorog, *Anal. Sci.*, 2004, **20**, 767–782.
9 A. Peepliwal, S. D. Vyawahare and C. G. Bonde, *Anal. Methods*, 2010, **2**, 1756–1763.
10 M. R. H. Nezhad, J. Tashkhourian, J. Khodaveisic and M. R. Khoshi, *Anal. Methods*, 2010, **2**, 1263–1269.
11 M. G. A. Korn, A. C. Ferreira, L. S. G. Teixeira and A. C. S. Costa, *J. Braz. Chem. Soc.*, 1999, **10**, 46–50.
12 J. J. Berzas, J. Rodríguez and G. Castañeda, *Anal. Sci.*, 1997, **13**, 1029–1032.
13 E. Sauri, H. Jalalizadeh, H. Farsam, R. Ghadiri and M. Amanlou, *Chem. Pharm. Bull.*, 2005, **53**, 949–951.
14 J. J. Berzas, F. J. Rodrigues and G. Castañeda, *Analyst*, 1997, **122**, 41–44.
15 F. R. P. Rocha and L. S. G. Teixeira, *Quim. Nova*, 2004, **27**, 807–812.
16 P. Nagaraja, H. S. Yathirajan, H. R. Arunkumar and R. A. Vasantha, *J. Pharm. Biomed. Anal.*, 2002, **29**, 277–282.
17 A. M. Romero, C. G. Benito and J. M. Calatayud, *Anal. Chim. Acta*, 1995, **308**, 451–456.
18 M. R. Herrero, A. M. Romero and J. M. Calatayud, *Talanta*, 1998, **47**, 223.
19 P. Nagaraja, H. R. A. Kumar, R. A. Vasantha and H. S. Yathirajan, *Int. J. Pharm.*, 2002, **325**, 113–120.
20 M. A. Eldawy, A. S. Tawfik and S. R. Elshabouri, *J. Pharm. Sci.*, 1975, **64**, 1221.
21 K. Horita, G. F. Wang and M. Satake, *Analyst*, 1997, **122**, 1569.
22 *The United States Pharmacopeia*, Mack Publishing Co., Easton, PA, 1995, 23rd Rev., pp. 881–883.