DRUG FORMULATIONS AND CLINICAL METHODS

Development and Validation of Spectrophotometric and High-Performance Column Liquid Chromatographic Methods for the Simultaneous Determination of Dienogest and Estradiol Valerate in Pharmaceutical Preparations

MEHMET GÖKHAN ÇAĞLAYAN, ISMAIL MURAT PALABIYIK,[1] and FEYYAZ ONUR
University of Ankara, Faculty of Pharmacy, Department of Analytical Chemistry, Tandogan, Ankara, Turkey

Simultaneous determination of dienogest (DIE) and estradiol valerate (EST) in sugar-coated tablets was performed by using HPLC and spectrophotometry. In HPLC, the separation was achieved on an ACE C8 column using the mobile phase acetonitrile–NH_4NO_3 (0.03 M, pH 5.4; 70 + 30, v/v) at a flow rate of 2 mL/min. The detection wavelength was 280 nm, and cyproterone acetate was selected as an internal standard. The linearity range was 3.0–45.0 g/mL for DIE and 18.0–100.0 g/mL for EST. As spectrophotometric methods, two chemometric methods, principal component regression and partial least-squares, were developed. In the chemometric techniques, the concentration data matrix was prepared by using mixtures containing these drugs in methanol–water (3 + 1, v/v). The absorbance data matrix corresponding to the concentration data matrix in these methods was obtained by the measurement of absorbances in their zero-order spectra; then, the calibration was obtained by using the data matrix for the prediction of unknown concentrations of DIE and EST in their binary mixture. Working ranges were found as 2.0–24.0 g/mL for DIE and 20.0–270.0 g/mL EST in the methods. These three developed methods were validated and successfully applied to a pharmaceutical preparation, a sugar-coated tablet, and the results were compared with each other.

Dienogest (DIE), 17 -cyanomethyl-17 -hydroxy-4,9-estradien-3-one (CAS No. 65928-58-7; Figure 1a), is a novel progestin combining the typical pharmacokinetic and pharmacodynamic properties of the 19-norprogestins with those of progesterone derivatives. It exhibits high receptor selectivity, binding only to the progesterone receptor with strong progestational activity (1, 2). Estradiol valerate (EST), 1,3,5(10)-estratriene-3,17 -diol 17-pentanote (CAS No. 979-32-8; Figure 1b), is used in hormone replacement therapy for treatment of climacteric complaints in menopause (2). The binary combination of DIE and EST is widely used as an effective oral contraceptive.

Determination of the active ingredients in a pharmaceutical preparation becomes more difficult because of the increasing number of components and possible interference effects from excipients in dosage forms. Chemometric techniques (multivariate calibration techniques) in spectrophotometry, based on computer-aided instrumentation and algorithms, are employed for the resolution of mixtures of analytes with overlapped spectra in quantitative determinations. There are a number of available calibration methods using commercial software. Principal component regression (PCR) and partial least-squares (PLS) have some advantages over other multivariate calibration techniques, such as using the whole spectrum for the simultaneous analysis of the mixture component to minimize the effects of noise peaks. Furthermore, these methods, as in other chemometric techniques, also provide shorter analysis time, lower cost, and use of simple instrumentation in an analysis without the need for chemical pretreatment.

A literature survey revealed that several methods were used for the determination of DIE in pharmaceutical preparations or in biological fluids, including HPLC (3), radioimmunoassay (4, 5), and GC (6). Several methods have also been described for the determination of EST, such as GC (6), spectrophotometry (7–9), HPLC (10–13), TLC (14), electrophoresis (15), and electrochemistry (16–18). Only a GC method was reported for the simultaneous determination of DIE and EST (6), and it was used for the analysis of these drugs in biological fluids. To our knowledge, there have been no reports of the simultaneous determination of DIE and EST in pharmaceutical preparations.

The aim of this study was to develop spectrophotometric methods using the chemometric techniques PCR and PLS and an HPLC method that allow the simultaneous determination of DIE and EST in pharmaceutical preparations.

Experimental

Apparatus

A Shimadzu (Columbia, MD) 1601 PC double-beam spectrophotometer with a fixed slit width (2 nm) connected to

Received November 8, 2008. Accepted by SW February 9, 2009.
[1] Corresponding author's e-mail: mpala@pharmacy.ankara.edu.tr

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

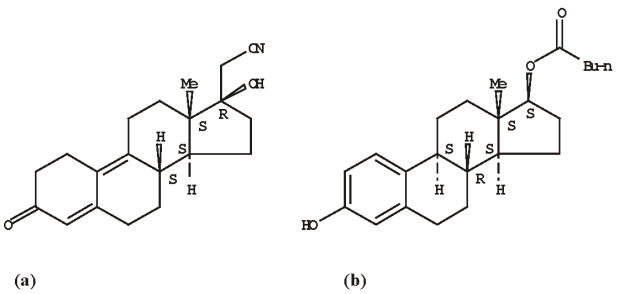


Figure 1. The structures of (a) DIE and (b) EST.

a computer loaded with Shimadzu UVPC software was used for the spectrophotometric measurements. Standard quartz cuvets (10 mm) were used for absorbance measurements.

An Agilent Technologies HP 1100 system (Wilmington, DE), equipped with a G1379A degasser, G1311A quaternary pump, 61313A autoinjector, and G1315B diode array detector was used. An ACE octylsilyl bonded (C8) column (Advance Chromatography Technologies, Aberdeen, Scotland), 250 × 4.6 mm, 5 m particle size, was used. The chromatograms were recorded and the peaks were quantified using an automatic integrator.

In the chemometric procedures, Multivariate Analysis Add-in for Excel v1.3 software (19) was used.

Materials and Reagents

DIE, EST, and cyproterone acetate internal standard (IS) were kindly donated by Schering Alman Ilaç ve Ecza Tic. Ltd Sti (Istanbul, Turkey). Pharmaceutical preparation containing DIE and EST (Climodien, Batch No. 71112 E), 2 mg DIE and 2 mg EST in a sugar-coated tablet was obtained from local pharmacies. HPLC grade acetonitrile and methanol were purchased from LabScan, Analytical Sciences (Dublin, Ireland) and NH_4NO_3 from Merck Inc. (Darmstadt, Germany). Water for preparation of solutions was purified by a Purelab-UHQ (Elga Labwater, Marlow, UK) water purification system. The mobile phase and the injection solution were degassed in an ultrasonic bath and filtered through a 0.45 m nylon membrane before use.

Standard Solutions

Solutions of DIE (200 g/mL) and EST (500 g/mL) were prepared in methanol–water (3 + 1, v/v) for spectrophotometric measurements. For HPLC, solutions of DIE (100 g/mL), EST (250 g/mL), and cyproterone acetate (50 g/mL) IS solution were prepared in acetonitrile. Various aliquots of standard solutions were then diluted to 25 mL with methanol–water (3 + 1, v/v) for spectrophotometric measurements and with acetonitrile for HPLC method to give the final analyte concentrations desired.

Sample Preparation

(**a**) *For spectrophotometry*.—The contents of six sugar-coated tablets were accurately weighed and powdered in a mortar. A weight equivalent to one sugar-coated tablet was dissolved in 10 mL methanol–water (3 + 1, v/v) in a 25 mL volumetric flask and diluted to volume with the same solvent. After 30 min of mechanical shaking, the solution was filtered through 0.45 m Whatman No. 42 filter paper into a 25 mL volumetric flask. Then 6.25 mL of this filtrate was

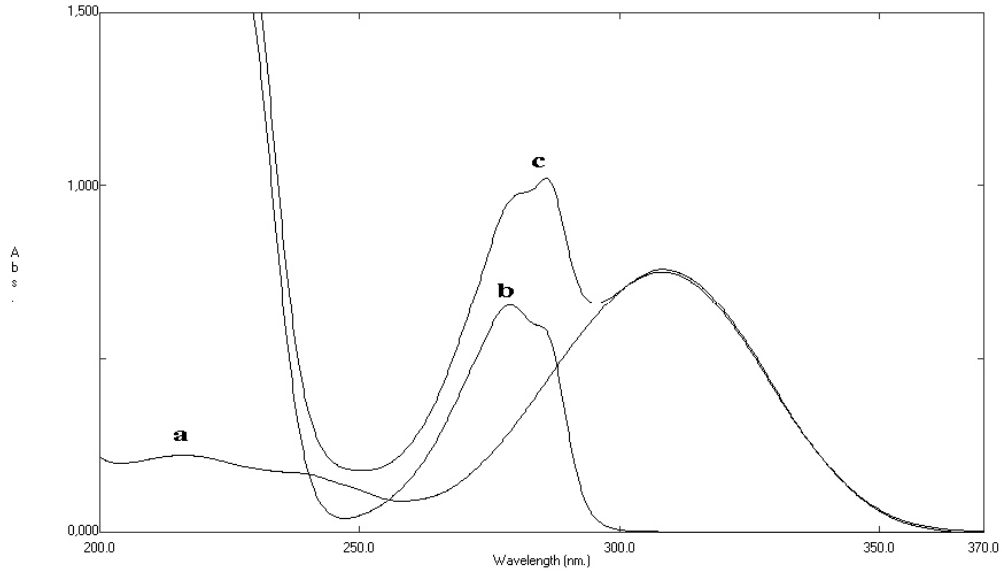


Figure 2. Zero-order absorption spectra of (a) 12 g/mL solution of DIE, (b) 120 g/mL solution of EST, and (c) solution containing 12 g/mL DIE and 120 g/mL EST in methanol–water (3 + 1, v/v).

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

Table 1. Training sets used in the PCR and PLS methods for DIE and EST in their mixtures

Mixture No.	DIE, g/mL	EST, g/mL
1	16.0	120.0
2	2.0	120.0
3	2.0	20.0
4	24.0	20.0
5	8.0	270.0
6	24.0	60.0
7	16.0	270.0
8	8.0	120.0
9	8.0	60.0
10	20.0	60.0
11	24.0	200.0
12	20.0	270.0
13	16.0	200.0
14	24.0	120.0
15	24.0	270.0
16	2.0	270.0
17	20.0	20.0
18	2.0	200.0
19	16.0	20.0
20	20.0	120.0
21	20.0	200.0
22	8.0	200.0
23	2.0	60.0
24	8.0	20.0
25	16.0	60.0

diluted with methanol–water (3 + 1, v/v) to volume in a 25 mL volumetric flask. The developed spectrophotometric methods were applied to this final solution.

(**b**) *For HPLC*.—The contents of six sugar-coated tablets were accurately weighed and powdered in a mortar. A weight equivalent to one sugar-coated tablet was dissolved in 10 mL acetonitrile in a 25 mL volumetric flask, and after addition of 0.625 mL cyproterone acetate standard solution, diluted to volume with the same solvent. After 30 min of mechanical shaking, the solution was filtered through 0.45 m Whatman No. 42 filter paper into a 25 mL volumetric flask. Then 10 mL of this filtrate was diluted with acetonitrile to volume in a 25 mL volumetric flask. This final solution was injected into the chromatographic system.

Results and Discussion

PCR and PLS Spectrophotometric Methods

The zero-order absorption spectra for DIE and EST in methanol–water (3 + 1, v/v) are illustrated in Figure 2. The spectra of the two components were overlapped in the range 200–300 nm. Therefore, it was not possible to simultaneously determine DIE and EST in their binary mixtures in this spectral range using classical spectrophotometry. PCR and PLS techniques were employed for the simultaneous determination of these components in their binary combination using the zero-order absorption spectra of their mixture without any prior separation technique. For these methods, a training set was established first with 25 mixture solutions in methanol containing different concentrations of these active ingredients (Table 1). Then, optimal conditions for this technique were investigated, and the absorbance data matrix corresponding to the training set mentioned above was obtained by the absorbance values measured at 51 wavelengths with = 1 nm between

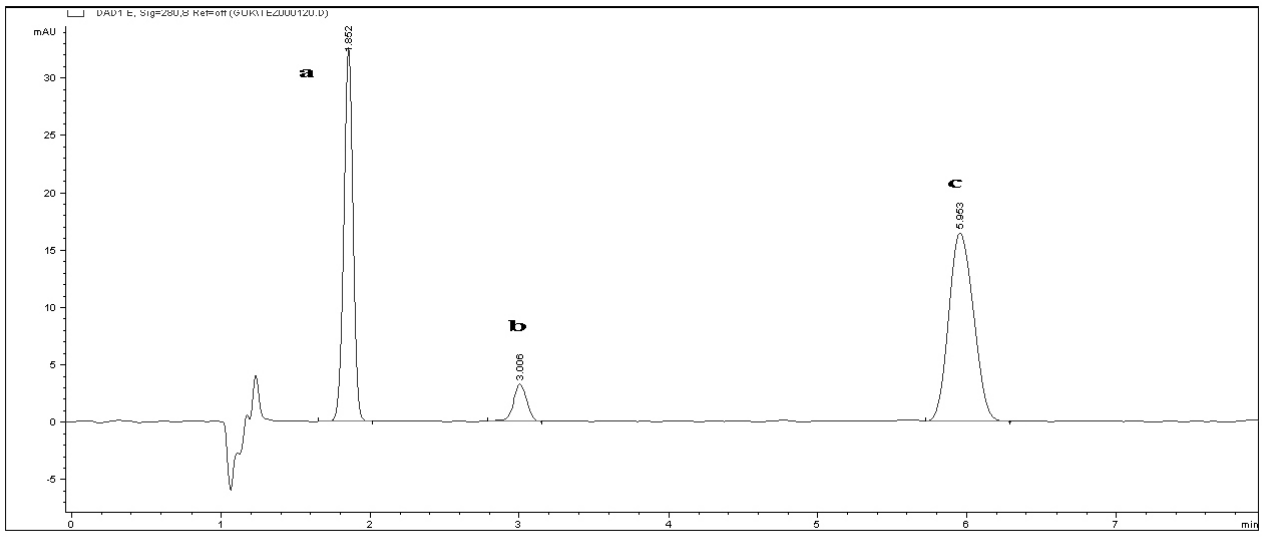


Figure 3. Typical chromatogram of a solution containing (a) 8 g/mL DIE, (b) 1 g/mL cyproterone acetate (IS), and (c) 64 g/mL EST in acetonitrile.

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

Table 2. Linearity parameters and system suitability test results for DIE and EST in the HPLC method

Parameter	DIE	EST
Linearity range, g/mL	3.0–45.0	18.0–100.0
Slope of the calibration curve ± SE[a]	0.794 ± 0.0010	0.1413 ± 0.0003
Intercept of the calibration curve ± SE[a]	–0.0052 ± 0.014	0.0774 ± 0.025
r	0.999	0.999
Separation factor ( )	6.178	6.178
Resolution (R)	11.54	19.64
Asymmetry (A_s)	1.00	1.01
Theoretical plates (N)	5487	14175

[a] SE = Standard error.

300–351 nm for DIE and at 41 wavelengths with = 1 nm between 260–300 nm for EST in their binary mixtures. We observed that good results were obtained by using mean-centered data for these drugs in calculation procedures. Working ranges were found to be 2.0–24.0 g/mL for DIE and 20.0–270.0 g/mL for EST. The LOD was found to be 0.2 g/mL for DIE and 2.5 g/mL for EST; the LOQ was 2.0 g/mL for DIE and 20.0 g/mL for EST in both of the methods.

To select the number of components for modeling the system without overfitting the concentration data in the PCR and PLS algorithm, a cross-validation method, leaving out one sample at a time, was employed using training data sets. For DIE, two principal components in PCR and one principal component in PLS were found to be optimal for the determination while five principal components in PCR and three principal components in PLS were suitable for the determination of EST. In these conditions, we observed that the root mean squares value was minimum. Multivariate Analysis Add-in for Excel v1.3 software was used for all of the calculations described above (19).

Liquid Chromatographic Method

A pharmacopeia survey revealed that there is one official method for determination of EST by HPLC (20), and method development and optimization studies were realized based on this method. During method development and optimization studies, the effect of the NH_4NO_3 solution's pH in the mobile phase from 4.8 to 6.0 was investigated, and NH_4NO_3, NH_4Cl, and $CH_3COO\ NH_4$ solution in mobile phase systems were tested to achieve optimum separation conditions. The NH_4NO_3–acetonitrile system was selected. Following this, concentration and pH of NH_4NO_3 and percentage of acetonitrile in the mobile phase were optimized for the separation on an ACE C8 column, and acetonitrile–NH_4NO_3 (0.03 M, pH = 5.4; 70 + 30, v/v) was found to be the optimal mobile phase for good separation. The flow rate, column temperature, and injection volume were optimized as 2.0 mL/min, 25 C, and 20 L, respectively. Cyproterone acetate could be successfully separated from the other compounds, so it was used as the IS in this study. The detection wavelength was selected as 280 nm.

Under these chromatographic conditions, DIE, cyproterone acetate (IS), and EST peaks were well-resolved, and their retention times were found to be 1.852, 3.006, and 5.953 min, respectively. A typical chromatogram of the drugs and IS is illustrated in Figure 3. System suitability tests including separation factor, resolution, asymmetry, and theoretical plates were carried out; the results are shown in Table 2.

The calibration curves were established with different concentrations in the range of 3.0–45.0 g/mL for DIE and 18.0–100.0 g/mL for EST. Each solution was injected three times, and the areas of peaks, measured at 280 nm, were integrated. The ratios of the peak areas of the investigated substances to that of the IS peak area were calculated for each injection. Regression equations were established by plotting the ratio of peak areas versus the concentration of each substance. The linearity was evaluated by linear regression analysis, which was calculated by least-squares regression. The results were shown in Table 2.

LOQ values were calculated (21) as 0.18 g/mL for DIE and 1.76 g/mL for EST, according to the equation:

$$\mathrm{LOQ} = 10(\mathrm{SD}/m)$$

where SD = standard deviation of the intercept and m = slope of the calibration curve. LOD values were calculated as 0.06 g/mL for DIE and 0.58 g/mL EST, according to the equation:

$$\mathrm{LOD} = 3.3(\mathrm{SD}/m)$$

Accuracy and Precision

Accuracy and precision were studied using three different solutions containing 8.0, 12.0, and 20.0 g/mL for DIE and 90.0, 120.0, and 150.0 g/mL for EST in PCR and PLS, and 8.0, 16.0, and 32.0 g/mL for DIE and 24.0, 48.0, and 72.0 g/mL for EST in HPLC. These solutions were analyzed on the same day (intraday) and on 3 consecutive days (interday). Table 3 shows the results obtained for intraday and

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

Table 3. Accuracy and precision data for DIE and EST

Added, g/mL	Intraday Found, g/mL[a]	Intraday Precision (RSD, %)	Intraday Accuracy (bias, %)[b]	Interday Found, g/mL[a]	Interday Precision (RSD, %)	Interday Accuracy (bias, %)[b]
			PCR			
DIE						
8.00	8.04 ± 0.00	0.03	0.47	8.04 ± 0.02	0.64	0.51
12.00	11.90 ± 0.01	0.19	–0.86	11.91 ± 0.01	0.27	0.79
20.00	20.01 ± 0.03	0.28	0.03	20.02 ± 0.02	0.22	0.11
EST						
90.00	87.77 ± 0.17	0.33	–2.48	88.15 ± 0.14	0.49	–2.05
120.00	121.09 ± 0.35	0.51	0.91	121.37 ± 0.18	0.45	1.13
150.00	152.65 ± 0.11	0.12	1.77	152.54 ± 0.21	0.42	1.69
			PLS			
DIE						
8.00	8.01 ± 0.03	0.66	0.16	8.00 ± 0.02	0.76	–0.01
12.00	11.88 ± 0.01	0.14	–1.00	11.91 ± 0.01	0.36	–0.76
20.00	19.94 ± 0.02	0.13	–0.32	20.03 ± 0.02	0.30	0.14
EST						
90.00	87.78 ± 0.08	0.15	–2.46	87.94 ± 0.12	0.42	–2.28
120.00	121.34 ± 0.18	0.26	1.11	121.63 ± 0.14	0.42	1.36
150.00	152.33 ± 0.17	0.19	1.56	152.59 ± 0.19	0.36	1.73
			LC			
DIE						
8.00	8.09 ± 0.03	0.73	1.09	8.18 ± 0.03	0.90	2.19
16.00	16.23 ± 0.02	0.20	1.43	16.37 ± 0.04	0.73	2.34
32.00	32.25 ± 0.05	0.25	0.77	32.50 ± 0.07	0.64	1.55
EST						
24.00	24.37 ± 0.19	1.35	1.52	24.15 ± 0.08	1.05	0.61
48.00	47.82 ± 0.13	0.47	–0.38	48.09 ± 0.11	0.67	0.19
72.00	71.88 ± 0.19	0.46	–0.17	71.90 ± 0.07	0.31	–0.14

[a] Mean ± standard error.

[b] Bias, % = [(found – added)/added] x 100.

interday accuracy and precision. The low RSD values and high recovery values indicate good precision and accuracy of the method, respectively.

The accuracy of the proposed methods was also tested by recovery experiments. According to official validation guidelines (21) in cases where it is impossible to obtain samples of all drug product components, it may be acceptable to add known quantities of the analyte to the drug product for determining recovery. For this reason, in order to know whether the excipients in the pharmaceutical preparation showed any interference with the analysis, the recovery test was done by the standard addition method. The recoveries obtained after three repeated experiments are summarized in Table 4. Recovery values were close to 100%, indicating that there was no interference from the excipients in the sugar-coated tablet.

Analysis of Pharmaceutical Preparations

Sample solutions were prepared according to the *Sample Preparation* method above, and the developed methods were applied for the simultaneous determination of DIE and EST in pharmaceutical preparations. Results that were found to be in agreement with the label claims were obtained for each compound (Table 5). The results obtained by the methods described above were compared with each other statistically, and no significant difference was observed for the amount of drugs using Student's *t*-test and the Fisher *F*-test at the $P = 0.05$ level in the commercial formulations (Table 5).

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

Table 4. Recovery results for DIE and EST

	DIE			EST		
Parameter	PCR	PLS	HPLC	PCR	PLS	HPLC
Mean ± SE[a]	103.17 ± 0.49	102.98 ± 0.49	100.71 ± 0.38	102.18 ± 0.50	102.05 ± 0.51	99.97 ± 0.58
RSD	1.51	1.50	1.19	1.54	1.58	1.85

[a] SE = Standard error.

Table 5. Assay results for commercial preparations (Climodien sugar-coated tablet containing 2 mg DIE and 2 mg EST)

	DIE			EST		
Parameter	PCR	PLS	LC	PCR	PLS	LC
Mean ± SE	2.06 ± 0.00	2.06 ± 0.01	2.05 ± 0.01	2.01 ± 0.02	2.00 ± 0.02	1.91 ± 0.01
RSD, %	0.50	0.60	0.60	0.70	0.08	1.35
Bias, %	3.16	3.17	0.74	2.31	3.09	–1.67
	DIE			EST		
Test	PCR-PLS	HPLC-PCR	HPLC-PLS	PCR-PLS	HPLC-PCR	HPLC-PLS
t-Test[a]	0.02	0.10	2.11	0.48	2.16	1.72
F-test[b]	1.42	2.12	1.49	1.23	3.08	2.50

[a] Theoretical value for t at $P = 0.05$ level = 2.26.
[b] Theoretical value for F at $P = 0.05$ level = 5.05.

Conclusions

In this study, an HPLC method and PCR and PLS methods as chemometric techniques in spectrophotometry could be applied with great success for the simultaneous determination of DIE and EST in binary mixtures and in a sugar-coated tablet pharmaceutical formulation. Spectrophotometric methods described in this work do not require any prior separation procedure. Satisfactory results were obtained by these methods, but the PCR and PLS methods need software for mathematical calculations. Using only zero-order spectra in the procedures, without any other graphical mode, such as derivative and ratio spectra derivative, is one of the advantages of the chemometric methods. Without any time-consuming sample preparation procedures and using only methanol–water (3 + 1, v/v) as the solvent, the spectrophotometric methods proposed in this article are easier and cheaper when compared with the HPLC method. Shorter running time (about 6 min) for the simultaneous determination of DIE and EST is the advantage of the HPLC method. All of the statistical values were within acceptable limits. Statistically, no significant difference was observed for the amount of drugs found with the three methods when compared to each other by the *t*-test and *F*-test at the $P = 0.05$ level for the commercial formulation. These developed methods were proven to be sensitive, accurate, and precise for application in routine analysis of these drugs in pharmaceutical formulations.

References

(1) Sitruk-Ware, R. (2004) *Maturitas* **47**, 277–283

(2) Gräser, T., Müler, A., Mellinger, U., Mück, A.O., Lippert, T.H., & Oettel, M. (2000) *Maturitas* **35**, 253–261

(3) Wehrberger, K., Ritter, P., Hillesheim, H.G., & Hobe, G. (1985) *Anal. Chem. Symp. Ser.* **23**, 511–518

(4) Khac Hieu, D., Wehrberger, K., & Hobe, G. (1986) *Pharmazie* **41**, 711–714

(5) Hobe, G., Klinger, G., Reddersen, G., Erdmann, A., & Klinger, G. (1986) *Pharmazie* **41**, 772–774

(6) Zimmerman, H., Thebault, J.J., Duvauchelle, T., Mignot, A., Renoux, A., & Gualano, V. (2000) *Clin. Drug Invest*. **20**, 123–134

(7) Dinc, E., Yücesoy, C., Palabıyık, İ.M., Üstündağ, Ö., & Onur, F. (2003) *J. Pharm. Biomed. Anal.* **32**, 539–547

(8) Yücesoy, C. (2000) *Ankara Üniversitesi Eczacılık Fakültesi Dergisi* **29**, 9–19

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021

(9) Yücesoy, C., & Erol, S. (2000) *FABAD J. Pharm. Sci.* **25**, 85–90

(10) Carignan, G.B., Lodge, A., & Skakum, W. (1984) *J. Chromatogr. A* **301**, 292–296

(11) Carignan, G.B., Lodge, A., & Skakum, W. (1980) *Can. J. Pharm. Sci.* **15**, 10–12

(12) Segall, A., Hormaechea, F., Vitale, M., Perez, V., & Pizzorno, M.T. (1999) *J. Pharm. Biomed. Anal.* **19**, 803–808

(13) Li-Xia, J., Zai-Jie, W., & Matlin, S.A. (1990) *J. Liq. Chromatogr. Relat. Technol.* **13**, 3473–3479

(14) Amin, M. (1989) *Pharm. Ind*. **51**, 109–112

(15) Tripodi, V., Flor, S., Carlucci, A., & Lucangioli, S.D. (2006) *Electrophoresis* **27**, 4431–4438

(16) Duan, J.P., Chen, G.N., Chen, M.L., Wu, X.P., & Chen, H.Q. (1999) *Analyst* **124**, 1651–1655

(17) Yan-yi, S., Kang-bing, W., & Sheng-shui, H. (2002) *Wuhan Daxue Xuebao* **48**, 685–688

(18) Wen-sheng, H., Chun-hai, Y., & Sheng-hui, Z. (2002) *Hubei Minzu Xueyuan Xuebao* **20**, 52–54

(19) Brereton, R.G. (2002) *Chemometrics, Data Analysis for The Laboratory and Chemical Plant*, John Wiley & Sons, New York, NY, pp 449–457

(20) U.S. Pharmacopeia 23 National Formulary 18 (1995) U.S. Pharmacopeial Convention, Rockville, MD, pp 625–626

(21) *ICH Harmonized Tripartite Guideline, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Validation of Analytical Procedure: Methodology (1996) International Conference on Harmonization, Geneva, Switzerland

Downloaded from https://academic.oup.com/jaoac/article/93/3/862/5655647 by ANKARA UNIVERSITY user on 11 December 2021