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Direct Quantitative Thin-layer Chromatographic Determination of Levonorgestrel and Ethinyloestradiol in Oral Contraceptives by Diffuse Reflection and Fluorescence Methods

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Two thin-layer chromatographic methods are described for the determination of levonorgestrel (D-norgestrel) and ethinyloestradiol in oral contraceptives. Development was carried out on both silica gel 60 F_{254} pre-coated commercial plates and high-performance thin-layer chromatography silica gel 60 F_{254} pre-coated commercial plates. By this means the two substances can be determined together directly, by means of a chromatogram - spectrophotometer, using either the diffuse reflection or the fluorescence method.

In the diffuse reflection method, the levonorgestrel spots are measured in the ultraviolet range on the silica gel plate at 248 nm. The ethinyloestradiol spots can be measured only after the plate has been sprayed with 5% methanolic sulphuric acid and after heating for 10 min at 120 °C (red spots appear in the visible range at 530 nm).

Using the fluorescence method, the spots are sprayed separately on a silica gel plate with 0.5% methanolic sulphuric acid and heated at 120 °C for 10 min. Fluorescent spots appear for both substances, which are excited at a wavelength of 365 nm and measured at $\lambda_{max.}$ 485 nm for levonorgestrel and 560 nm for ethinyloestradiol.

These methods for the determination are suitable for control and stability tests and for tests on content uniformity of these substances in drug forms. They are rapid and specific, and can be readily reproduced. The relative standard deviation for the diffuse reflection method is 0.44–2.6% and for the fluorescence method 3.4–5.2%, depending on the type of plates used.

Keywords: *Levonorgestrel determination*; *ethinyloestradiol determination*; *thin-layer chromatography*; *diffuse reflection*; *fluorescence*

There are several descriptions in the literature[1–11] of the determination of levonorgestrel and ethinyloestradiol, very small amounts of which form the active substances in preparations for hormone contraception. The methods described, which include the use of radioactively labelled derivatives,[1,2] dansyl or other fluorescent derivatives,[3–5] spectrophotometry or photometry[6–9] or gel or column chromatography,[10,11] are complicated. This paper describes an attempt to find a less complicated, rapid, specific and reproducible method of analysis for these two substances.

Experimental

Substances and Preparations Investigated

Levonorgestrel (D-*norgestrel*) (13-*ethyl*-17α-*ethinyl*-17β-*hydroxy*-4-*gonen*-3-*one*).

Ethinyloestradiol [1,3,5(10)-*oestratrien*-17α-*ethinyl*-3,17β-*diol*].

Preparation A. Dragées containing 250 μg of levonorgestrel and 50 μg of ethinyloestradiol per dragée.

Preparation B. Dragées containing 30 μg of levonorgestrel per dragée.

Preparation of Solutions

One dragée of each preparation is pulverised and shaken for about 20 min with chloroform - methanol (1 + 1), then the solution is separated from the undissolved components by filtration. The undissolved components are shaken twice with chloroform - methanol (1 + 1) and the solution is filtered. The combined filtrates are concentrated in a rotary evaporator

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under a vacuum at 30 °C, and the residue is dissolved in 1 ml of chloroform - methanol (1 + 1). The concentrations of the substances in the solutions are therefore 250 or 30 μg ml^{-1} for levonorgestrel and 50 μg ml^{-1} for ethinyloestradiol.

With the fluorescence method on a high-performance thin-layer chromatographic (HPTLC) pre-coated plate the residue is dissolved in 5 ml of chloroform - methanol (1 + 1). The concentrations of the substances in the solutions are then 6 μg ml^{-1} for levonorgestrel and 10 μg ml^{-1} for ethinyloestradiol.

In our laboratory, the extraction of the active ingredients from the formulated products is conducted completely automatically in an electronically controlled extraction apparatus.[12]

The reference solutions are prepared from pure substances in such a way that their concentrations are similar to those of the test solutions.

Thin-layer Chromatography

Silica gel 60 F_{254} pre-coated commercial plates (size 20 × 20 cm and layer thickness 0.25 mm) and HPTLC silica gel 60 F_{254} pre-coated commercial plates (size 10 × 10 cm) (both from E. Merck, Darmstadt) were used. There is virtually no difference in performance between fluorescent and non-fluorescent plates, but the former have the advantage of the visibility of the spots in ultraviolet light. The plates were divided into strips 1.5 cm wide and solute spots were applied using 1.2- and 5-μl micropipettes. In order to establish the calibration graphs for the diffuse reflection method, amounts between 1 and 5 μg were applied to the standard plates and amounts between 50 and 250 ng to the HPTLC plates. For the fluorescence method, amounts between 10 and 250 ng per spot were applied. After saturation of the atmosphere in the chambers, cyclohexane - diethyl ether - acetic acid (50 + 50 + 1) was used as the developing solvent. The length of the run was 2 × 20 cm on the standard plates and 2 × 10 cm on the HPTLC plates. Development took 60 min with the standard plates and 10 min with the HPTLC plates. Determinations of the contents of the active ingredients in the preparations investigated and the reproducibility of the methods of determination were checked with 11 or 6 dragées, with 11 or 6 similiar applications on each standard or HPTLC plate, respectively. The content uniformity of the substances was checked on the basis of investigations of 40 different doses.

Measurements and Evaluation

The levonorgestrel and ethinyloestradiol spots are measured directly on the silica gel plate using a PMQ II chromatogram - spectrophotometer (Zeiss, Oberkochen, Germany) by either the diffuse reflection or the fluorescence method.

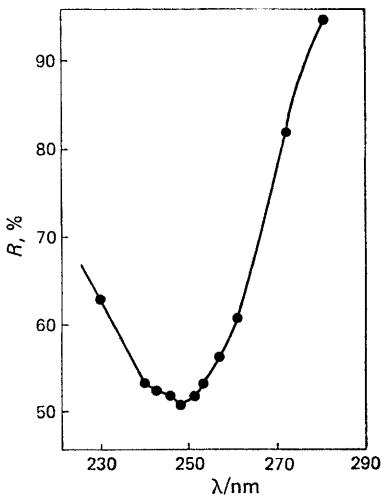

Fig. 1. Diffuse reflection spectrum of 5 μg of levonorgestrel measured on the silica gel plate. $\lambda_{max.}$ = 248 nm.

Diffuse reflection method

Levonorgestrel is measured directly in the ultraviolet range at 248 nm. Ethinyloestradiol is measured in the visible range at 530 nm, after spraying the silica gel plate with 5% methanolic sulphuric acid and heating in an oven for 10 min at 120 °C. The spots appear red on a white background. The quantitative evaluation is carried out either on the basis of calibration graphs applied to the same silica gel plate, or by comparison with the degree of reflection of a reference spot of known concentration.[13]

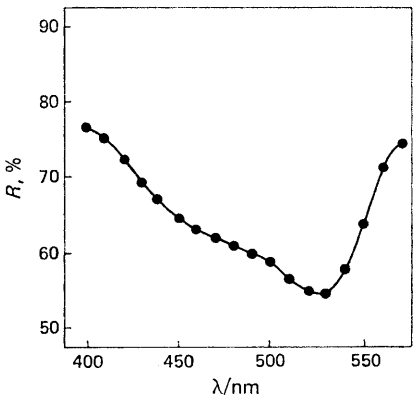

Fig. 2. Diffuse reflection spectrum of 5 μg of ethinyloestradiol measured on the silica gel plate, after reaction with 0.5% methanolic sulphuric acid and heating at 120 °C. $\lambda_{max.}$ = 530 nm.

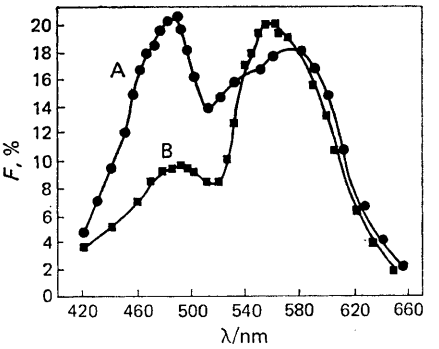

Fig. 3. Emission spectrum of (A) 300 ng of levonoregestrel ($\lambda_{max.}$ = 485 nm) and (B) 300 ng of ethinyloestradiol ($\lambda_{max.}$ = 560 nm) measured on the silica gel plate. Excitation wavelength, 365 nm.

Fluorescence method

Both levonorgestrel and ethinyloestradiol are converted into fluorescent spots after the plate has been sprayed with 0.5% methanolic sulphuric acid and heated for 10 min at 120 °C. They are determined with an excitation wavelength of 365 nm and an emission wavelength of 485 nm for levonorgestrel and 560 nm for ethinyloestradiol. The quantitative evaluation has been described earlier.[14]

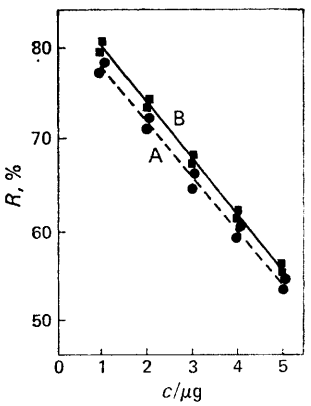

Fig. 4. Diffuse reflection calibration graph of (A) levonorgestrel ($\lambda_{max.}$ = 248 nm) and (B) ethinyloestradiol (λ_{max} = 530 nm) measured on the silica gel plate.

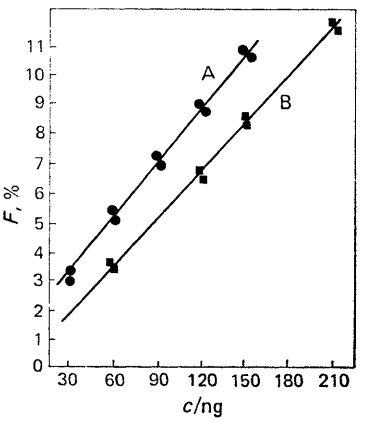

Fig. 5. Emission calibration graph of (A) levonorgestrel ($\lambda_{max.}$ = 485 nm) and (B) ethinyloestradiol ($\lambda_{max.}$ = 560 nm) measured on the silica gel plate. Excitation wavelength = 365 nm.

Results and Discussion

Quantitative Determination

The diffuse reflection and emission spectra and the calibration graphs for levonorgestrel and ethinyloestradiol are shown in Figs. 1–3. The linear relationship between the concentration and the relative reflection or emission can be seen in Figs. 4 and 5. The calibration graphs were measured in the range 1–5 μg (Fig. 4) and 30–210 ng (Fig. 5). For the determination of smaller amounts (as in Table III), calibration graphs are required for lower ranges (50–500 ng) for the diffuse reflection method and (5–30 ng) for the fluorescence method. The linearity of both compounds by reflection and fluorescence is also satisfactory in this range.

Fig. 6 shows the separated spots of the two substances from formulated products on the silica gel plate. Tables I and II summarise the results of the determination of the active substances in the formulated products and their statistical data. Table III gives the limits of determination of the substances investigated.

TABLE I

RESULTS OF THE DETERMINATION OF ACTIVE SUBSTANCE CONTENT IN PREPARATIONS A AND B ON SILICA GEL 60 F_{254} PRE-COATED COMMERCIAL PLATES

Values from 11 measurements.

Parameter	Diffuse reflection method			Fluorescence method		
	Preparation A		Preparation B: levonorgestrel	Preparation A		Preparation B: levonorgestrel
	Levonorgestrel	Ethinyloestradiol		Levonorgestrel	Ethinyloestradiol	
Amount applied per spot/μg	5	5	3	0.140	0.150	0.150
Arithmetic mean of the degree of reflection or emission ($\bar{X}$), with approximate amount (μg) per dragée in parentheses ..	49.9 (250.0)	54.7 (49.5)	68.6 (29.6)	6.1 (252.0)	6.2 (51.1)	6.8 (31.0)
Standard deviation of the single values (SD)/μg ..	1.08	1.44	1.09	0.26	0.32	0.23
Coefficient of variation (CV), %	2.16	2.63	1.59	4.26	5.16	3.38

Considering Table II, with diffuse reflection measurements it should be noted that better results can be obtained if the calculation of the coefficient of variation is based on data from HPTLC plates rather than from standard plates. The reason for this might be explained by (a) the thinner and, therefore, more uniform silica gel layers of the HPTLC plates and/or (b) the shorter development time (10 min), which causes less diffusion of the spots on the plates. With the fluorescence method there is no difference in performance between the two types of plates, presumably because the amounts applied are very small.

TABLE II

RESULTS OF THE DETERMINATION OF ACTIVE SUBSTANCE CONTENT IN THE PREPARATIONS A AND B ON HPTLC SILICA GEL 60 F_{254} PRE-COATED COMMERCIAL PLATES

Values from 6 measurements.

Parameter	Diffuse reflection method			Fluorescence method		
	Preparation A		Preparation B: levonorgestrel	Preparation A		Preparation B: levonorgestrel
	Levonorgestrel	Ethinyloestradiol		Levonorgestrel	Ethinyloestradiol	
Amount applied per spot/μg	0.250	0.250	0.250	0.050	0.025	0.050
Arithmetic mean of the degree of reflection or emission ($\bar{X}$), with approximate amount (μg) per dragée in parentheses	94.1 (249.5)	90.1 (48.9)	92.8 (28.9)	4.8 (250.5)	5.6 (50.5)	4.7 (30.6)
Standard deviation of the single values (SD)/μg ..	0.41	0.66	0.71	0.25	0.26	0.22
Coefficient of variation (CV), %	0.44	0.73	0.77	5.21	4.64	4.68

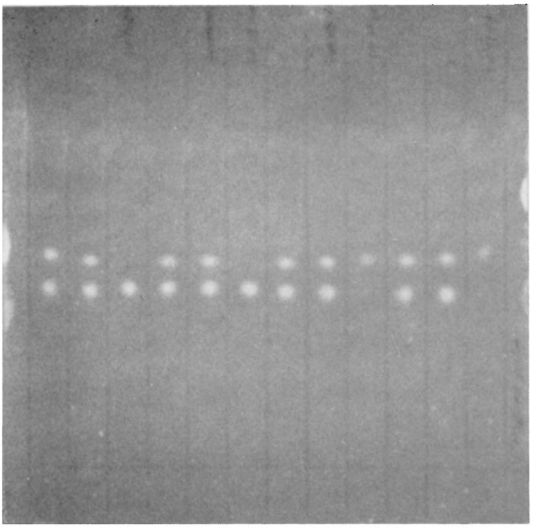

Fig. 6. Thin-layer chromatogram of preparation B. Bands 1, 2, 4, 5, 7, 8, 10 and 11: test solutions each containing 300 ng of levonorgestrel and 60 ng of ethinyloestradiol. Bands 3 and 6: reference solution with 300 ng of levonorgestrel. Bands 9 and 12: reference solution with 60 ng of ethinyloestradiol. Solvent: cyclohexane - diethyl ether - acetic acid (50 + 50 + 1).

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Content Uniformity and Statistical Analysis

Results for the content uniformity of preparation A, determined by the fluorescence method, are given in Table IV.

TABLE III

LIMITS OF DETERMINATION OF ETHINYLOESTRADIOL AND LEVONORGESTREL ON SILICA GEL 60 F_{254} PRE-COATED PLATES AND HPTLC PLATES BY THE DIFFUSE REFLECTION AND FLUORESCENCE METHODS

Quantitative determination is not recommended below the limits given, as the ratio of interference to signal is greater than 1:10.

	Diffuse reflection method		Fluorescence method	
Steroid	Silica gel 60 F_{254} plates	HPTLC silica gel 60 F_{254} plates	Silica gel 60 F_{254} plates	HPTLC silica gel 60 F_{254} plates
Levonorgestrel/μg	0.100	0.060	0.012	0.006
Ethinyloestradiol/μg ..	0.175	0.120	0.025	0.007

TABLE IV

CONTENT UNIFORMITY OF DRAGÉES BY THE FLUORESCENCE METHOD

	Levonorgestrel	Ethinyloestradiol
No. of determinations	40	40
Range, % of theory	95–105	95–105
Mean recovery, %	99.02	98.50
Standard deviation, %	2.79	2.85
Coefficient of variation, %	2.81	2.90
95% confidence limits, %	92.9–105.2	92.4–104.7
99% confidence limits, %	90.5–107.6	90.0–107.0

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