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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF STEROID HORMONES IN ORAL CONTRACEPTIVES

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SUMMARY

Reversed-phase high-performance liquid chromatography with UV detection is studied for the determination of both progestogenic and oestrogenic components of oral contraceptive formulations. The applicability of the assay is demonstrated for a number of different progestogen–oestrogen combinations in both conventional tablet and novel "paper" formulations. The results show that the method developed is a versatile technique for the routine assay of these pharmaceutical formulations.

INTRODUCTION

Oral contraceptive preparations that are commonly prescribed at the present time contain a combination of two different steroid hormones as the "active ingredients", they are a progestogen such as norgestrel and an oestrogen such as ethynyl oestradiol. The progestogen–oestrogen formulations available not only differ in the type of steroid hormone but also in the ratio and amount of steroid hormone. Generally the combination will vary between 50–1000 μg of progestogen and 30–50 μg of oestrogen. This potentially disproportionate ratio may pose a non-trivial problem to the analyst seeking a simple rapid method for simultaneously determining both steroid hormones in these pharmaceutical preparations.

Previously published methods of determining the oestrogenic component have relied upon time consuming derivatisations for gas–liquid chromatographic and colorimetric determinations[1]. Fluorometric analysis has also been utilised both in natural samples and after reaction with sulphuric acid–methanol reagents[2,3]. Derivatisation procedures and natural fluorescence methods of analysis are limited however in combined progestogen–oestrogen formulations because of the interference from the relatively large excess of progestogen[4].

High-performance liquid chromatography (HPLC) with a suitable detection mode would seem to be an ideal approach to the problem of mutual interference by

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the steroid hormones in the analysis of oral contraceptives. The HPLC of steroid hormones has been recently reviewed[5] and many applications of this technique to the determination of these types of compounds has appeared in the literature[6]. Bagon and Hammond[7] have used reversed-phase high-performance liquid chromatography (RP-HPLC) for the determination of ethynyl oestradiol at the 10 μg per tablet level. Although this work was primarily concerned with ethynyl oestradiol the applicability of this approach to a general method for the analysis of oral contraceptives was noted.

Johnston[8] in his work on normal phase HPLC of oral contraceptives correctly pointed out that the reversed-phase system used by Bagon and Hammond[7] was unable to resolve an important progestogen–oestrogen combination of oral contraceptives, norethisterone and ethynyl oestradiol. His approach of using normal-phase or adsorption chromatography whilst resolving the norethisterone/ethynyl oestradiol pair could not resolve chromatographically another equally important combination, norgestrel and ethynyl oestradiol. The resolution was achieved at the detection stage by using a fluorescence detector in series with a UV detector.

The use of organic modifiers in the mobile phase of RP-HPLC separations has been shown to give altered selectivity for various compounds[9,10]. Lee *et al.*[11] has shown that the addition of small percentages of ethers to a binary mobile phase in RP-HPLC can provide resolution between hitherto difficult to separate steroid groups including a viable separation scheme for norethisterone and ethynyl oestradiol by RP-HPLC.

The benefits of using RP-HPLC in a routine laboratory situation are well known. The solvent(s) used are comparatively less environmentally hazardous and cheaper than their normal phase counterparts making the "system" simpler and therefore easier to use. A single mode of detection would also simplify any proposed means of analysis.

It was with these facts in mind that a study was undertaken into the RP-HPLC determination of various progestogen–oestrogen formulations with the view to the development of a simple, rapid means for the quality assurance of oral contraceptives.

EXPERIMENTAL

Materials

All chromatographic mobile phase solvents were prepared from Waters Assoc. (Milford, MA, U.S.A.) HPLC-grade methanol and tetrahydrofuran (THF) together with distilled deionised water and were filtered through a 0.45-μm filter before use. Samples of reference steroids were generously donated by the manufacturers listed in the acknowledgements.

Apparatus

The RP-HPLC system comprised a Waters Assoc. Model 6000A dual piston pump together with a Model U6K injector fitted with a 50-μl sample loop. The column used was a Waters Assoc. radial compression module (RCM) fitted with a μBondapak C_{18} cartridge. The variable-wavelength detector was a Waters Assoc. Model 450 connected to a Model 730 data module.

Extraction procedure

(i) Coated and uncoated tablets. A single tablet was placed in a 50-ml round bottom flask to which was added 1 ml of water and a small PTFE-coated stirring bar. The tablet disintegrated upon stirring and 20 ml of methanol was added. The solution was raised to a temperature of 60°C and stirred for a further 10 min. The resulting solution was quantitatively transferred and filtered into another 50-ml round bottom flask with an additional 10 ml of methanol. The solution was evaporated to dryness by passing a stream of nitrogen over the surface of the solution with gentle heating. The sample was redissolved in 5 ml of the appropriate chromatography solvent and 50 μl taken for analysis.

(ii) Paper formulation. A single "square" of rice paper impregnated with a daily dosage of steroid hormones, was taken for analysis and placed in a 50-ml round bottom flask together with 30 ml of methanol. The solution was refluxed for 30 min then treated as described for the coated and uncoated tablets from the filtration stage on.

Recovery experiments

The extraction efficiency and precision of the experimental procedure for tablet formulations was determined as follows. Two placebos were crushed and a homogenous sample, representative of a single tablet, was taken for analysis. To this sample was added, by microburette, a methanolic solution of progestogen/oestrogen combination suitable for the formulation under study. The sample was dried and then subjected to the experimental procedure as outlined in extraction procedure (i) with the results listed in Table Ia.

A satisfactory method of determining the recovery of the steroid hormones from the paper formulations proved difficult to develop. Placebo formulations were unobtainable and therefore exhaustive extraction of real samples was undertaken.

TABLE I

EXTRACTION EFFICIENCIES FROM TABLET AND PAPER FORMULATIONS

Combination	*Recovery**
(a) Tablets	
Norgestrel	98.5 ± 0.7%
Ethynyl oestradiol	99.3 ± 1.5%
Norethisterone	101.1 ± 0.8%
Ethynyl oestradiol	98.7 ± 2.7%
Norethisterone acetate	101.8 ± 0.5%
Ethynyl oestradiol	99.4 ± 1.6%
(b) Paper formulation	
Norethisterone	99.2 ± 0.8%
Ethynyl oestradiol	99.5 ± 1.5%
Mergestrel acetate	100 ± 0.7%
Ethynyl oestradiol	99.3 ± 1.3%

* Mean and relative standard deviation of ten separate determinations.

The "exhausted" sample sheets were then "spiked" with known quantities of progestogen/oestrogen combinations then subjected to the extraction procedure outlined previously (ii) with the results as listed in Table Ib.

RESULTS AND DISCUSSION

Chromatography of steroid hormones

The various types of oral contraceptives chosen for this study were comprised of four different combinations of progestogen–oestrogen in a variety of dosage regimes. They were (i) norgestrel–ethynyl oestradiol, (ii) norethisterone acetate–ethynyl oestradiol, (iii) mergestrel acetate–ethynyl oestradiol and (iv) norethisterone–ethynyl oestradiol. The chromatographic resolution of the first three combinations presents a trivial problem on a C_{18} bonded reversed-phase column. Table II presents the steroid hormones under consideration together with their respective capacity factor, k', in various solvent mixtures on a C_{18} column. Inspection of this table reveals that for combinations i, ii and iii, a simple binary mixture of methanol–water will provide sufficient resolution of these pairs to permit quantitation as evident in the chromatogram of combination i, Fig. 1. The fourth combination, norethisterone–ethynyl oestradiol, whilst proving intractable in the simpler binary solvents, is readily resolved in a ternary methanol–water–THF mixture, see Fig. 2. These results are in agreement with previous published in this area[7,11].

Detection

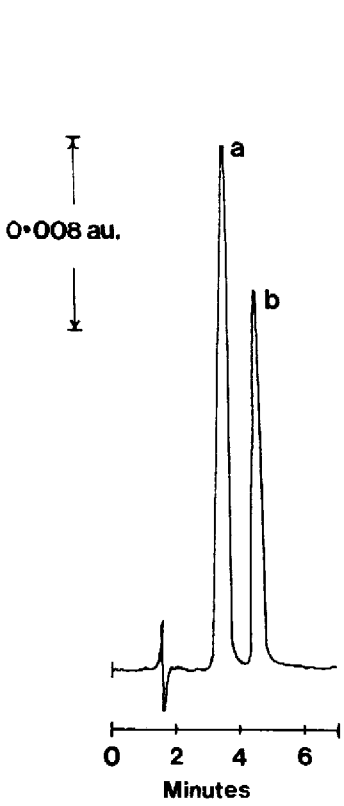
The disproportionate ratio of progestogen to oestrogen together with different UV characteristics of each compound in oral contraceptives requires a flexible approach to the problem of UV detection of these steroid hormones. A UV variable-wavelength detector was employed in this instance to give the flexibility demanded of the analytical technique with a single mode of detection.

Table III lists the compounds under study together with their respective UV maximum and molar extinction coefficient, ε. From this data it is clear that the "ideal" wavelengths to choose, from the viewpoint of sensitivity, would be 240 nm for the $\Delta 4$ unsaturated ketones norgestrel, norethisterone and norethisterone acetate; 290 nm for the $\Delta 4,6$ unsaturated ketone mergestrel acetate and 212 nm for the phenolic oestrogen ethynyl oestradiol. However, in keeping with the underlying premise of a simple and rapid assay procedure either 212 nm or 280 nm as the UV wavelengths

TABLE II

CAPACITY FACTORS (k') OF STEROID HORMONES IN VARIOUS SOLVENT MIXTURES

Solvent	*Norgestrel*	*Norethisterone*	*Norethisterone acetate*	*Mergestrel acetate*	*Ethynyl oestradiol*
Methanol water (85:15)	0.62	0.55	1.27	1.19	0.47
Methanol–water (75:25)	1.74	1.24	2.86	2.89	1.13
Methanol–water (60:40)	8.25	5.12	16.27	11.94	5.06
Methanol–water–THF (60:30:10)	2.68	1.68	3.88	5.06	2.44



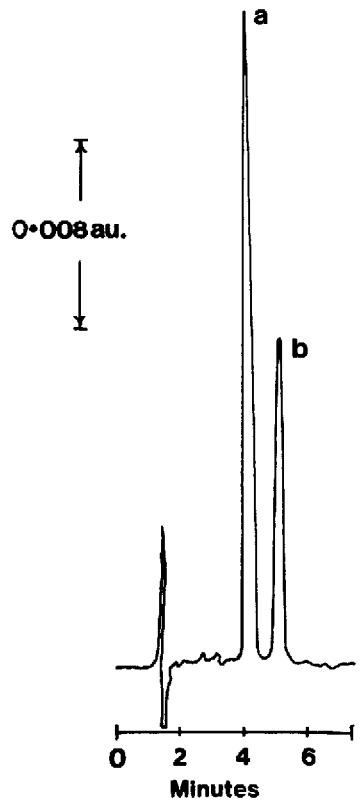


Fig. 1. Analysis of norgestrel–ethynyl oestradiol tablets using 212 nm detection and a mobile phase of methanol–water (75:25) at a flow-rate of 1.5 ml min^{-1}. Peaks: a = ethynyl oestradiol; b = norgestrel.

Fig. 2. Analysis of norethisterone–ethynyl oestradiol tablets using 280 nm detection and a mobile phase consisting of methanol–water–THF (60:30:10); flow-rate = 2.0 ml min^{-1}. Peaks: a = norethisterone, b = ethynyl oestradiol.

were selected for the determination of steroid hormones in oral contraceptives depending upon the amount and type of progestogen in the formulation.

The rationale behind these choices is that at these wavelengths the UV maximum absorption occurs for ethynyl oestradiol, which is the most difficult steroid to detect due to the inherently low extinction coefficient, ε and low pharmaceutical dosage. The progestogenic component on the other hand is formulated in comparatively larger amounts and has a significantly larger ε_{max}. The corollary of the choice of wavelengths is the maximisation of the detectability of the oestrogenic component and the minimisation of the detectability of the progestogenic component.

The judicious choice of either 212 nm or 280 nm allows the simultaneous quantification of both steroidal components, of all oral contraceptives studied, in a single chromatogram.

Interferences

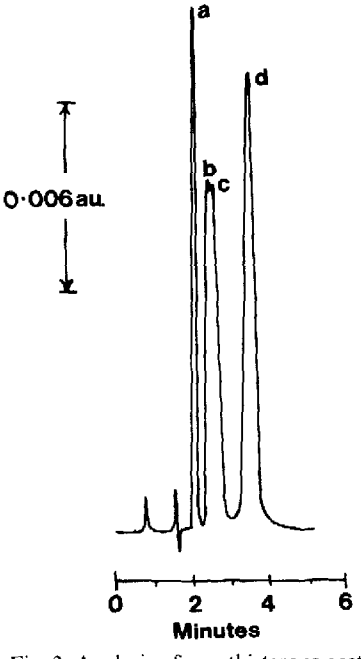
Of the nine different formulations investigated in this study only one presented any perceivable interference. The formulation in question nominally contained 1000

TABLE III

UV MAXIMA AND MOLAR EXTINCTION COEFFICIENTS OF VARIOUS STEROIDS

Steroid	*UV maxima (nm)*	ε_{max}
Norgestrel	240	≈17,000
Norethisterone	240	≈17,000
Norethisterone acetate	240	≈17,000
Mergestrel acetate	290	≈28,000
Ethynyl oestradiol	280	≈ 2000
	212	≈ 8000

μg of norethisterone acetate and 50 μg of ethynyl oestradiol per tablet. From the results summarised in Table II an efficient chromatographic solvent to resolve this pair of steroid hormones on a C_{18} column would be an 85:15 mixture of methanol–water. Fig. 3 shows the resultant chromatogram of an extract from this oral contraceptive run in methanol–water (85:15) solvent. The chromatogram shows that whilst separation of ethynyl oestradiol (c) from norethisterone acetate (d) is accomplished within 4 min there is a co-eluting compound (b) obscuring the ethynyl oestradiol response. The interferent(s) absorbed at both 212 nm and 280 nm and therefore could not be resolved with the proposed detection method. The chromato-



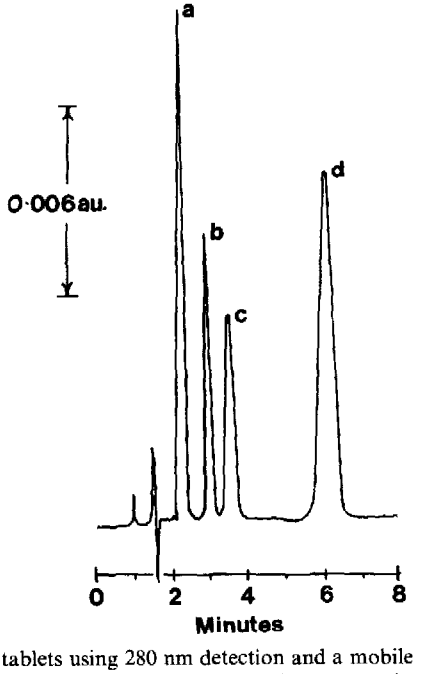


Fig. 3. Analysis of norethisterone acetate–ethynyl oestradiol tablets using 280 nm detection and a mobile phase of methanol–water (85:15); flow-rate = 1.5 ml min^{-1}. Peaks: c = ethynyl oestradiol; d = norethisterone acetate.

Fig. 4. Same as for Fig. 3 except mobile phase consists of methanol–water (75:25). Peaks: a, b = unknown interferents; c = ethynyl oestradiol; d = norethisterone acetate.

TABLE IV

ASSAY RESULTS FOR TABLET AND PAPER FORMULATIONS

Formulation	*Conditions*			*Nominal content (μg)*	*Assay results* (μg)*
	Solvent	*Flow-rate (ml min^{-1})*	*Wavelength (nm)*		
(a) Tablets					
Norethisterone	Methanol–water–THF (60:30:10)	2.0	280	2000	1950 ± 1.8%
Ethynyl oestradiol				50	50.0 ± 3.0%
Norethisterone	Methanol–water–THF (60:30:10)	2.0	280	1000	995 ± 1.1%
Ethynyl oestradiol				35	34.7 ± 2.5%
Norgestrel	Methanol–water (75:25)	1.5	212	150	152 ± 1.8%
Ethynyl oestradiol				30	30.5 ± 2.8%
Norgestrel	Methanol–water (75:25)	1.5	212	125	126 ± 2.4%
Ethynyl oestradiol				35	34.5 ± 3.0%
Norgestrel	Methanol–water (75:25)	1.5	212	75	74.1 ± 3.0%
Ethynyl oestradiol				30	31.5 ± 3.0%
Norethisterone acetate	Methanol–water (75:25)	1.5	280	1000	995 ± 1.8%
Ethynyl oestradiol				50	49.5 ± 2.4%
(b) Paper formulations					
Norethisterone	Methanol–water–THF (60:30:10)	2.0	212	600	610 ± 2.1%
Ethynyl oestradiol				30	30.5 ± 2.6%
Mergestrel acetate	Methanol–water (85:15)	1.5	212	1000	1010 ± 1.9%
Ethynyl oestradiol				35	35.5 ± 2.6%
Mergestrel acetate	Methanol–water (85:15)	1.5	212	600	590 ± 2.5%
Ethynyl oestradiol				35	34.6 ± 3.1%

* Mean and relative standard deviation of 20 separate determinations.

graphic solvent was decreased in strength to methanol–water (75:25) and the resulting chromatogram, Fig. 4, clearly shows that the resolution of the interfering peak from the peak of interest has been accomplished within 7 min. The unidentified peaks (a, b) display maximum UV absorption at 255 nm. Both peaks were present in extracts of placebos and therefore presumed not to be associated with the steroid hormone component of the oral contraceptive.

Assay results

Nine different formulations were chosen for this study on the basis of the progestogen–oestrogen type, amount and ratio. This was to give a diverse range of this pharmaceutical product examination with the view of determining the suitability of this method as a general method of analysis.

Calibration of the method was achieved using appropriate concentrations of external standards and the comparison of peak height was used for subsequent determination of sample levels.

The results of the recovery experiments indicate that, for tablet preparations, a suitable extraction procedure has been developed for both progestogenic and oestrogenic components of oral contraceptives. The procedure adopted for the recovery experiments in the novel paper formulation, whilst giving excellent recovery values, is not entirely satisfactory from the viewpoint of ensuring identical matrix composition. It is however a practical alternative and from a pragmatic viewpoint is a viable method in view of the results in Table IV.

The results obtained from single tablet determinations of the various formulations are summarised in Table IV together with the chromatographic conditions employed in each determination. The assay results are obtained from 20 individual determinations of each formulation. The mean and relative standard deviation results of each steroid show excellent agreement with the manufacturers nominal content and adequately meet the content uniformity requirements of the *British Pharmacopeia* and *United States Pharmacopeia*.

CONCLUSION

The RP-HPLC method described in this report enables the simultaneous determination of both progestogenic and oestrogenic components of a variety of oral contraceptive formulations. The method is sufficiently simple and rapid yet sensitive and accurate enough for the quality assurance of these types of pharmaceuticals in a routine laboratory situation.

REFERENCES

1 S. Görög and Gy. Szasz (Editors), *Analysis of Steroid Hormone Drugs*, Akademiai Kiado, Budapest, 1978, p. 372.
2 T. James, *J. Pharm. Sci.*, 61 (1972) 1306.
3 R. J. Templeton, W. A. Arnett and I. M. Jakouljevic, *J. Pharm. Sci.*, 57 (1968) 1168.
4 J. H. McB. Miller and P. Duguid, *Proc. Anal. Div. Chem. Soc.*, 13 (1976) 93.
5 E. Heftmann and I. R. Hunter, *J. Chromatogr.*, 165 (1979) 283.
6 M. Kautsky (Editor), *Steroid Analysis by HPLC, Recent Applications, Chromatographic Science Vol. 16*, Marcel Dekker, New York, 1980, and references cited therein.
7 K. R. Bagon and E. W. Hammond, *Analyst (London)*, 103 (1978) 156.
8 M. A. Johnston, *J. Chromatogr.*, 216 (1981) 269.
9 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
10 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 149 (1978) 431.
11 G. Jia-long Lee, R. M. K. Carlson and S. Kushinsky, *J. Chromatogr.*, 212 (1981) 108.