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GENERAL METHOD FOR THE ASSAY OF ORAL CONTRACEPTIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

This paper describes a method which employs the technique of high-performance liquid chromatography to determine the content of each progestrogenic and oestrogenic ingredient in currently available oral contraceptive formulations. The procedure involves a simple solvent extraction followed by analysis on a silica column with cyclohexane and 2-propanol as the mobile phase. The method has been used for the determination of content uniformity of thirty-two oral contraceptive products available in Australia.

INTRODUCTION

Oral contraceptives are marketed in Australia in the form of coated or uncoated tablets containing a progestogen and an oestrogen. The progestogen component may be norethisterone, norethisterone acetate, lynoestrenol, ethynodiol diacetate or norgestrel and is usually present in milligram amounts while the oestrogen may be ethinyloestradiol or mestranol in microgram quantities.

A number of procedures for determining the content of progestogen or oestrogen in therapeutic goods have been reported. These include analysis by gas–liquid chromatography (GLC) after suitable derivatisation[1,2], or following direct extraction[3] where quantitation of the progestogen in an oral contraceptive tablet formulation was the prime concern. Thin-layer chromatographic (TLC) systems have been developed to achieve separation[4] of the progestogen and oestrogen for subsequent UV or colorimetric determination. Column chromatography has been used as a clean up procedure with subsequent dansyl derivative formation[5] or reaction with acetic anhydride and sulphuric acid[6] followed by fluorescence detection. However, the fluorimetric methods are not applicable to formulations containing both progestogen and oestrogen, as the progestogen inhibits or quenches the fluorescence. The same limitation applies to colorimetric work based on the Kober reaction, where the progestogen and oestrogen are analysed separately to avoid interference from both the relatively large amount of progestogen present and from formulation excipients which interfere with colour formation[7–9]. The colorimetric determination of oestrogen in combination formulations has other inherent problems. For example ethi-

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nyloestradiol has poor solubility and hence the methods for its quantitation invariably involve numerous transfers and extractions.

Colour development is dependent on solvent purity, moisture and method of reagent preparation thus making the method cumbersome and time consuming.

More recently quantitative determinations have been performed using high-performance liquid chromatography (HPLC). The early HPLC work of Roos[10] involved separating oestrogen sulphate esters. The esters were hydrolysed and the free oestrogens separated by further chromatography. The method was considered to be too time consuming. Dansyl derivative formation following chromatographic separation[11] also has the problem of potential interference or quenching from progestogens present in the combined formulation. The reversed-phase chromatographic system of Bagon and Hammond[12] encountered problems with sugar coated tablets and was unable to separate norethisterone and ethinyloestradiol, a widely used combination in contraceptive formulations.

In the past this laboratory has assayed contraceptive tablets using numerous methods described in the British Pharmacopoeia (BP), the United States Pharmacopoeia (USP) and the literature. None of these methods have been found to be sufficiently reproducible or generally applicable for our purposes. In particular the actual extraction and subsequent quantitation of ethinyloestradiol has proved a problem.

As a regulatory laboratory a method that would allow testing of contraceptive formulations for compliance with quality control specifications would be of considerable value. The method would need to take into consideration the limitations imposed by the low levels of oestrogen present and the problems associated with determining uniformity of content for tablets containing an oestrogen and a progestogen, the relative amounts of which may differ by a factor of 100. The method would need to have good sensitivity over the oestrogen content range (current formulations contain between 10 μg and 1 mg) and should not be subject to interference from tablet excipients.

EXPERIMENTAL

Materials

Unless otherwise specified the solvents were all Spectrograde obtained from Ajax Chemicals (Sydney, Australia). Anhydrous sodium sulphate was analytical grade (AnalaR) obtained from BDH (Sydney, Australia). Water was freshly distilled.

All progestogen and oestrogen reference substances were generously donated by the following manufacturers: ethynodiol diacetate and mestranol (Searle Labs., North Sydney, Australia), levonorgestrel (Schering, Berlin, Tempe, Australia), lynoestrenol (Parke Davis & Co., Caringbah, Australia), norethisterone and norethisterone acetate (Ethnor, North Ryde, Australia), ethinyloestradiol and D-norgestrel (Wyeth Pharmaceuticals, Parramatta, Australia).

Apparatus

The HPLC system comprised a Waters Assoc. M45 solvent pump, a Perkin-Elmer LC55 variable-wavelength spectrophotometer and a Perkin-Elmer MPF44 spectrofluorimeter coupled in series.

The chromatographic column employed was a Waters μPorasil silica column 30 cm × 3.9 mm I.D., average particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.).

Initially the injection system comprised an Altex injector Model 210 fitted with a 100-μl loop. This was subsequently replaced by a Waters auto-injector WISP 710B programmed for a 100-μl sample-injection volume.

A Hewlett-Packard 3380A integrator was connected to the Perkin-Elmer LC55 for quantitating the progestogen while a Waters Data Module was connected to the fluorimeter for determing oestrogen content. A chart speed of 5 mm/min was used for each integrator.

Chromatographic conditions

The mobile phase consisted of 2-propanol and cyclohexane, the ratio used being dependent on the steroid combination under examination (depicted in Fig. 1). Norethisterone, norethisterone acetate, norgestrel and levonorgestrel were analysed at 240 nm while lynoestrenol and ethynodiol diacetate were analysed at 213 nm. The oestrogens, mestranol and ethinyloestradiol, were analysed by fluorimetry, excitation wavelength 280 nm and emission 310 nm. All the HPLC separations were carried out using a solvent flow-rate of 1.5 ml/min.

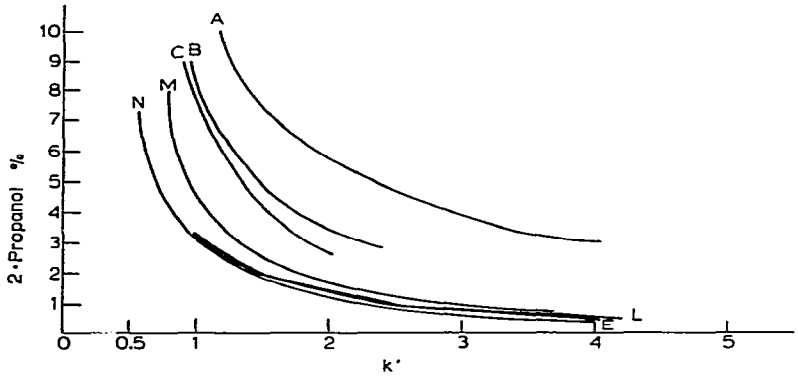


Fig. 1. Relationship between percentage of 2-propanol in the mobile phase and k' for a number of contraceptive steroids. A = Norethisterone; B = norgestrel; C = norethisterone acetate; E = ethynodiol diacetate; L = lynoestrenol; M = ethinyloestradiol; N = mestranol.

Tablet sampling and preparation

Both uncoated and sugar coated tablets were treated by the same procedure. A 25-ml Pyrex conical flask containing a single tablet and 1 ml of distilled water was placed in an ultrasonic bath until the tablet disintegrated (generally less than 5 min). The contents of the flask were quantitatively transferred to a 125-ml separation funnel with the aid of 5 ml distilled water. The sample was extracted with 3 × 25 ml chloroform or methylene chloride (the choice of solvent was dependent on the progestogen of interest). The organic solvent was filtered through a funnel containing approximately 1.0 g anhydrous sodium sulphate, into a 100-ml actinic volumetric flask, and made up to volume.

TABLE I

RESULTS OF RECOVERY EXPERIMENTS

Steroid combination	*Expected amounts*	*Mean recovery (%)*	*Standard deviation (%)*	s_T
Norethisterone/ethinyloestradiol	1.0 mg/50 μg	99/98.9	1.01/2.1	1.4/2.3
Norethisterone acetate/ethinyloestradiol	1.0 mg/50 μg	98.4/99.4	1.3/0.9	2.4/1.0
Norethisterone acetate/mestranol	1.0 mg/50 μg	99/98.9	0.8/3.2	1.2/3.2

Thirty-two oral contraceptive formulations were examined by the described method. A minimum of twenty individual tablets were assayed for each formulation.

Recovery experiments

The samples for the recovery analysis were made up using a typical manufacturer's formulation. Taking a nominal tablet formulation as containing povidone (2 mg), maize starch (5 mg), magnesium stearate (0.25 mg) and lactose (42 mg), a bulk tablet mix equivalent to forty tablets was weighed out and blended. Unit weights of 50 mg were weighed from the bulk mix, and the individual steroids added by pipette from standard solutions made up in chloroform. The "tablet" sample was mixed, evaporated to dryness and then processed using the sample preparation procedure. Ten samples of each steroid mixture were examined.

The results of the recovery experiments are shown in Table I. The mean recovery for the thirty samples with respect to each steroid was 99% with a standard deviation of 2.1%. The extraction of each of the progestogen and oestrogen steroids from contraceptive formulations was therefore considered to be quantitative and reproducible.

RESULTS AND DISCUSSION

Method development

All the steroids of interest are soluble in chloroform but insoluble in water while most excipients encountered in the manufacturers formulations are insoluble in chloroform. Chloroform, however, has a strong UV absorbance at 213 nm which interferes with the quantitation of lynoestrenol and ethynodiol diacetate, both of which have a low k' value. This problem was overcome through the use of methylene chloride as extraction solvent for formulations containing lynoestrenol or ethynodiol diacetate. Due to the low absorptivity of these two compounds, however, the final dilution volume was reduced to 50.0 ml.

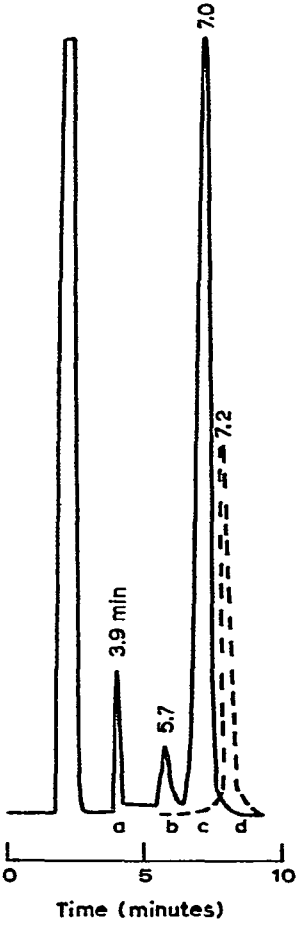
Fluorimetric detection was used to achieve the sensitivity required for the low levels of oestrogen. The native fluorescence of the phenolic group in the A ring (excitation 280 nm, emission 310 nm) was sufficient to permit quantitation of the oestrogens present at the microgram level. The linear response range for mestranol and ethinyloestradiol on the MPF44 spectrofluorimeter are given in Table II. The narrow linear response range of the oestrogens and consequent large dilution factor used resulted in the need to analyse the progestogens at maximum sensitivity.

TABLE II
LINEAR RESPONSE RANGE FOR CONTRACEPTIVE STEROIDS STUDIED

Progestogens	*Linear response range*
Ethinyloestradiol	0.05–1.0 μg/ml
Mestranol	0.05–1.4 μg/ml
Norethisterone	0.002–0.1 mg/ml
Norethisterone acetate	0.002–0.1 mg/ml
Norgestrel	0.001–0.1 mg/ml
Ethynodiol diacetate	0.001–0.08 mg/ml
Lynoestrenol	0.001–0.08 mg/ml

Norethisterone, norethisterone acetate, norgestrel and levonorgestrel were analysed at 240 nm (where their molar absorptivity is at a maximum). Ethynodiol diacetate and lynoestrenol, which both exhibit low intensity absorption at 240 nm, were analysed at 213 nm. The linear response ranges for the respective progestogens are also given in Table II.

To validate further the method, 10-μg ethinyloestradiol tablets were examined. The standard deviations of the single tablet assays were of the same order as those obtained for formulations of higher oestrogen concentration. The only alteration to the method was to decrease the final dilution by a factor of 10. It is considered that this method is sufficiently sensitive and selective for the detection of cross contamination of products by minute quantities of ethinyloestradiol. The method has sub-



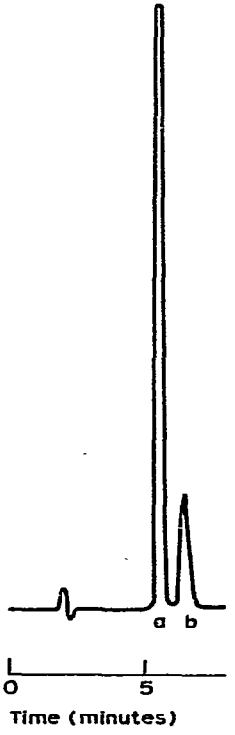
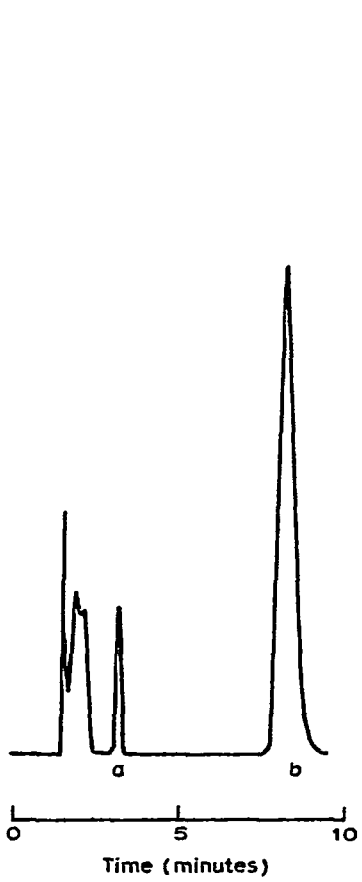


Fig. 2. Analysis of norgestrel–ethinyloestradiol tablets using 240 nm detection (———) and fluorescence detection (– – –), respectively. Mobile phase: 2-propanol–cyclohexane (1.5:98.5). Peaks: a = methyl-*p*-hydroxybenzoate; b = propyl-*p*-hydroxybenzoate; c = norgestrel; d = ethinyloestradiol.

Fig. 3. Analysis of lynoestrenol–mestranol tablet. Mobile phase: 2-propanol–cyclohexane (1:99). Peaks: a = lynoestrenol at 213 nm; b = mestranol, fluorescence.



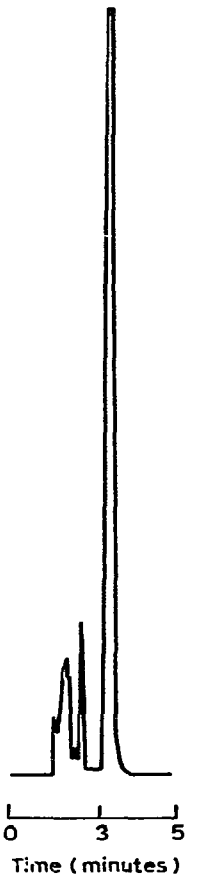


Fig. 4. Analysis of norethisterone–mestranol tablet. Mobile phase: 2-propanol–cyclohexane (3:97). Peaks: a = mestranol, fluorescence; b = norethisterone at 240 nm.

Fig. 5. Analysis of norethisterone–mestranol tablet. Mobile phase: 2-propanol–cyclohexane (10:90). Peaks: norethisterone at 240 nm, and mestranol part of solvent front.

sequently been used in a study of dissolution characteristics of contraceptive formulations.

As an aid to optimising the selection of the mobile phase ratio a study was conducted using the steroids encountered in the contraceptive formulations surveyed. The results of the study are summarised in Fig. 1, a plot of percent 2-propanol against change in k'. By choosing appropriate ratios from this graph, a suitable mobile phase composition can be selected for each contraceptive steroid combination. The use of this graph is demonstrated in Table III where the operating conditions for the laboratory program are presented.

One formulation contained methyl and propyl parabens as preservatives and alteration of the theoretical ratio was necessary to obtain appropriate resolution (Fig. 2). The only other deviation from the expected solvent ratio was found with norgestrel–ethinyloestradiol mixtures where steroid resolution was very poor. However each steroid could be quantitated using the different detectors, UV at 240 nm and fluores-

cence, respectively. No interference between the oestrogen and progestogen or quenching effects were noted in the analysis of the thirty-two formulations available to the laboratory.

To test the reproducibility of the injection volume twelve injections of one mixture were made. The standard deviation of the peak areas for progestogen and oestrogen was determined to be 1.0 and 0.8%, respectively. If an internal standard is required, a choice can be made on the basis of the data given in Fig. 1 where a steroid is selected to give appropriate peak resolution for an appropriate mobile phase ratio.

By using the mobile phases shown in Table III all the formulations examined were assayed with the analysis time and peak resolution being optimised by appropriate choice of the mobile phase ratios. Examples of the separations obtained are shown in Figs. 2–5.

TABLE III

OPERATING CONDITIONS FOR LABORATORY SAMPLING PROGRAMME

Formulation	*UV detection wavelength (nm)*	*Mobile phase (2-propanol–cyclohexane)*	*Retention time (min)*
Ethynodiol diacetate/ethinyloestradiol*	213	0.5:99.5	7.0/10.0
Ethynodiol diacetate/mestranol*	213	0.5:99.5	7.0/9.0
Lynoestrenol/ethinyloestradiol	213	1:99	5.3/8.2
Lynoestrenol/mestranol	213	1:99	5.4/6.3
Norethisterone/ethinyloestradiol	240	8:92	4.0/3.2
Norethisterone/mestranol	240	3:97	10.0/3.5
Norethistorone acetate/ethinyloestradiol	240	4:96	4.0/3.8
Norgestrel/ethinyloestradiol	240	1.5:97.5	7.0/7.2

* Mestranol and ethinyloestradiol were quantitated by fluorescence detection, excitation 280 nm, emission 310 nm.

The results obtained for the single tablet assays are shown in Table IV. The standard deviations are expressed as percent deviation about the mean content while the values of the statistic s_T refer to the percent deviation about the stated or target content. The results obtained from the contraceptive formulations tested indicate the content uniformity for most products is very good. The only exception is the formulation containing 500 μg of ethynodiol diacetate and 50 μg ethinyloestradiol where the mean ethynodiol diacetate content was determined to be 96.5% of the label content with a standard deviation of 12.5%. The progestogen and oestrogen contents as determined in the survey were well within the general requirements for content uniformity of the BP and the specific requirements of the USP.

CONCLUSION

The HPLC method described allows the concurrent determination of progestogen and oestrogen concentrations in contraceptive formulations. The procedure offers excellent sensitivity, selectivity and accuracy for low dose formulations. No problems were encountered with interference from tablet excipients or coating ma-

TABLE IV
RESULTS FROM LABORATORY SURVEY OF CONTRACEPTIVE FORMULATIONS

Formulation	*Label stated content*	*Mean label content (%)*	*Std. deviation as a % of stated content*	s_T
Ethynodiol diacetate–ethinyloestradiol	500 μg/50 μg	96.5/101.2	12.5/7.54	12.7/7.4
	1 mg/50 μg	98.9/101.2	4/6.12	4.0/6.0
Ethynodiol diacetate–mestranol	500 μg/100 μg	97.9/ 98.9	4.2/5.3	4.7/5.23
	1 mg/100 μg	98.0/ 99.1	8/6.8	7.5/6.45
	2 mg/100 μg	101.2/101.0	8/6.5	7.9/6.45
Lynoestrenol–ethinyloestradiol	1 mg/50 μg	98.7/101.2	4.7/4.6	4.8/4.7
Lynoestrenol–mestranol	1 mg/100 μg	100.4/100.1	5/3.9	4.9/3.8
	2.5 mg/75 μg	100.0/ 98.5	2/2.3	2.1/2.5
	2.5 mg/75 μg	99.6/ 99.0	2/2.3	1.8/2.5
Norethisterone–ethinyloestradiol	500 μg/35 μg	105.8/101.1	3.5/2.8	6.6/2.8
	1 mg/35 μg	98.6/100.3	2.3/4.0	2.7/3.9
Norethisterone–mestranol	1 mg/50 μg	101.1/ 99.0	3.1/7.8	3.0/7.7
	1 mg/50 μg	99.3/101.2	4/4	4.1/4.2
	1 mg/50 μg	100.0/101.2	2/4	2.9/4.6
	1 mg/50 μg	98.0/102.0	6/2.7	6.1/3.2
	1 mg/80 μg	99.6/100.4	6/4.6	5.4/4.5
	2 mg/100 μg	103.0/ 97.3	5/3.0	5.5/4.7
	2 mg/100 μg	102.6/100.2	4/5.4	4.7/5.3
Norethisterone acetate–ethinyloestradiol	1.0 mg/50 μg	98.4/ 97.0	4/3.1	4.0/4.3
	1.0 mg/50 μg	98.4/103.3	3.4/3.4	3.8/3.6
	2.5 mg/50 μg	100.2/103.3	2.5/3.0	2.5/4.4
	3.0 mg/50 μg	98.5/100.3	3.3/5.1	3.6/5.0
	4.0 mg/50 μg	99.4/100.0	4.6/6.8	4.6/6.7
Norgestrel–ethinyloestradiol	500 μg/50 μg	99.2/99.6	2.55/3.9	2.6/3.9
Levonorgestrel–ethinyloestradiol	150 μg/30 μg	100.7/ 98.3	8.1/5.4	8.0/5.5
	250 μg/50 μg	101.5/101.8	6.9/4.8	7.0/5.0
Ethinyloestradiol	10 μg	93	4.0	4.2
	10 μg	101.6	5.4	5.7
	20 μg	106	4.9	5.2
	20 μg	111	1.6	1.5
	20 μg	103.3	5.5	5.9
	50 μg	95.4	3.0	3.1

terials in using the simple extraction procedure. With the exception of one contraceptive formulation, where the progestogen or oestrogen were detector separated, baseline resolution was achieved for all products analysed. The procedure provides a suitable method for determining uniformity of content for oral contraceptives available in Australia. The inherent flexibility with respect to mobile phase ratio selection provides good steroid resolution while the modes of detection offer a high degree of sensitivity.

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