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QUANTITATIVE ANALYSIS OF NORETHINDRONE IN MILK USING DEUTERATED CARRIER AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A specific assay has been developed to quantitate norethindrone (17α-ethinyl-17β-hydroxy-estr-4-ene-3-one) in human milk by multiple ion detection technique using gas chromatography-mass spectrometry. 14,15-D_2-norethindrone was synthesized and used as an internal standard and carrier. After addition of internal standard, the milk samples were extracted using Amberlite XAD-2 columns and further purified on Sephadex LH-20 columns. O-methoxime-trimethylsilyl ether derivatives of the purified samples were used for analysis. The analysis of known amounts of norethindrone added to control milk samples showed high precision and reproducibility. The assay method was used to measure levels of norethindrone in milk samples of five women inserted with single silastic implant D containing 40 mg nore-thindrone acetate. The mean level at three weeks post insertion was 208 pg/ml which then showed a slow and steady decline.

INTRODUCTION

During recent years several methods have been described for the quantitative determination of steroids in body fluids. Combined gas chromatograph-mass spectrometer (GC–MS) equipped with a multiple ion detector has been successfully used as a quantitative analytical method for steroid hormones [1–3]. In this technique, the selected ion response from the sample is measured in relation to that of a known amount of internal standard. This intermittent focusing on preselected ions, characteristic for the steroid to be detected and the internal standard chosen, provides a high degree of specificity to the assay. Several internal standards have been explored which include synthetic analogues of the steroids to be detected [4, 5] and deuterium labelled molecules [6]. The commercial non-availability of the deuterium labelled steroid hormones has restricted their use as internal standards. However, the present study describes the synthesis of 14,15-D_2-norethindrone and using this compound as internal standard and carrier an assay method has been developed for the quantitation of norethindrone. The assay was used to measure subnanogram concentrations of norethindrone in milk samples taken from women inserted with single silastic implant D [7] six weeks post-partum.

EXPERIMENTAL

Subjects. Five lactating women between 20–30 years of age were chosen and inserted with single silastic implant D containing 40 mg norethindrone acetate (NET-A), 6 weeks post partum. Each woman fed the baby on left breast at 7 a.m. and milk was collected from this breast at 9 a.m. while she was feeding the baby on the right breast. Milk was taken from right breast at 11 a.m. while she fed the baby on left breast. Both milk samples were pooled and kept frozen at −20°C until analysed.

Silastic implant. The single silastic implant D was prepared by filling 40 mg NET-A in a silastic tube of 22 mm length, 0.60 mm wall thickness and 3.18 mm outer diameter [7].

Chemicals & solvents. All organic solvents were obtained from E. Merck, Darmstadt, Germany and were re-distilled using on all glass fractionating column before use.

Amberlite XAD-2 (Rohms & Hass, Philadelphia) was washed with water, ethanol, acetone and water and stored in water until used. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden.

Synthesis of 17α-ethinyl-17β-hydroxy-(14,15-D_2)-4-estrene-3-one. The synthesis of 14,15-D_2-norethindrone is described in Fig. 1 and comprises of the following steps.

17β-Acetoxy-3-methoxy-1,3,5(10),8-(14,15-D_2)-estratetraene (**II**). 11.0 g (34 mmol) 17β-acetoxy-3-methoxy-1,3,5(10),8,14-estrapentaene [1] was deuterated in 25 ml deuterated methanol and 25 ml deuterated benzene over 1.1 g 10% palladium on calcium carbonate in an atmospheric-pressure apparatus. Before adding the steroid the catalyst was saturated with deuterium to exclude any contamination with hydrogen. During the deuteration the mixture was shaken vigorously to assure a rapid reaction. Total absorption of deuterium in the steroid was 99%.

After deuteration the reaction mixture was separated from the catalyst, washed with a small portion

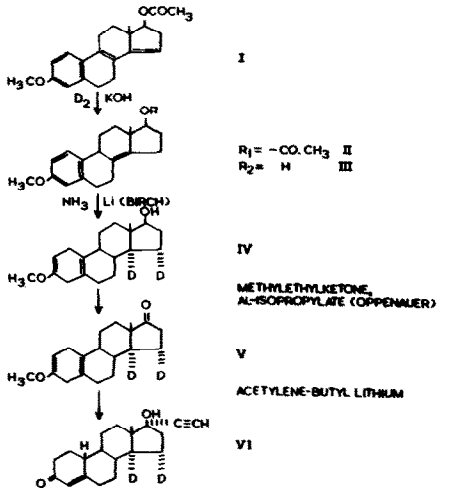

Fig. 1. Synthesis of 14,15-D_2-norethindrone.

of benzene and dried. The residue was recrystallized from 20 ml methanol, affording 6.25 g (56% of theoretical) pure substance melting at 114–117°C. Distribution of deuterium was found to be D_0 0.87%, D_1 7.92%, D_2 81.29%, D_3 9.69%, D_4 0.23%, rapid deuteration gave the best results.

3-Methoxy-17β-hydroxy-1,3,5(10),8-(14,15-D_2)-estratetraene **(III).** 21.6 g (65 mmol) steroid **(II)** was stirred with 12.64 g potassium hydroxide in 250 ml methanol under nitrogen for 60 min. After this time the reaction was complete. A thick light-coloured precipitate began to separate after 10 min.

Under nitrogen a solution of 282 ml water and 21 ml conc. hydrochloric acid was added dropwise with cooling, Stirring was continued for 60 min in an ice bath, the solid filtered, washed with water and dried over P_2O_5 *in vacuo*.

Yield: 18.55 g (98.5% of theoretical), crude material melting at 124/126–131°C. TLC with cyclohexane-ethyl acetate (90:60, V/V) showed no starting material.

3-Methoxy-2,5(10)-(14,15-D_2)-estradiene-17β-ol **(IV).** Twelve grammes of steriod **(III)** were dissolved in 400 ml tetrahydrofuran and added dropwise to about 360 ml ammonia at −33°C (inside temperature). Then 4.07 g lithium was added in one portion and stirred for 10 min. Sixty millilitres of ethanol were added within 60 min with vigorous stirring. The blue colouring of ammonia vanished and stirring was continued for 5 min. After adding 36 g ammonium chloride the cooling bath was removed and the ammonia evaporated slowly. The residue was treated with water, extracted with benzene–ether (1:1 V/V), washed with water, dried over sodium sulphate and the solvents distilled off. Yield: 11.25 g (91.5% of theoretical). TLC using cyclohexane–ethyl acetate (90:60, V/V) showed some impurities. For purification silica gel column was used (hexane–acetone–triethylamine, 94:5:1, by vol).

3-Methoxy-2,5(10)-(14,15-D_2)-estradiene-17-one **(V).** Five point six-five grammes (19.46 mmol) steroid **(IV)** was stirred for 24 h under nitrogen at reflux in a solution of 7.54 g aluminiumisopropylate in 38 ml methylethyl-ketone and 94 ml benzene (dried over Al_2O_3 and distilled over NaH). After cooling 44 ml 5% NaOH solution was added and the mixture was diluted with water. The benzene layer was separated and the mother liquor extracted three times with methylene chloride. The benzene and methylene chloride solutions were combined, washed with water, dried over magnesium sulfate, filtered and evaporated to dryness. The residue was treated with cold methanol, separated, washed with cold methanol and dried in air. Yield: 2.9 g (51.7% of theoretical) melting point 118/124–135°C. Starting material and a week impurity were observed after TLC with cyclohexane-ethyl acetate (90:60, by vol).

17α-Ethinyl-17β-hydroxy-(14,15-D_2)-4-estrene-3-one **(VI).** Sixty-eight point five millilitres (112.43 mmol) of a 51% solution of butyllithium in hexane (weight percent) was diluted with 250 ml tetrahydrofuran (dried over Al_2O_3 and distilled over NaH). Under cooling acetylene was passed in the solution for 45 min. A solution of 5.47 g steroid (V) in 72 ml tetrahydrofuran was added slowly to the reaction mixture and stirred for 30 min. Saturated ammonium chloride solution was carefully added dropwise. The mixture was diluted with ethyl acetate and evaporated after washing and drying. No starting material was observed, after TLC with chloroform–acetone (90:10, V/V).

To eliminate the protective group the crude product was dissolved in 137.5 ml methanol and 137.5 ml methylene chloride and stirred with 2.035 ml conc. hydrochloric acid over night at room temperature. The mixture was adjusted to pH 5–6 with sodium hydroxide solution and filtered. The residue was dissolved in methylene chloride, the solution treated with water, dried and evaporated. Yield: 5.73 g (100% of theoretical) crude material. TLC showed few impurities but no starting material. For purification, the substance was recrystallized from 50 ml ethanol yielding 3.35 g (59% of theoretical) solid, melting at 190/198–203°C. A second crop of 0.6 g could be isolated. A small amount of impurity was found on TLC with chloroform–acetone (90:10, V/V). Distribution of deuterium was D_0 0%, D_1 9%, D_2 78%, D_3 11%, D_4 1%.

Preparation of carrier. 14,15-D_2-norethindrone thus obtained was mixed with 15,16-^{3}H-norethindrone (57.14 Ci/mmol) to give an overall specific activity of 18.84 μci/μmol.

Gas chromatography–mass spectrometry. The gas chromatography–mass spectrometry was performed on an LKB 2091 instrument equipped with a multiple ion detector. Column packed with 1% OV-1 on supelcoport 80–100 mesh was used at 220°C. Molecular separators and ion source were maintained at 270 and 290°C respectively. Ionization energy was 22.5 eV and trap current 100 μA.

Extraction of milk samples. A column bed of Amberlite XAD-2 (150 × 14 mm) was prepared in dis-

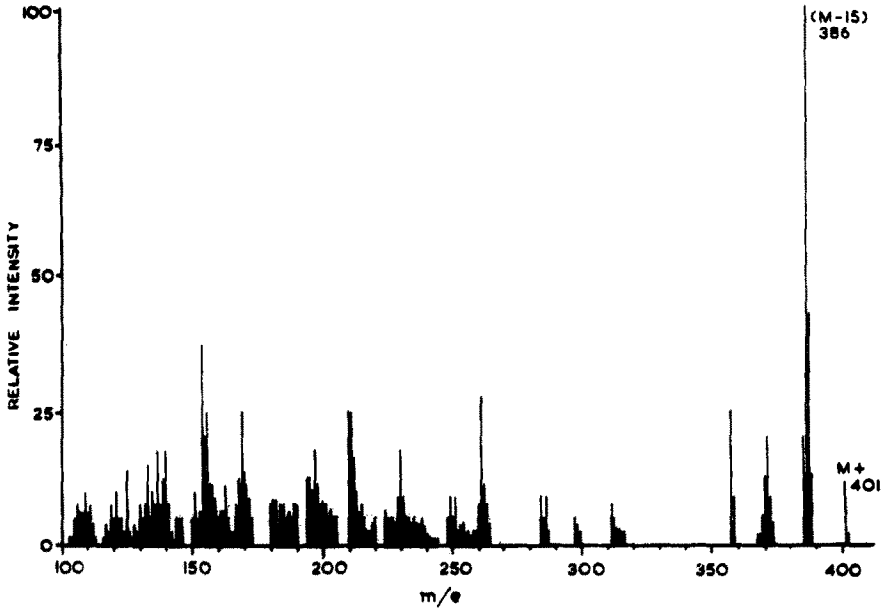

Fig. 2. Mass spectrum of 14,15-D_2-norethindrone MO-TMSi ether (electron energy 22.5 eV).

tilled water. Milk samples (10–12 ml) to which a known amount of carrier had been added (600–800 ng) was applied on the column. Initial elution was carried out with water (50–60 ml) and the steroid was eluted with methanol (25–30 ml). The methanol effluent was evaporated *in vacuo*.

Purification of steroid. The residue obtained above was dissolved in 0.5 ml methanol–toluene (5:95 V/V) and applied on a Sephadex LH-20 column (250 × 12 mm) packed by gravity flow in methanol–toluene (5:95 V/V). The column was washed with mobile phase before the sample application. Each fraction of 2 ml was collected and fractions containing norethindrone (checked by radioactivity determination of aliquots) were pooled. Solvents were evaporated to dryness under vacuo. The same column was used for at least three samples after washing the column with methanol–toluene (1:1 V/V) each time and equilibrating it with the mobile phase.

Derivatization. The residue containing norethindrone was dissolved in pyridine (200 μl) and 3–4 mg methoxyamine hydrochloride was added. After standing overnight the pyridine was evaporated, distilled water added and steroid derivative extracted with toluene. The toluene extract was evaporated to dryness. Pyridine 200 μl, hexamethyl disiliazane 50 μl and

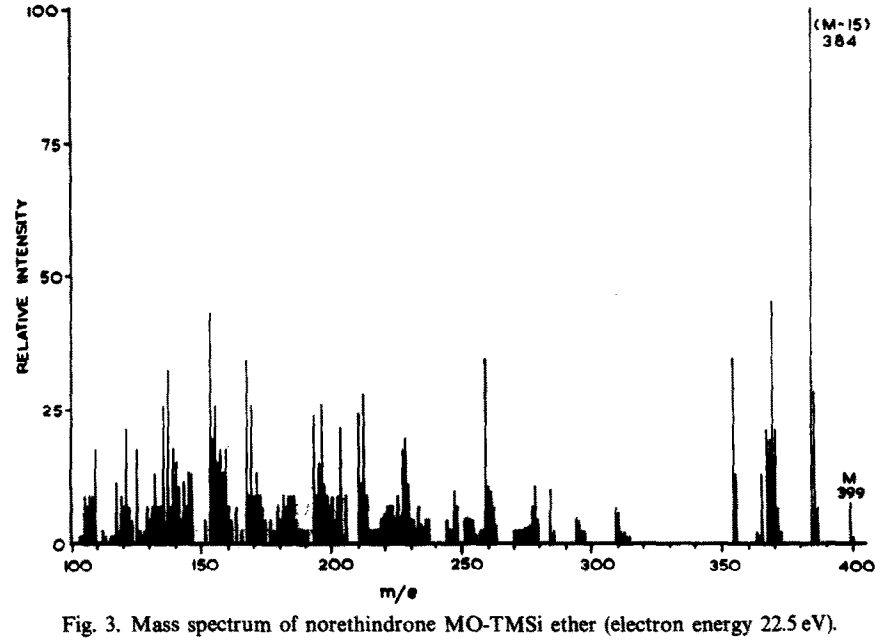

Fig. 3. Mass spectrum of norethindrone MO-TMSi ether (electron energy 22.5 eV).

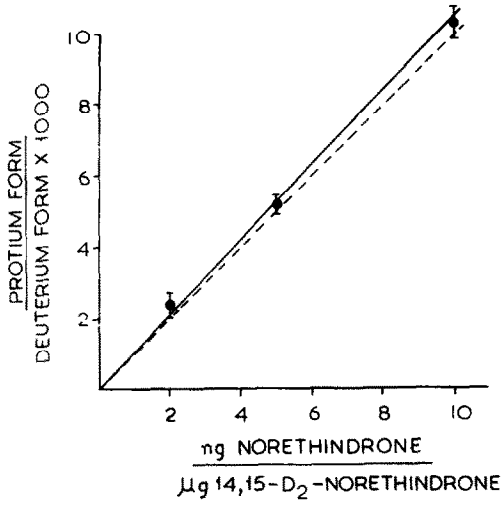

Fig. 4. Standard curve obtained from repetitive analysis of MO-TMSi ether derivatives of norethindrone and 14,15-D_2-norethindrone in the ratios 2/1000; 5/1000; 10/1000. The standard deviation for each standard and the theoretical slope is indicated.

trimethyl chlorosilane 20 µl were added to the residue. Next day the mixture was evaporated to dryness and residue dissolved in hexane for gas chromatographic–mass spectrometric analysis.

Quantitation of norethindrone. The mass spectra of 14,15-D_2-norethindrone MO-TMSi is shown in Fig. 2. The base peak appeared at *m/e* 386 (M-15). Similarly the base peak of norethindrone MO-TMSi derivative appeared at *m/e* 384 (Fig. 3). On the basis of response and background, the ions at *m/e* 386 and *m/e* 384 selected for measurement using multiple ion detection. Norethindrone was added to 14,15-D_2-norethindrone in ratios of 2:1000; 5:1000 and 10:1000, and the MO-TMSi ether derivatives were prepared. Aliquots of these mixtures were analyzed on GC–MS while the ions at *m/e* 384 and *m/e* 386 were focused on the electron multiplier using multiple ion detector. A standard curve obtained after injection of aliquots containing 100 ng of 14,15-D_2-norethindrone and 200–1000 pg norethindrone is shown in Fig. 4 (each point in the standard curve is a mean of ten observations).

Known amounts of norethindrone were added to the control milk samples (12–15 ml) taken from women before treatment, Following extraction and purification, the steroid was analysed on GC–MS. The recoveries are shown in Table 2.

RESULTS

Recovery experiments during extraction on Amberlite XAD-2 columns and purification on Sephadex LH-20 columns were made by monitoring the 15,16-^{3}H-norethindrone mixed with the 14,15-D_2-norethindrone. The recoveries were found to be 94.8 ± 2.70 (n = 10) and 69.1 ± 4.86 (n = 10) respectively.

Precision and sensitivity.

Repeated analysis of standard mixtures containing norethindrone (200–1000 pg) was carried out on different days. The coefficient of variation ranged between 4.4–13.7% (Table 1). Recoveries of known amounts of norethindrone added to control milk samples and their analysis on GC–MS are shown in Table 2. It may be seen that different amounts of norethindrone in a milk sample could be analysed with a mean recovery of 104.1% and a precision of 8.2% (range 7.8–8.6%). Thus 1 ng of norethindrone in a milk sample could be analysed with a precision of 7.8% and a mean recovery of 102%.

Table 1. Accuracy and precision of norethindrone analysis by GC–MS method using mid

Injected amount of 14,15-D_2-norethindrone carrier (ng)	Amounts of norethindrone (protein form) (ng)	Mean ± coefficient of variation n^* = 10
100	0.20	0.24 ± 13.7
100	0.50	0.52 ± 4.4
100	1.00	1.03 ± 4.7

n^* is the number of observations.

Table 2. Recovery of known amounts of norethindrone added to control milk samples

Amount of norethindrone added to milk (ng)	Number of experiments	Amount of norethindrone analysed by GC–MS (±SD)	Percentage recovery (±%CV)
1.0	5	1.02 ± 0.08	102 ± 7.8
1.5	5	1.58 ± 0.13	105.3 ± 8.2
2.0	5	2.10 ± 0.18	105 ± 8.6

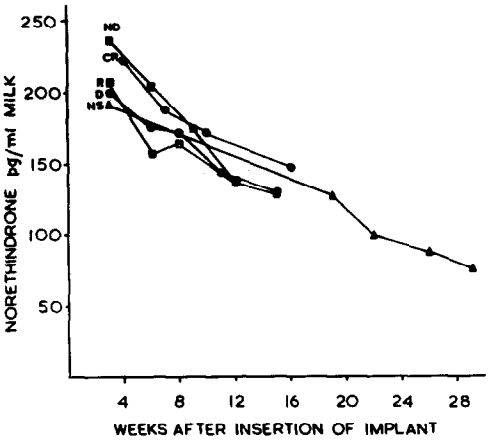

Fig. 5. Levels of norethindrone in milk of five subjects inserted with single silastic implant-D.

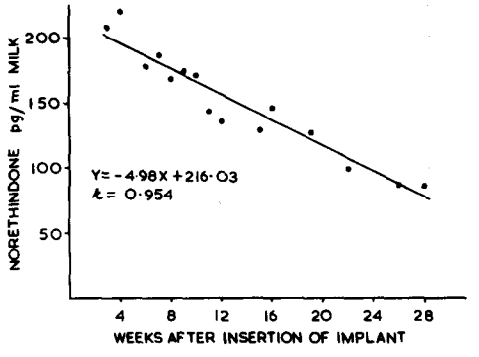

Fig. 6. Rate of decrease in norethindrone levels with time (weeks) after insertion of single silastic implant-D.

Levels of norethindrone in milk.

The concentration of norethindrone in the milk samples taken from five women inserted with single silastic implant D containing 40 mg NET-A, are shown in Fig. 5. The mean level of norethindrone at 3 weeks post insertion was 208 pg/ml. Each subject showed a slow and steady decline in the levels of norethindrone up to 29 weeks of observation. The mean rate of decline in norethindrone levels plotted against time showed a negative regression (Fig. 6).

DISCUSSION

The present study has revealed that gas chromatography–mass spectrometry technique using deuterated carrier and internal standard provides a good method for the quantitative estimation of norethindrone in milk. The results obtained showed that the assay was highly precise and sensitive for norethindrone determination. The percent coefficient of variation observed in this method was comparable to the variation obtained with other assay methods. Furthermore, this method is more specific because the non-specific effect of unexpected metabolites of steroid is eliminated during the extensive purification and gas chromatography of the sample.

In the present study the assay method developed above was used to measure milk levels of norethindrone in women inserted with a single silastic implant D releasing NET-A. The levels of norethindrone showed a steady decline over a period of six months. In another study [8] using the same implant D in lactating women our group has found the serum levels of norethindrone as measured by RIA, to be in the range of 1 ng/ml. Evaluation of our present studies on milk levels thus suggested that the levels of norethindrone in milk were approximately 12% of the serum levels. Similar results were recently reported by Saxena *et al.*[9] who showed that following the oral administration of norethindrone and D-norgestrel in women, the levels of these drugs in milk were about 10% of those in plasma. However, following the injection of medroxyprogesterone acetate the levels in milk were same as those in plasma. Nilson *et al.*[10] showed that oral d-norgestrel resulted in the milk levels which were fifteen percent of the plasma levels.

The present study on milk levels of norethindrone in women inserted with a single implant D showed that a maximum of 125 ng of norethindrone would be transferred into the infant with 600 ml of mother's milk daily. Following the oral administration of 250 μg and 150 μg of D-norgestrel, it has been shown [10] that the intake of the contraceptive by the infant was 500 ng and 300 ng respectively. Thus there would be a lesser intake of the drug by the infant through milk of the women using a single silastic implant D. However, the biological impact of this daily transfer of contraceptive with the mother's milk into the baby remains to be investigated.

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REFERENCES

1. Sweely C. C., Elliot W. H., Frios I. and Ryhage R.: Mass spectrometric determination of unresolved components in gas chromatographic effluents. *Analyt. Chem.* **38** (1966) 1549–1553.
2. Prost M. and Maume B. F.: Hormonal steroids in biological materials: A study by mass fragmentography. In *Mass Spectrometry in Biochemistry and Medicine* (Edited by A. Frigerio and N. Castagnoli). Raven Press, New York (1974) pp. 139–150.
3. Adlercreutz H., Martin F., Wahlroos O. and Soini B.: Mass spectrometric and mass fragmentographic determination of natural and synthetic steroids in biological fluids. *J. steroid Biochem.* **6** (1975) 247–259.
4. Stillwell W. G., Stillwell R. N. and Horning E. C.: Analysis of nanogram quantities of norethindrone in plasma using a GC–MS–COM selective ion detection procedure. *Steroids Lipids Res.* **5** (1974) 79–90.
5. Adlercreutz H., Nieminen U. and Ervast H. S.: A mass fragmentographic method for the determination of megestrol acetate in plasma levels after administration of the progestin to patients with carcinoma corporis uteri. *J. steroid Biochem.* **5** (1974) 619–626.

6. Siekmann L.: Mass fragmentography of steroid hormones. *J. steroid Biochem.* **5** (1974) 727–732.
7. Bhatnagar S., Srivastava U. K., Takkar D., Chandra V. L., Hingorani, V. and Laumas K. R.: Long-term contraception by steroid-releasing implants II. A preliminary report on long-term contraception by a single silastic implant containing norethindrone acetate (ENTA) in women. *Contraception* **5** (1975) 505–523.
8. Laumas V., Malik B. K., Jamal Krira, Seth, U., Agarwal N., Hingorani, V. and Laumas K. R.: Radioimmunoassay of norethindrone: Serum levels of norethindrone in lactating women after insertion of a single silastic implant releasing norethindrone acetate. *Contraception*, **18** (1978) 593–605.
9. Saxena B. N., Shrimanker K. and Grudzinskas J. G.: Levels of contraceptive steroids in breast milk and plasma of lactating women. *Contraception* **16** (1977) 605–614.
10. Nilsson S., Nygren K. G. and Johansson E. D. B.: D-Norgestrel concentrations in maternal plasma, milk and child plasma during administration of oral contraceptives to nursing women. *Am. J. Obstet. Gynec.* **129** (1977) 178–184.