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Isolation, identification and densitometric determination of norethisterone-4β,5β-epoxide after photochemical decomposition of norethisterone

A.G.J. Sedee, G.M.J. Beijersbergen van Henegouwen and H.J.A. Blaauwgeer

Department of Pharmacochemistry, Subfaculty of Pharmacy State University of Leiden, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden (The Netherlands)

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Summary

From the literature it is known that oral contraceptive pills in combination with light can cause side-effects. We have identified norethisterone-4β,5β-epoxide as one of the photoproducts of norethisterone (NE), a progestogenic component of the oral contraceptive pill, upon irradiation with UV light of mainly 300 nm. As a part of the research into the cause of those light-induced side-effects a method was developed for the quantification of this NE-4β,5β-epoxide. With the use of impregnated self-charring TLC plates a linear relationship was obtained between the densitometric signal and the spot content of NE, NE-4β,5β-epoxide and mestranol (ME) in the ranges of 0.5–20.0 μg, 0.5–20.0 μg and 1.2–30.0 μg, respectively. The relative standard deviation is about 2%. The detection limit is low, about 0.1 μg. This densitometric method will be appropriated to determine the oral contraceptive pill components NE and ME with some advantages over other assays.

After 30 min irradiation with 300 nm light at 37°C of a 1.67×10^{-4} M solution of NE in phosphate-buffered saline (pH 7.4) containing 10% ethanol about 23% of the total quantity of photoproducts was NE-4β,5β-epoxide. Kept in the dark for 4 h at 37°C the percentage was raised to 34% without further decomposition of NE. This NE-4β,5β-epoxide may be responsible for some light-induced side-effects of "the pill". There are some reasons to suspect this photochemically formed epoxide to be the cause of some of the non-dermatological side-effects of NE-containing oral contraceptives.

Introduction

Norethisterone (NE; Fig. 1) is given in the treatment of amenorrhoea, functional uterine bleeding and endometriosis in doses up to 30 mg daily, sometimes continued for 9–12 months.

Most frequently used is NE or its 17-acetoxy derivative in a dose of 350 μg to 4 mg, daily in a continuous regimen often as the progestogenic component in the "combined" oral contraceptive pill together with either ME (50–100 μg) or ethinyloestradiol (EE: 35 μg) as the estrogenic components (Fig. 1) (Martindale, 1978).

In the last few years an increasing number of articles have been published about norethisterone-4β,5β-epoxide (Fig. 1) as a possible metabolite of NE. The percentage of the metabolites being NE-4β,5β-epoxide was about 5%, when NE was incubated with the 10,000 *g* supernatant fraction from the livers of phenobarbital-treated beagles (Cook et al., 1974). Kappus showed that NE was enzymatically biotransformed to a reactive metabolite, which was irreversibly bound to proteins, as NE-4β,5β-epoxide did with and without metabolic transformation by hepatic microsomal enzymes of rats (Kappus and Remmer, 1975; Kappus and Bolt, 1976). On the contrary White (1980) could not demonstrate a conversion to NE-4β,5β-epoxide, either with or without 1,2-epoxytrichloropropane, an epoxide hydratase inhibitor, although NE was extensively metabolized in the presence of NADPH and rat liver microsomes. NE-4β,5β-epoxide was also formed in incubations of NE with liver microsomes from rats, pretreated with phenobarbital or 3-methylcholanthrene, to a maximum of about 3% of the total amount of incubated NE or to 15–25% of ethylacetate-extractable metabolites. NE-4β,5β-epoxide inhibited microsomal epoxide hydrolase and glutathione S-transferases, enzymes that are involved in the control of reactive metabolites of environmental carcinogens (Peter et al., 1981). The epoxide showed cytotoxic effects to Walker cells in culture and to rat liver in vivo (White and Suzengar, 1980). From the literature data it can be concluded that the epoxide might be involved in some systemic side-effects of the contraceptive pill such as liver cell tumours. Those contraceptive pills containing NE are also known

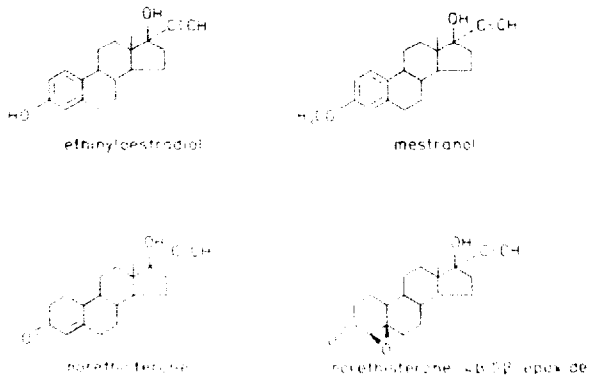

Fig. 1. Chemical structures of the steroids used.

generators of a variety of light-induced side-effects (Jelinek, 1970; Horkay et al., 1975; Mathison and Haas, 1970; Zaun, 1981). Very little is known about the mechanism of induction, about the responsible component (either NE or the estrogenic one, or both) as well as about the photochemical decomposition of the components (Sopirak, 1975 and refs. cited therein). Notwithstanding the smaller doses of the components used the last years, the frequency in occurrence of light-induced side-effects due to oral contraceptives increases (Van Weelden, 1981). Because the dose of NE is much higher than that of the estrogen and because it appeared from our own experiments that NE is photochemically more reactive than EE or ME, we considered NE as the first compound of choice in a research into the cause of light-induced side-effects of oral contraceptive pills. We found that the more so as we got an indication from preliminary experiments that NE can form epoxides on irradiation with UV-light.

In view of its definitive identification and to know to what extent it is formed on irradiation of NE, we have prepared NE-4β,5β-epoxide and developed a densitometric method for its determination. The epoxide was formed in vitro and constituted up to 23% of the total amount of photoproducts at pH 7.4 and 37°C. The epoxide appeared also to be formed subsequently without light from instable photochemical decomposition products. NE-4β,5β-epoxide might be responsible for some light-induced side-effects of oral contraceptives, visible on the skin. Besides, the possibility remains that this photochemically formed epoxide is responsible for some systemic side-effects.

Materials and Methods

Materials

Norethisterone (Sigma), mestranol (Sigma) and ethinyloestradiol (Sigma) were used as purchased without further purification. Research grade ethanol and methanol (Merck), dichloromethane, diethylether, acetone, ethyl acetate and cyclohexane (all "Baker" grade), hexane-mixed isomers ("Baker" Chemicals) and demineralized water were distilled before use. Dodecylsodium sulphonate (Merck), sodium chloride ("Baker" Analyzed Reagent), di-sodium hydrogen phosphate-2-hydrate (Merck), potassium dihydrogen phosphate ("Baker" Analyzed Reagent) were purchased and used as such.

Norethisterone-4β,5β-epoxide was synthesized from NE by H_2O_2 in alkaline methanol and was identified by NMR and mass spectrometry (Cook et al., 1974).

Thin layer chromatography

DC-plastikfolien Kieselgel 60F254 (Merck) with the eluents cyclohexane–ethylacetate (1 : 1, v/v) and hexane–acetone–diethylether (4 : 1 : 1, v/v) were used for the identification and Safety-Kotes self-charring plates (Applied Science) with the eluent hexane–acetone–diethylether (4 : 1 : 1, v/v) were used for the quantification. Before the development the plates were pre-eluted with the eluents to prevent reactions of the photoproducts with impurities on the plates.

Column chromatography

A Lobar Fertigsäule Grösse B (310-25) Lichroprep Si 60 (40–63 μm) (Merck) was used. A flow rate of 3.5 ml/min was applied with a Eldex-Labs inc B-94-2 pump. Eluent was hexane–acetone–diethylether (4 : 1 : 1 v/v).

High-pressure liquid chromatography

A Waters M 45 Solvent Delivery System with a column (20 × 0.28 cm, i.d.) filled with 7 μm RP-2 (Lichrosorb) was used. The solvent system was methanol–water (11 : 9, v/v) containing dodecylsodium sulphonate (40 mg · l^{-1}) at a flow rate of 1.3 ml · min^{-1} (Schüsler-van Hees and Beijersbergen van Henegouwen, 1980). Detection was performed with a LKB 2138 Uvicord-S UV detector (LKB, Sweden) fixed at 206 nm. A Kipp BD 40 recorder (Kipp and Zonen, Delft, The Netherlands) recorded the detector signal.

Absorption spectrometry

The absorption measurements were made at 248 nm on a Zeiss M4Q III spectrometer (Zeiss, F.R.G.) fitted with a digital registration apparatus Optilab Multilog 311 and Multiblank 171 (Optilab AB, Sweden).

Densitometry

After charring at 170°C during 45 min, the spots were measured by reflectance measurements on a Shimadzu CS-920 densitometer (Shimadzu, Japan) at a wavelength of 404 nm.

Irradiation of NE

A solution of 1.67×10^{-4} M NE in water or in phosphate-buffered saline (0.04 M phosphate, 0.4% NaCl, pH 7.0 or pH 7.4) containing 10% or 50% ethanol was divided in quantities of about 13 ml over a number of quartz tubes (15 × 1 × 1 cm). Subsequently the tubes were placed in a merry-go-round and irradiated at room temperature or at 37°C in the Rayonet Photochemical Reactor (RPR-208, Southern New England Ultraviolet) with two lamps (RUL 3000 Å). The distance between the lamps and the tubes was 8 cm. The UV energy on the tubes was about 1250 μW · cm^{-2} as measured with a UVX-31 radiometer sensor (UV Products, U.S.A.).

Preparative irradiation of NE

A solution of NE (20 mg/100 ml = 6.67×10^{-4} M) in saline (pH 7.4)–ethanol (1 : 1, v/v) was irradiated after being divided over quartz tubes with 2 lamps during 45 min.

Isolation and identification of NE-4β,5β-epoxide

After preparative irradiation, the solution was extracted two times with dichloromethane. The solvent was evaporated at a temperature below 15°C and the residue was resolved in 10 ml of the eluent, filtered through a 5 μm filter (Millipore, London) and applied to the Lobar column. After the first 175 ml, which did not contain NE or its photoproducts, fractions of 3.5 ml were collected and analyzed by

TLC in a saturated tank by developing twice with a solvent of the same composition as mobile phase. Spots were visualized by spraying with H_2SO_4–methanol (8 : 2, v/v) followed by heating at 110°C during 5 min. After 210 ml of the eluent, pure NE-4β,5β-epoxide came from the column in about 25 ml.

Quantification of NE-4β,5β-epoxide and NE

During the irradiation, which was performed as described, a tube was taken out of the Rayonet after fixed time intervals. A quantity of 1.00 ml of the irradiated solution was diluted with 20% ethanol in H_2O and the concentration of NE was determined on the Zeiss M4Q III spectrometer at 248 nm. 10.00 ml of the irradiated solution was extracted with 1.00 ml of a solution of the internal standard (0.25×10^{-3} M mestranol) in dichloromethane on a Vortex Mixer. Also 10.00 ml of two solutions with known concentrations of NE and its epoxide (0.60×10^{-4} M NE, 0.30×10^{-4} M epoxide and 0.30×10^{-4} M NE, 0.15×10^{-4} M epoxide, respectively) were extracted. Dependent on the content of a steroid, a quantity of each organic layer was spotted on the plate; it was developed twice and charred. The plate was equilibrated with the atmosphere during an hour after which densitometric determination of the spots was performed.

Results and Discussion

After spotting the isolated epoxide and the synthesized epoxide, developing the plate twice in one direction and visualization, two spots result with the same R_f, and the same rose colour and blue fluorescence at 360 nm. A mixture of the isolated and synthesized epoxide yields one single spot on two-dimensional TLC after developing twice in both directions.

The mass spectra of the epoxides are identical. From these results it is concluded, that NE-4β,5β-epoxide is formed photochemically.

To quantify the epoxide we have developed a densitometric determination because there is no such method described in the literature that is suitable for our purpose. Gas chromatography cannot be used because of the thermolability of the products and of the necessity of derivatization, possibly attended by unknown chemical shifts. Although separation of NE, its epoxide, other photoproducts and EE as internal standard is possible with HPLC (Fig. 2), this method is not appropriate because of the lack of a sensitive detector for the used concentrations.

With TLC we got a good separation between the photochemical products. Moreover, steroids in less complicated mixtures have also been quantified densitometrically after TLC separation (Cullen et al., 1968; Albers and Lisboa, 1979; Amin and Hassenbach, 1979; Huf et al., 1979; Jarzębiński et al., 1979; Shroff and Shaw 1972; Touchstone et al., 1972a, 1972b) even after derivatization (Penzes and Oertel, 1970).

By using self-charring, impregnated plates, the best reproducibility is obtained (Touchstone et al., 1972a; Albers and Lisboa, 1979). In our case the separation with the impregnated plates is still better than with normal silica plates. The intensity of

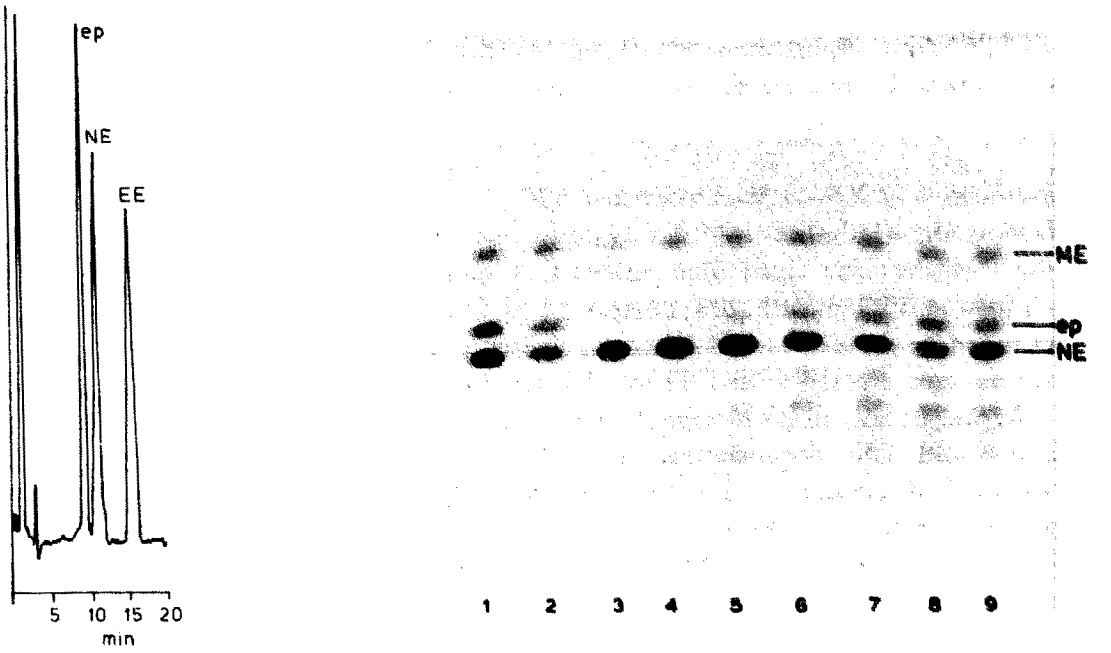

Fig. 2. HPLC chromatogram of 100 µl of a solution of 3×10^{-4} M norethisterone-4β,5β-epoxide, 2×10^{-4} M NE and 5×10^{-5} M EE.

Fig. 3. Thin-layer chromatogram of an irradiation experiment of 1.67×10^{-4} M solutions of NE (pH 7.4 and 37°C). Bands 1 and 2: reference solutions with known concentrations. Bands 3–9: solutions after 0, 2.5, 7.5, 15, 20, 30 and 60 min irradiation. 100 µl of each organic layer was spotted with the exception of band 3 (40 µl), band 4 (75 µl) and band 5 (75 µl).

each spot does not change over a period of hours in contrast to other TLC detection methods used (Albers and Lisboa, 1979; Cullen et al., 1968). In one week the decrease in intensity is only a few percent. This causes no inconvenience, because solutions of known concentrations of the compounds being determined are also applied to the plate and the spots with unknown amounts are related to them.

The spots, being circular after application, appear as narrow bands, well separated from each other, making the determination of each compound easy (Fig. 3). Moreover, by the formation of these narrow bands the compounds are concentrated on a small area giving a low detection limit of about 0.1 µg for NE and its epoxide and of about 0.2 µg for ME.

Applying various amounts of NE, NE-4β,5β-epoxide and ME in dichloromethane, linear calibration curves between signal and concentration were obtained in the range 0.5–20.0 µg, 0.5–20.0 µg and 1.2–30.0 µg, respectively (Fig. 4). By means of the internal standard, corrections are possible for differences in charring capacity in the plate, for evaporation of dichloromethane and for errors in taking and spotting the 100 µl quantity.

Determination of 8 quantities of 10.00 ml of a solution of 2×10^{-5} M NE and 2×10^{-5} M epoxide in the same way as the irradiated solutions gives densitometric

integration values of 25794 ± 502 and 22043 ± 465, respectively. The results show that with this method accurate values of the quantities of NE and its epoxide in the irradiated solutions can be obtained.

The UV-spectrometric data are in accordance with the densitometric ones indicating that the decomposition products do not interfere. As expected from a photochemical point of view, the chromophore of the α,β-unsaturated ketone, absorbing at 248 nm, is destroyed. So the non-interference of the products in the UV-spectrometric method is comprehensible. Because of its rapidity and simplicity this UV-spectrometric method has been also used to follow the decomposition of NE, as time went on, and to determine NE at its highest concentrations. At those concentrations the response of the densitometer to the spot content of NE is not entirely linear.

Although outside the aim of this study it is noteworthy that this densitometric method will be appropriate to determine both components of the oral contraceptive pill, NE and ME. Evidently another internal standard has to be used. This method has some advantages over other assays. Both components can be quantified by the same method of detection as distinct from other TLC methods (Shroff and Shaw, 1972; Amin and Hassenbach, 1979). Moreover, the intensity of each spot is stable.

In a test on content uniformity of "the pill" this method will also be suitable to detect and to determine small amounts of steroidal impurities and decomposition products (including NE-4β,5β-epoxide), which is not possible with HPLC methods (Sundaresan et al., 1981; Görög, 1981). Applying this densitometric method to solutions of NE irradiated under different circumstances, it has appeared that a number of factors affected the yield of 4β,5β-epoxide, e.g. the percentage ethanol, the pH, the temperature during irradiation, the amount of oxygen in the solution and the manner of handling the samples. For some reaction conditions the results are presented in Fig. 5. Being interested in the cause of the photosensitivity of women due to the oral contraceptive pill, it is important to choose the reaction conditions in such a way that the data obtained has some relevance to the in vivo situation. Irradiation was performed with UV-B light ($\lambda_{max} = 300$ nm), which is responsible for processes such as, e.g., sunburn and vitamin D_3 production. The intensity applied was 1250 $\mu W/cm^2$, quite normal for a sunny day in May in Holland.

When the experiment is done at pH 7.4 and 37°C, the amount of 4β,5β-epoxide is about 23% of the total quantity of photoproducts being formed after 30 min of irradiation. When the solution after 30 min irradiation is kept at 37°C for 4 h in the dark even more NE-4β,5β-epoxide is formed, up to an amount of 34%, while NE does not decompose further. This epoxide, photochemically formed to such a high extent under circumstances relevant to the situation in vivo, may be responsible for some light-induced side-effects of "the pill".

There are reasons to suspect the epoxide of other side-effects. Compounds photosynthesized in the skin in a very low concentration can have an important biological effect elsewhere in the body (Beijersbergen van Henegouwen, 1981). This is demonstrated by the formation and activity of the endogenous compounds, vitamin D_3, a compound with a steroid-related structure (Holick, 1981). That exogenous chemicals such as drugs can also have a tremendous influence on the

Fig. 4.

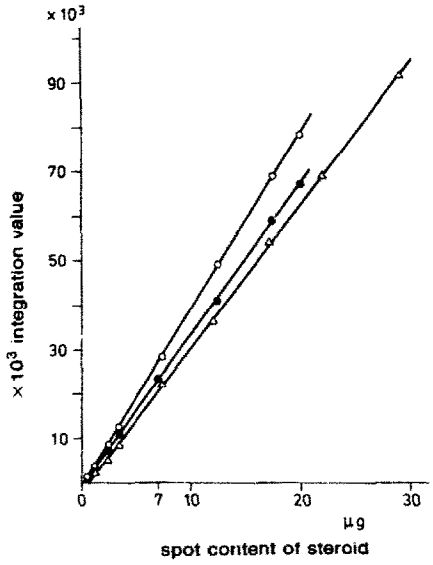

Fig. 5.

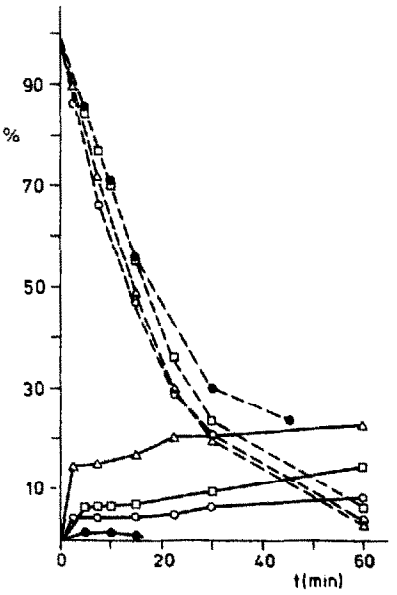

Fig. 4. Plots illustrating the linearity in response of the densitometer to the spot content of the steroids.

Compound	Line	LR *	Slope (μg^{-1})	Intercept (μg)	n
ME	△——△	0.9996	0.00030	0.8	8
NE	○——○	0.9997	0.00025	0.4	8
NE-4β,5β-epoxide	●——●	0.9998	0.00029	0.3	8

* LR = linear regression (correlation coefficient) = r^2.

Fig. 5. Irradiation (λ_{max} = 300 nm, UV-energy = 1250 $\mu W \cdot cm^{-2}$) of NE (1.67×10^{-4} M) under different circumstances.

	pH	Temp. (°C)	n
●——●	unbuffered solution	20	2
○——○	7.0	20	2
□——□	7.4	20	2
△——△	7.4	37	3

n is the number of experiments. Percentage of NE (dotted curves) and NE-4β,5β-epoxide as percentage of the total quantity of photoproducts (solid curves).

inner organs after being irradiated in the skin is illustrated by the research of Bakri (1982). Rats, treated with chlordiazepoxide and irradiated with UV light of 350 nm show, e.g., a quantitatively different pattern of urinary metabolites and covalent

binding of this compound in the liver leading to dysfunction and necrosis of this organ. There is evidence that the effects are caused by reactive oxaziridines formed from photoexcited chlordiazepoxide or its N_4-O metabolites, present in the skin (see also Cornelissen, 1979, 1980). NE-4β,5β-epoxide is stable in aqueous phosphate buffer, pH 7.4 at 37°C, for at least one hour (White, 1980).

The lifetime in vivo of NE-4β,5β-epoxide is probably long enough to leave the skin and to be transported to the inner organs, because skin microsomes have only 6% of the corresponding liver microsomal epoxide hydratase activity and skin cytosol has 15% of the hepatic S-transferase activity in the non-pretreated rat (Mukhtar and Bickers, 1981). So it is possible that NE-4β,5β-epoxide, reaching the inner organs, provokes some of the non-dermatological side-effects of NE-containing oral contraceptives.

In connection with the above about the reactive oxaziridines, we are currently investigating the possibility of covalent bonding to DNA, to protein and to glutathione upon photoexcitation of NE as well as the nature of its other photoproducts.

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