Irreversible Binding of Norethisterone to Human Serum Protein Induced by UV-B Light

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Abstract: In as much as oral contraceptive ingestion has been associated with photoallergic effects, the irreversible binding of 4-^{14}C-norethisterone to proteins catalyzed by UV-B light (290–320 nm) was investigated. Irreversible binding to a human plasma protein mixture and to albumin of the radiolabeled progestogen was linear for 40 min, when mixtures of protein and steroid were irradiated with UV-B light. Approximately 16 % of the radiolabel was irreversibly bound under the conditions of the assay. Irreversible binding also occurred, when proteins were added several hours after irradiation. Preliminary experiments in rats administered 4-^{14}C-norethisterone also indicate the formation in skin and liver of irreversibly bound adducts under photoreactive conditions. Such reactions may in part be responsible for some of the side-effects associated with oral contraceptive use.

Adverse effects may be caused by the combined exposure of the body to xenobiotics and sunlight (1). The contraceptive pill is an example of an agent that can cause a variety of light-induced side-effects (2, 3, 4). Both the mechanism of induction and the component of the pill that is responsible for the effect (either the progestogenic or the estrogenic one, or both) are unknown.

A frequently used progestogenic component is norethisterone. Norethisterone is also a principal metabolite of some of the most important 19-norprogestogens (5). Another frequently used progestagen, norgestrel, and the most important metabolite (6) of desogestrel, a new progestational compound, have the same conjugated ketone function as norethisterone (Fig. 1). It is this chromophore that is the photoreactive part of the molecule. Norethisterone is applied in a higher dose and it is more photolabile than the estrogenic components, ethinylestradiol and mestranol. We therefore consider norethisterone as the first component of choice in an investigation of the cause of light induced side-effects of oral contraceptive pills.

One of the main photochemical decomposition products of norethisterone in a phosphate buffered solution at pH 7.4 is norethisterone-4β, 5β-epoxide (7). This epoxide is stable at 37° C and pH 7.4 for at least one hour (8) and it binds irreversibly to proteins, the more so after enzymic activation (9). Furthermore, products (10) are formed by addition of solvent molecules to the conjugated ketone group upon excitation by UV-B light (280–310 nm). Steroids also form reversible complexes with serum proteins, receptor proteins of target tissue, and steroid metabolizing enzymes. The conjugated ketone function of norethisterone is essential for this binding (11). The possibility that reactive products are photogenerated in the presence of proteins has prompted us to investigate whether or not norethisterone binds irreversibly to proteins under the influence of UV-B light. Such binding could provide an explanation for allergic side-effects and possibly some systemic side-effects of the oral contraceptive pill (3, 4, 12, 13).

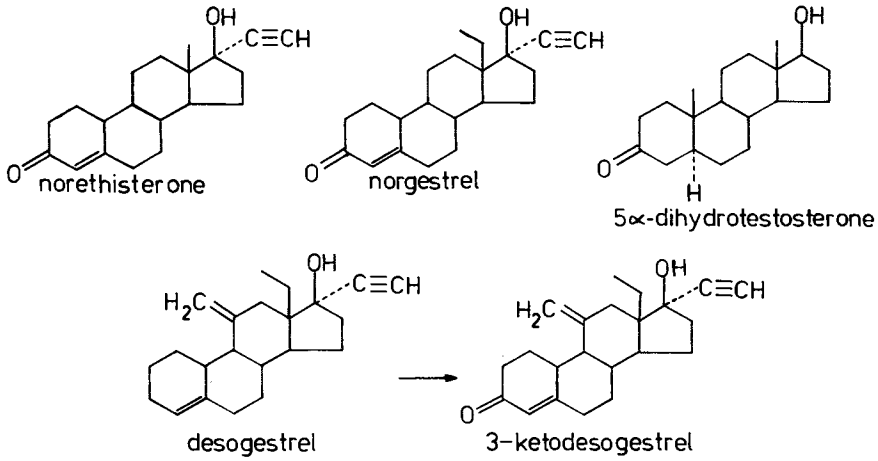

Fig. 1.

Materials and Methods

Materials

Norethisterone and 5α-dihydrotestosterone (both from Sigma) were used as purchased without further purification. 4-^{14}C-Norethisterone (specific activity 1.998 GBg.mmol^{-1}), a generous gift from Schering AG Berlin, was purified by means of TLC (DC-Fertigplatten Kieselgel 60 F_{254} [Merck], hexane-acetone-diethylether, 4 : 1 : 1, v/v) (> 96.5 % radiochemical purity) and diluted with norethisterone to a specific activity of 2.5×10^{11} Bq.mol^{-1}. Petroleum ether, 60–80° (Brocacef), research grade ethanol and methanol (Merck), dichloromethane, diethylether and acetone (all "Baker" grade), hexane mixed isomers ("Baker" chemical) and demineralized water

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were distilled before use. Ammonium sulphate (Merck), disodium hydrogen phosphate 2-hydrate (Merck), potassium dihydrogen phosphate (Baker analyzed reagent) were purchased and used as such.

Pasteurized plasma solution was a gift from the Central Laboratories of Blood Transfusion Services of the Dutch Red Cross, Amsterdam. It was distributed in quantities of 1.0 ml into 2.0 ml ampoules and stored at −20° C. Crystallized and lyophilized albumin was bought from Sigma, Sephadex G-25 (20–80 μ) from Pharmacia (Uppsala, Sweden), and Amberlite XAD-2 (300–840 μ) from BDH Chemicals Ltd. (Poole, England). The DC-Fertigplatten Kieselgel 60 F_{254} (Merck) were pre-eluted with the eluent (hexane-acetone-diethylether, 4 : 1 : 1, %).

Irradiation

The pasteurized plasma solution (1.0 ml), albumin solution (38 mg/ml, 1.0 ml) or buffer (pH 7.4, 1.0 ml) were added to 4.0 ml of a 5×10^{-7} M solution of ^{14}C-norethisterone in phosphate buffer (0.04 M phosphate, pH 7.4) in a quartz tube (15 × 1×1 cm). The tubes were incubated at 37°C for half an hour and irradiated with 2 UV-B lamps at a distance of 10 cm as described before (7).

Column chromatography

Glass columns (40 × 2 cm I.D.) were filled to a height of 9 cm with Sephadex G-25 that had been allowed to swell overnight in the eluent methanol-water (15 : 85). The upper surface of the gel bed was protected with a layer of seasand (Merck, zur Analyse). After a steroid-protein sample of 2.0 ml was applied, the column was eluted under pressure (2 cm Hg). The elution of protein was detected by monitoring the absorbance at 254 nm with a LKB 8300 Uvicord II photometer. Fractions of 7.0 ml were collected and added to 5 ml Instagel (Packard-Becker, Groningen, The Netherlands). The amount of radioactivity in the fractions was determined with a Packard Tricarb Liquid Scintillation Spectrometer, model 3310. The efficiency was calculated by the ISCR method. To the collected fractions containing free steroid Amberlite XAD-2 (5 g, dry weight) was added. After stirring for 30 min the suspension was filtered via a glass column (50 × 1 cm I.D.) provided with a piece of sintered glass. A quantity of 7 ml petroleum ether dispelled the remaining water from the column (14). Ethanol (60 ml) was used to elute the steroids from the Amberlite resin.

Determination of the decomposition of norethisterone

The ethanol was evaporated and the residue was redissolved in dichloromethane. After thin layer chromatography (DC Fertigplatten Kieselgel 60 F_{254} [Merck], hexane-acetone-diethylether, 4 : 1 : 1, %) and localization of the radioactive spots by scanning the plate on a Berthold Thin Layer Radioactivity Scanner, type LB 2722, the spots containing norethisterone and the rest were scraped off separately. By counting these two parts and the rest of the residue the ratio between norethisterone and the decomposition products as well as the total radioactivity of the residue could be calculated. Because the efficiency of the XAD column is not the same for all steroids, the efficiency for norethisterone was determined (92.7 ± 0.4 %; n = 6). Knowing this efficiency, the quantity of norethisterone in the residue and the extent of irreversible binding, the decomposition of norethisterone could be determined.

Dialysis experiments

Samples (2.0 ml) of a solution of norethisterone in plasma irradiated for 30 min were dialysed in cellulose tubing (Visking no. 16) during three days. The bulk volume of 15 % methanol in water (5 l) was changed three times. The radioactivity remaining in each dialysis bag was determined.

Competitive binding assay

Samples of a norethisterone-protein solution irradiated for 50 min to which a 40 or 75 fold excess of dihydrotestosterone was added, were incubated 4, 24 or 60 hours at 22° C. Each sample was filtered on the Sephadex G-25 column, and the radioactivity of the protein fraction and of the fraction containing free steroid was determined.

Globulin precipitation

An equal volume of a saturated ammonium sulphate solution was added to a known quantity of a norethisterone-plasma solution at 0° C and mixed by vortexing. The sample was centrifuged, and 2.0 ml of the supernatant was pipetted on to the Sephadex column to determine the binding.

Results

Filtration on Sephadex G-25 of a protein solution that contained labeled norethisterone resulted in one peak of radioac-

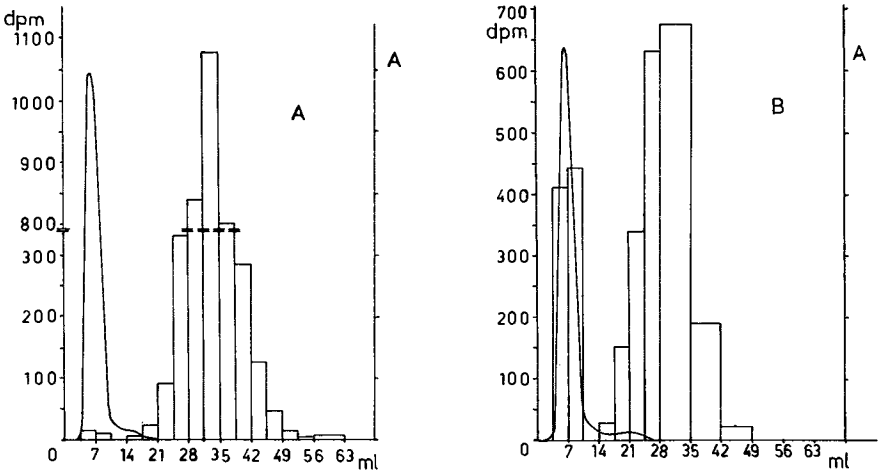

Fig. 2 Sephadex G-25 column chromatography of a 4-^{14}C-norethisterone-plasma solution before irradiation (A) and after irradiation for 40 min (B). The continuous lines indicate absorption of proteins at 254 nm. The histograms represent disintegrations per minute per ml eluent in sequential fractions of the column effluent.

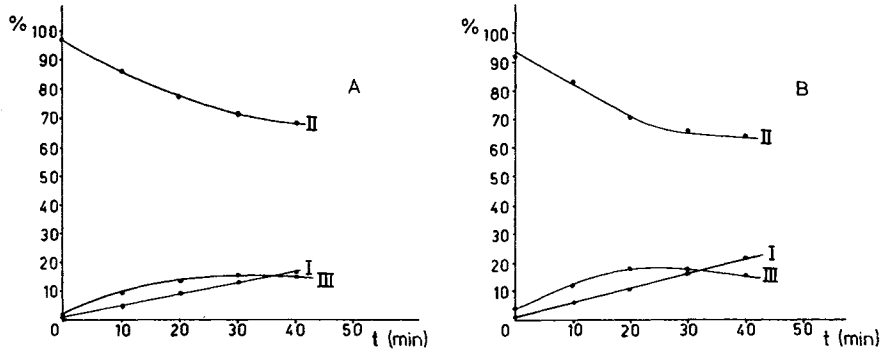

Fig. 3 Extent (%) to which norethisterone is decomposed and bound irreversibly to plasma proteins (A) and to albumin (B) as a function of the time (min) of irradiation.
I – norethisterone and decomposition products irreversibly bound
II – norethisterone non-reacted
III – photoproducts and impurities

tivity before irradiation and in two well separated peaks after irradiation of the solution (Fig. 2). The first of these two peaks was coincident with the peak of the protein fraction which indicates that the steroid was irreversibly bound to protein. The second peak, which was also obtained when steroids were chromatographed after irradiation without protein, represented free and reversibly bound norethisterone as well as reaction products. The recovery on the Sephadex column was 99 ± 2 % (n = 11). The extent to which steroid had been irreversibly bound to protein as a function of the irradiation time is represented in Figure 3. After 40 min of irradiation 16 % of the radioactivity was irreversibly bound to the protein fraction and 15 % was present as photochemical decomposition products and impurities. Chromatography on Sephadex G-25 of samples irradiated for 30 min gave a value of 12.9 ± 0.4 % (n = 5) binding to protein; the radioactivity in the dialysis bags was 11.9 ± 0.4 % (n = 7) after dialysis.

Statistical evaluation of the results showed that Sephadex G-25 column chromatography and dialysis gave the same results. The distribution of radioactivity between the protein and the steroid fraction after addition of dihydrotestosterone was approximately the same as before (Table 1). To distinguish between the irreversible binding of norethisterone to either albumin or sex hormone binding globulin, ammonium sulphate was added. The binding found in the supernatant was 14.6 % instead of the original 16.4 %.

An albumin-norethisterone solution (38 mg/ml, pH 7.4) showed 20 % irreversible binding of the norethisterone radioactivity to the protein after 40 min irradiation (Fig. 3). Addition of dihydrotestosterone did not give a diminution of this association after Sephadex G-25 column chromatography (Table 1). The solution of norethisterone without protein (4.0 ml) was irradiated with UV-B light during 30 min and resulted in a decomposition of 58 %. Addition of 1.0 ml of pasteurized plasma solution immediately after irradiation followed by one hour incubation at 37° C gave an irreversible binding of 8.9 % of norethisterone. This binding was reduced to 8.3 %, 7.6 % and 6.7 % if the protein solution was added 20 min, 90 min and 22 hours, respectively, after irradiation.

Table 1 Influence of addition of dihydrotestosterone on the irreversible binding of norethisterone to protein after irradiation for 40 min.

	percentage of decomposition	percentage of irreversible binding	addition of dihydrotestosterone: excess	incubation time (h)	percentage of irreversible binding
I	32.8	16.7	75 ×	4	16.9
I	33.1	16.6	75 ×	24	16.2
I	31.4	16.4	40 ×	60	16.3
II	36.2	20.3	40 ×	60	22.4
III	71	12.3	40 ×	60	11.5

I – norethisterone plasma sample
II – norethisterone albumin sample
III – norethisterone solution to which plasma is added immediately after irradiation

Discussion

Using a variety of techniques we have demonstrated that irradiation of the progestogen, norethisterone, leads to products that irreversibly bind to plasma proteins. The bound product(s) were not released by chromatography over Sephadex G-25, dialysis, or displacement by concentrations of dihydrotestosterone that are known to displace reversibly bound progestogens from sex hormone binding globulin (15), which is the major protein that reversibly binds norethisterone in plasma (16). Ammonium sulphate precipitation of globulins from plasma samples that contained ^{14}C-norethisterone irreversibly bound to protein, showed that the majority (~90 %) of this binding was to the albumin fraction rather than the globulin fraction. Interestingly, this irreversible binding of reactive photo-products of norethisterone can occur to proteins even when the proteins are added several hours after irradiation. Thus, photoproducts formed in one tissue may react with proteins in other tissues.

UV-B light is a normal part of sunlight, and approximately 5 % at 290 nm, 30 % at 300 nm, and 40 % at 320 nm reaches the capillaries in the dermis (17). The energy output of the lamps used was 1250 μW cm^{-2} as measured near the tubes with a UVX-31 radiometer sensor (UV Products, Inc., USA). This situation is comparable with a sunny day in May in Holland (52° North latitude). Therefore, irreversible binding of norethisterone could occur *in vivo* (in capillary blood) in the dermis, and photoproducts formed there might react with endogenous compounds in inner organs. To test this hypothesis we carried out preliminary experiments in rats which were injected intraperitoneally with 100 μg of ^{14}C-norethisterone. Those animals that were irradiated for 3 hours with UV-B light immediately after injection had significantly more radiolabel irreversibly bound to proteins in both skin and liver than animals that were not irradiated.

At the present time the significance of the irreversible binding of norethisterone to proteins catalyzed by UV-B light is unknown. However, irreversible binding of other xenobiotics to proteins is known to cause allergic reactions (18), and even liver damage in rats has apparently resulted from combined exposure to the drug, chlordiazepoxide, and UV-A light

(19). Furthermore, the photoproduct, cholesterol-5α,6α-epoxide, is suspected of inducing skin cancers (20), and 7-dehydrocholesterol rearranges in the skin under photoirradiation to vitamin-D_3 which effects calcium stasis (21). Thus, our results on the irreversible binding of the commonly used progestogen, norethisterone, to plasma proteins may be important in the further delineation of side-effects of oral contraceptives.

Acknowledgements

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Site Specific Rectal Drug Administration in Man with an Osmotic System: Influence on "First-Pass" Elimination of Lidocaine

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Abstract: Lidocaine was administered to healthy volunteers at different sites in the rectum. Unchanged drug and monoethylglycinexylidide (MEGX) concentrations were measured in plasma with a newly developed gas chromatographic method. Lidocaine was given rectally by means of an osmotic system (Osmet®) which delivered 25 mg/h at zero-order rate. In a pilot experiment in two subjects it was shown that lidocaine administration close to the anus for 5 h resulted in higher lidocaine plasma levels as compared to administration at 15 cm from the anus. Six other subjects participated in three separate experiments, in which lidocaine was administered rectally close to the anus and at 7.5 and 15 cm from the anus. A zero-order infusion plasma level profile was found for both the parent compound and its metabolite. The MEGX/lidocaine plasma concentration ratio was calculated for all experiments. After administration most proximal to the anus the mean metabolite/parent drug concentration ratio was significantly less than that obtained after administration at 15 cm from the anus, whereas at approximately 7.5 cm from the anus the values were in-between. Comparison of the AUC lidocaine/AUC MEGX ratios gave similar results; the highest value, 3.2 ± 1.3 (mean ± S. D.), was found after administration close to the anus, while at 15 cm from the anus the ratio was 1.6 ± 0.3 ($p < 0.01$). The terminal elimination half-lives of lidocaine and MEGX did not differ for the three sites of administration, and the mean values were 110 and 180 min respectively. The results of this study demonstrate that the site of drug administration in the human rectum determines the degree of hepatic "first-pass" elimination of high-clearance drugs. Maximal avoidance of presystemic elimination is achieved when administration takes place close to the anus.

Lidocaine is subject to extensive hepatic "first-pass" elimination when given orally, which gives rise to low and variable systemic availability (1, 2). A more appropriate route for high-clearance drugs like lidocaine, propranolol, some narcotic analgesics and nitroglycerin, should be non-hepatic and non-invasive. Rectal, dermal and buccal routes of drug administration have been suggested as alternatives (3, 4, 5). We have been particularly interested in the rectal route, because it has been recently shown in man that it is in principle possible, at least partly, to bypass the liver. When lidocaine was administered rectally as an enema, the systemic availability was doubled as compared to oral administration to the same subjects (6). In rats complete avoidance of hepatic "first-pass" elimination was found rectally with propranolol and lidocaine (7, 8). The partial avoidance of hepatic presystemic elimination in man can be explained by the venous drainage of the rectum (9). The lower and probably also the middle rectal hemorrhoidal veins pass the absorbed drug directly into the inferior vena cava. More upwards in the rectum the drug passes into the upper hemorrhoidal vein which is connected to the

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