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The isolation and identification of some degradation products of flurandrenolide in Cordran cream *

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Summary

Isolation and identification of the major degradation products of flurandrenolide occurring in its commercially marketed cream base are reported. The syntheses of these agents are described. An extraction procedure and reversed-phase HPLC assay are presented, the basis of the assay being separation of acidic degradation products using an ion pairing agent. Comparison of the HPLC assay with the blue tetrazolium (BT) assay showed the chromatographic assay to be superior to the colorimetric assay. This was principally due to a major degradation product giving a positive response to the B.T. assay. The major degradation products observed were the C_{20}-aldehyde of flurandrenolide along with the C_{20}-carboxylic acid and the C_{17}-carboxylic acid.

Introduction

In the course of developing a high-performance liquid chromatographic (HPLC) assay for flurandrenolide (I) in its commercially marketed cream formulation (Cordran, Lilly), comparison was made with the blue tetrazolium (BT) assay. While agreement between the two methods was excellent using freshly prepared cream, the

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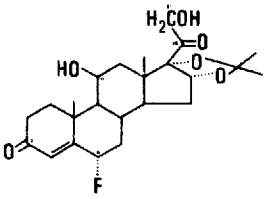


Flurandrenolide, I

HPLC assay indicated lower levels of drug present when aged cream samples (t > 1 year) were analyzed.

Examination of the HPLC traces of extracts of aged cream showed several additional peaks which were not present in freshly prepared cream. An investigation of the stability of flurandrenolide in the cream base was thus undertaken.

While numerous studies have investigated the degradation of corticosteroids in vitro (Guttman and Meister, 1958; Marcus, 1960; Osterling and Guttman, 1964; Pitman et al., 1972) only recently have the products of these reactions been isolated and identified (Hansen and Bundgaard, 1979a and b, 1980a and b).

Degradation of corticosteroids in actual formulations has been addressed by several authors (Allen and Gupta, 1974; Gupta, 1978; Yip and Po, 1979; Hidaka et al., 1980; Shek et al., 1982); however, isolation and identification of degradation products from aged cream samples has not been reported.

The present work describes the isolation and identification of the major degradation products of flurandrenolide in its commercially marketed cream base [1]. The approach taken in elucidating the degradation profile of this compound was 2-fold. The first phase involved the identification of compounds present in aged cream samples after separation by preparative high-performance liquid chromatography. The second required the selective synthesis of potential degradation products and identifying the presence of such compounds in aged cream via analytical HPLC.

Materials and Methods

Synthetic procedures

All materials used were of reagent grade. Flurandrenolide was obtained through Lilly raw material stores. Each compound synthesized was characterized via elemental analysis, NMR (Bruker model WH 360), IR (Beckman model IR 13) and mass spectral analysis (Finnigan-MAT model 731 with Field Desorption Probe).

(6α,11β,16α)-6-Fluoro-11,21,21-trihydroxy-16,17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3,20-dione (III)

The procedure followed was based upon that of Lewbart and Mattox (1963) utilizing the divalent metal ion oxidation of the α-ketoalcohol. Flurandrenolide, 600

[1] Cordran cream, also marketed as Haelan or Sermaka in Europe, contains propylene glycol, polyoxyl 40 stearate, stearic acid, cetyl alcohol, mineral oil, citric acid, sodium citrate and purified water.

mg (1.4×10^{-3} moles), was dissolved in 150 ml of methanol. To this was added 31.5 mg (1.7×10^{-4} moles) of cupric acetate and the mixture was stirred until a solution formed. Air was bubbled through the solution via a gas dispersion tube for 60 min, the reaction being followed on HPLC. The reaction was quenched by the addition of 50 mg of disodium EDTA dissolved in 20 ml of water. The solution was evaporated to 30 ml under reduced pressure and the product extracted with methylene chloride. The methylene chloride was dried over anhydrous magnesium sulfate, filtered and evaporated. Recrystallization from aqueous methanol gave fine white needles in excess of 80% yield—m.p. 168–170°C; mass spec M^+ 434, 377 (M^+ corresponds to the anhydrous glyoxal); NMR and IR were consistent with the structure. Analysis calculated for $C_{24}H_{33}O_7F$—theory: C 63.70, H 7.35; found: C 63.88, H 7.65.

(6α,11β,16α)-6-Fluoro-11-hydroxy-16,17-[(1-methylethylidene)bis(oxy)]-3,20-dioxopregn-4-en-21-oic acid (IV)

The procedure was adapted from the report of Shamma and Rodriguez (1968). To a solution of 500 mg (1.1×10^{-3} moles) of III in 50 ml of absolute ethanol silver nitrate, 450 mg (2.65×10^{-3} moles), in 2 ml of water was added. A solution of 800 mg of potassium hydroxide in 30 ml of water was prepared and added dropwise to the ethanolic solution with stirring. A whitish precipitate formed rapidly which changed to a brown color during the addition process. HPLC indicated the reaction to be complete within 10 min of the last addition. The precipitate was removed from the solution by filtration after being washed with an equal volume of water. The basic filtrate was evaporated under vacuum to about 30 ml and extracted twice with ether to remove any remaining starting material. The pH of the aqueous phase was lowered to pH 2 and the unionized acid extracted with chloroform. This solution was dried over anhydrous magnesium sulfate. Evaporation of the filtered solution yielded a yellow gummy resin. Recrystallization from methanol–isopropyl ether gave fine white crystals in a 40% yield—m.p. 144–146°C; mass spec M^+ 450, 407, 377; NMR and IR were consistent with the structure. Analysis calculated for $C_{24}H_{31}O_7F$—theory: C 63.99, H 6.94; found: C 64.07, H 7.00.

(6α,11β,16α,17β)-6-Fluoro-11-hydroxy-16,17-[(1-methylethylidene)bis(oxy)]-3-oxoandrost-4-ene-17-carboxylic acid (V)

The method was derived from that of Pesez and Bartos (1962). Flurandrenolide 500 mg (1.1×10^{-3} moles) was dissolved in 100 ml of methanol. To this was added 120 mg (2.1×10^{-3} moles) of potassium hydroxide. After 1 h, an additional 60 mg (1.1×10^{-3} moles) of potassium hydroxide were added and the depletion of flurandrenolide was monitored by HPLC. Additional amounts of potassium hydroxide were added to force the reaction further toward products. The volume of solution was reduced to about 50 ml under vacuum, and 30 ml of water were added. The basic solution was washed with 3 × 20 ml of ether to remove any starting material or unionized products. The pH of the aqueous phase was lowered to 2 units and the mixture extracted with chloroform. The chloroform was dried with anhydrous magnesium sulfate, filtered and evaporated. Recrystallization from isopropyl ether–chloroform yielded a fine white powder in a 60% yield—m.p. 286–288°C;

mass spec M^+ 423, 407, 377; NMR and IR were consistent with the structure. Analysis calculated for $C_{23}H_{31}O_6F$—theory: C 65.39, H 7.40; found: C 65.46, H 7.43.

(6α,16α)-6-Fluoro-21-hydroxy-16,17-[(1-methylethylidene)bis(oxy)]pregn-4-ene-3,11, 20-trione (VI)

The method was adapted from Corey and Suggs (1975). Pyridinium chlorochromate, 400 mg (1.9×10^{-3} moles), was suspended in 7 ml of dichloromethane. A solution of flurandrenolide, 550 mg (1.3×10^{-3} moles) in 20 ml of dichloromethane, were added and the mixture was stirred for 2 h.

The dark brown mixture was reduced in volume to about 5 ml and passed through a prewetted Florisil pad in a 60 ml sintered glass funnel. The pad was washed with several portions of dichloromethane until all of the green band was collected, leaving behind a dark brown layer on the surface of the Florisil pad.

The green solution was evaporated under vacuum to yield a greenish gum. This gum was dissolved in acetone–methanol and treated with several portions of decolorizing charcoal resulting in an almost colorless solution. Evaporation of the solvent yielded an off-white, slightly greenish precipitate with a faint pyridine odor.

This soiid was dissolved in dichloromethane, 10 ml, and washed with two 5 ml portions of hydrochloric acid, 0.1 M. The organic phase was washed with water, dried over anhydrous magnesium sulfate, filtered and evaporated, yielding a fluffy white powder.

This material was recrystallized from methanol–water in a 72% yield—m.p. 228–230°C; mass spec M^+ 434, 375. Analysis calculated for $C_{24}H_{31}O_6F$—theory: C 66.34, H 7.19; found: C 66.45, H. 6.98.

Extraction and analysis

Apparatus

The high-performance liquid chromatography (HPLC) system consisted of a Waters 6000 A pump, a 20 μl loop injector (Rheodyne Model 7120), a variable wavelength detector (Spectra-Physics model 770) set at 240 nm, and a strip chart recorder (Laboratory Data Control, model 3401-M). A Waters μBondapak C_{18} column (30 cm × 3.9 mm i.d.) was used. The flow rate was 2 ml/min.

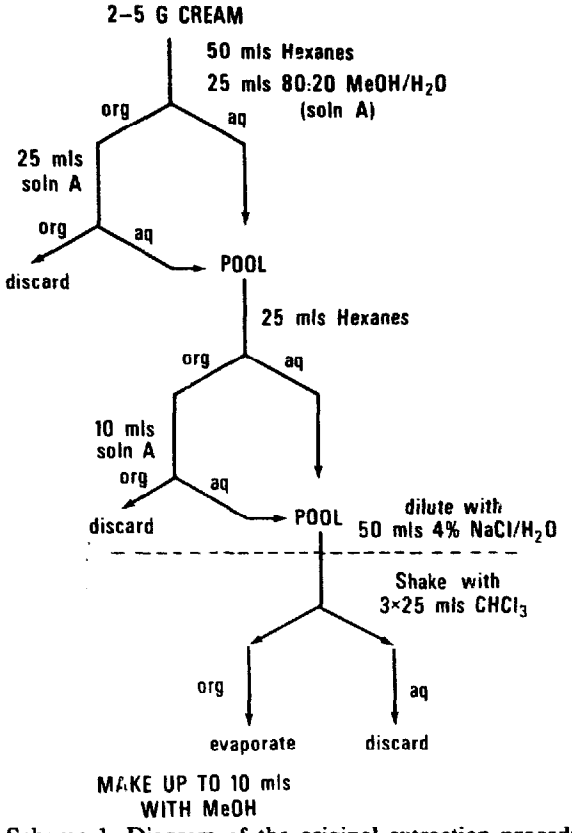
Semi-preparative HPLC was performed on the same equipment, modified to include a 1.0 ml injector loop and a semi-preparative column (μBondapak, C_{18} 30 cm × 7.8 mm i.d.) with the flow rate increased to 3 ml/min. Thin-layer chromatography was done on pre-coated silica gel 60 F-254 plates (E. Merck). Mass spectral data was obtained as described above.

Analytical procedures

Sample preparation

2–5 g of Cordran cream and 50 ml of hexanes were shaken in a separator with two 25 ml portions of methanol–water (80 : 20). The hexane layer was discarded; the

aqueous layers were combined and washed with an additional 25 ml hexanes. The hexane layer was backwashed with 10 ml methanol–water (80 : 20) and then discarded. All aqueous portions were combined. 50 ml of water and 2 g of sodium chloride were added. The flurandrenolide and its degradation products were extracted with three 25 ml portions of chloroform. The chloroform was drained through a layer of anhydrous sodium sulfate and evaporated to dryness with mild heat and a nitrogen stream. The sample residue was dissolved in 10 ml of the mobile phase solution (Scheme 1).



Scheme 1. Diagram of the original extraction procedure for flurandrenolide cream. An 80% methanol solution is used to extract the flurandrenolide, the hexanes are employed to remove the lipoidal matrix. The dashed line indicates where the procedure is modified by acidification of the aqueous phase as described in the text.

Standard preparation

25 mg of flurandrenolide was dissolved in 25 ml methanol. 1 ml of this solution was evaporated to dryness, and the residue was dissolved in 10 ml of the mobile phase solution. Quantitation was accomplished by comparison of peak heights for sample and standard.

Isolation of the major degradation product by semi-preparative HPLC

60 g of aged Cordran cream were extracted in 10 g portions using the analytical procedure. The extracts were combined and injected onto the semi-preparative

HPLC column. The peak eluting just after the flurandrenolide peak was collected from each injection. The collected fractions were combined and concentrated. The sample was streaked on several TLC plates, and the plates were developed twice in benzene–ethyl acetate (1 : 1). The zones were located by viewing under short wavelength UV light (254 nm), then scraped off the plates and eluted with chloroform. After concentration, the samples were filtered and evaporated to dryness for mass spectral analysis.

Blue tetrazolium assay

The assay was performed according to the procedure listed in the USP XX as assay for steroids. The assay was performed using 10 ml of extract derived from 5 g of cream sample compared with a solution of flurandrenolide prepared from reference standard. The reactivity of the C_{20} aldehyde to this assay was performed on 10 mg of crystalline hydrate material compared with 10 mg of flurandrenolide reference standard. All assays were carried out in triplicate.

Modified extraction procedure and HPLC

The extraction procedure was similar to that described under sample preparation with the following modification. Just prior to the final 3 chloroform washings a sufficient quantity of hydrochloric acid solution was added to adjust the aqueous phase to a pH of 1–2. Modification of the HPLC method involved the addition of 0.005 M tetrabutylammonium hydroxide (TBAH) in pH 7.0 buffer to the aqueous phase of a (60 : 40) methanol–buffer mobile phase. In order to further verify peak identity, the solvent composition was altered to (55 : 45) methanol–0.005 M TBAH in buffer.

TABLE I

RETENTION TIMES FOR FLURANDRENOLIDE AND SEVERAL POTENTIAL DEGRADATION PRODUCTS IN 3 HPLC SOLVENT SYSTEMS ON A C_{18} COLUMN

Compound	Solvent system	Retention time (min)
Flurandrenolide I	A	6.2
C_{20} aldehyde II	A	7.5
C_{20} acid IV	A	Solvent Front
C_{20} acid IV	B	3.0
C_{20} acid IV	C	4.0
C_{17} acid V	A	Solvent Front
C_{17} acid V	B	2.3
C_{17} acid V	C	3.1
Flurandrenolone	A	4.6
C_{11} ketone VI	A	4.2

Solvent systems:
A = 60 : 40 methanol–water
B = 60 : 40 methanol–0.005 M TBAH in phosphate buffer at pH 7.0
C = 55 : 45 methanol–0.005 M TBAH in phosphate buffer at pH 7.0

Results and Discussion

Isolation of the C_{20} aldehyde (II)

Fig. 1A shows the HPLC tracing of an extract of a 3-year-old cream sample. The peak eluting at 6.2 min corresponds to flurandrenolide, the other peaks are seen in aged cream samples. These samples consisted of standard production lots, stored in vinyl-lined metal tubes stored at 30°C. In order to obtain analyzable quantities of degradation products, the original extraction procedure was scaled up to work with 60 g of cream. This extract was injected onto a semi-preparative column in several portions so as not to overload the column. The material eluting just after the flurandrenolide peak was subjected to mass spectral analysis using field desorption (FD) as an ionizing source. The spectrum showed a molecular ion at m/e 434 with a

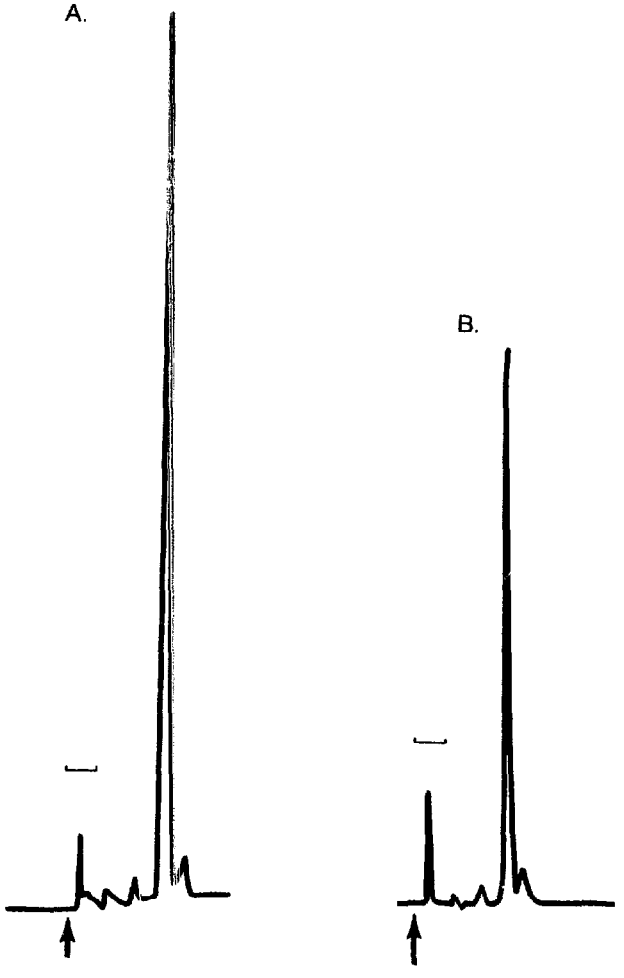
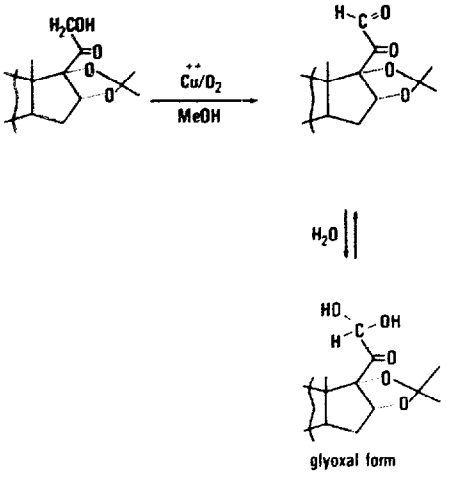


Fig. 1. HPLC (60:40 methanol–water mobile phase and C_{18} column) tracings of an extraction of a 2-year-old sample of flurandrenolide cream. Chromatogram A was for a sample utilizing water in the extraction procedure while the sample yielding chromatogram B utilized aqueous hydrochloric acid (pH 2) in the extraction. Chromatogram B has been photo-reduced by a factor of 0.67 relative to chromatogram A. 2 min = ⊔.

base peak at 377. Tentative identification was made that this material was the C_{20} aldehyde—i.e. two mass units less than the parent alcohol.

Positive identification of the material was made when II was synthesized (Scheme 2) and characterized and produced an identical mass spectrum. The synthesized material isolated after recrystallization was the aldehyde in the hydrated form III, as evidenced by NMR and elemental analysis. The field desorption mass spectrum yielded a parent peak with a m/e ratio corresponding to the non-hydrated aldehyde, due to the loss of water during ionization of the sample.



Scheme 2. Reaction scheme for the preparation of the C_{20} aldehyde II, isolated as the glyoxal III.

The HPLC retention time of the synthesized material was identical to that of the peak designated as the aldehyde. It is interesting to note that although the aldehyde is isolated as the hydrate, it is not in this form under the chromatographic conditions. This is because methanol in the mobile phase may form the hemi-acetal and acetal adducts, thus causing elution after flurandrenolide peak. Hansen and Bundgaard (1980a) referred to this possibility when methanol is present in the mobile phase when studying such systems by HPLC.

The hydrate was subjected to the blue tetrazolium assay yielding a result of 449.0 mg/g of steroid present compared to 1030 mg/g of standard. Thus, one of the degradation products of flurandrenolide responds to the assay for steroids, possibly leading to erroneous conclusions regarding corticosteroid present in samples.

Determination of remaining degradation products

Examination of the HPLC chromatograms of extracts of aged cream samples indicated the regular decrease in flurandrenolide concentration with time. However, this decrease was not accompanied by the formation of new peaks in the spectra. The aldehyde peak, once formed, maintained a constant concentration independent of the age of the cream extract.

The two most likely explanations for these findings were either that the degradation products that formed lacked a chromophore at 240 nm, or that these products were somehow not being extracted from the aged cream samples. The first postulate

can be dismissed since the α,β-unsaturated ketone of the A ring, which gives rise to the chromophore, is a reasonably stable moiety (Görög and Szász, 1978). The latter explanation would be feasible if very polar degradation products formed, since the analyte is extracted into chloroform prior to analysis (Scheme 1). Typically, polar compounds would be the corresponding acidic oxidation product of flurandrenolide or its aldehyde yielding the C_{20} acid IV, or the oxidative degradation product, namely the C_{17} acid V. Analogous compounds have been observed to occur with other corticosteroids (Johnson, 1982; Hansen and Bundgaard, 1980a and b), so the possibility exists that such acidic products could form with flurandrenolide. In an environment above their pK_a, these compounds would be ionized and hence possess a very polar nature.

In the extraction scheme, the final backwash step has no pH control, therefore acidic degradation products would not readily partition into the chloroform layer. By lowering the pH of the aqueous phase to about 1–2 pH units during this backwash, protonation of any carboxylic acids and thus their transfer into the chloroform layer was ensured.

The HPLC tracing of a sample subjected to this modified extraction procedure is shown in Fig. 1B. The large 'solvent peak' indicates the presence of material eluting with the solvent front. This is consistent with ionized material experiencing negligible retention in the reverse-phase system. In order to quantify and identify the

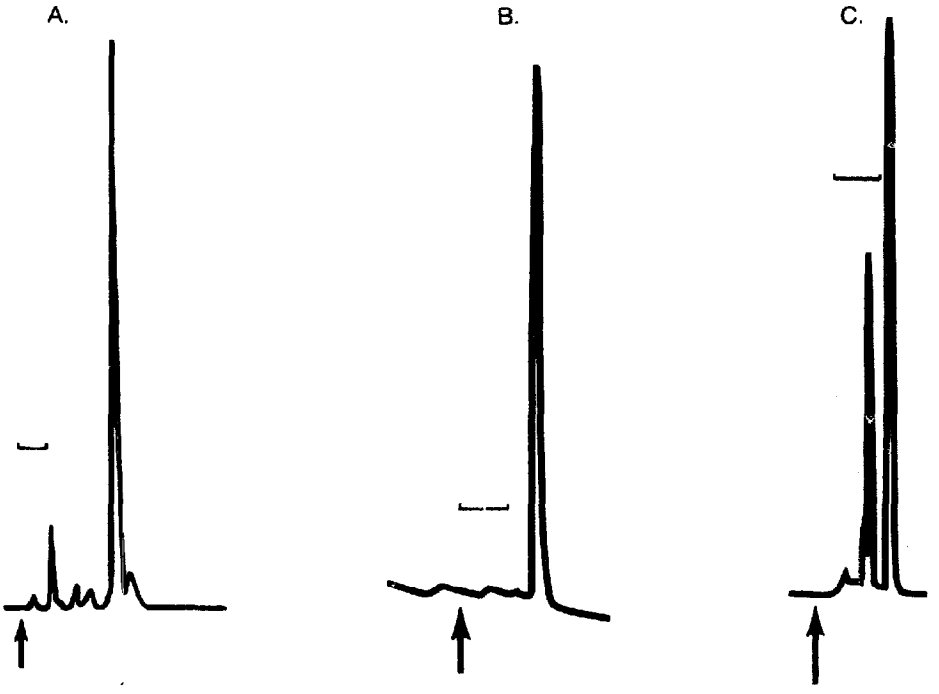


Fig. 2. HPLC tracings obtained using a C_{18} column and a mobile phase of 60:40 methanol–water (containing 5×10^{-3} M tetrabutylammonium hydroxide at pH 7 in the water). A: an extract (using the acid wash extraction procedure) of an aged flurandrenolide cream sample. B: sample of IV (retention time = 3.0 min). C: a mixture of IV and V. 2 min = ⊔.

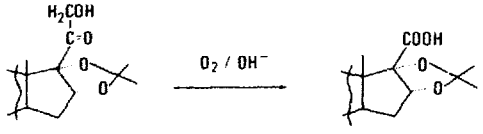
material eluting with the solvent front, a modified chromatographic system had to be developed.

Since the compounds in question were presumed to be one or more ionized acidic species, the most telling analysis would occur upon inclusion of a suitable ion-pairing agent. The ion-pairing agent would increase the retention time of the anionic species and leave the neutral components unaffected. Thus the modified mobile phase, incorporating 0.005 M tetrabutylammonium hydroxide in pH 7 buffer (40 parts), and (60 parts) methanol was employed.

Fig. 2A depicts a chromatogram of an extract of aged cream subjected to the modified HPLC system. The large peak formerly eluting with the solvent front now appears with a retention time of 2.3 min, the other peaks remaining relatively unaffected. This result was consistent with the conclusions of Hansen and Bundgaard (1980a) regarding an acidic degradation product; however, in order to prove the nature of the material, synthesis of the C_{20} acid IV and the C_{17} acid V were performed.

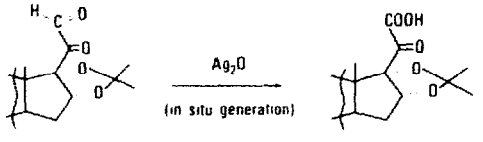
Synthesis of potential degradation products

Preparation of V was a relatively straightforward procedure, employing the oxidative degradation of the C_{17} steroid side-chain according to the method of Pesez and Bartos (1962). This reaction involved the oxidation of flurandrenolide in basic methanol, bubbled with air (Scheme 3). The reaction was followed by HPLC which showed the formation of the aldehyde followed by a new peak eluting at the solvent front. Addition of more base forced the reaction to form more of this material, with the concomitant decrease in flurandrenolide. The material was purified, recrystallized and identified as described in the experimental section.



Scheme 3. Reaction scheme for preparation of the C_{17} acid V.

Synthesis of IV required more selective conditions, since it could conceivably oxidize further to the C_{17} acid. The C_{20} aldehyde was used as a starting point, and mild oxidation of this compound to produce the acid was pursued (Scheme 4). The method chosen was based on a technique used by Shamma and Rodriguez (1968) who employed the in situ generation of an oxidizer—silver oxide—to cause the oxidation of cyclohexyl aldehydes to their corresponding acids.

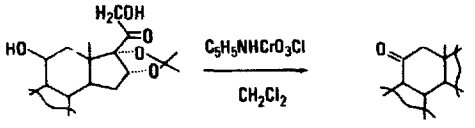


Scheme 4. Reaction scheme for preparation of the C_{20} acid IV.

The method employs an ethanolic solution of aldehyde containing silver nitrate.

Generation of the sparingly soluble silver oxide is achieved by the dropwise addition of potassium hydroxide. The potassium salt of the acid is formed making isolation from the other reaction components a relatively simple task.

Preparation of the C_{11} ketone (VI) was performed via selective oxidation of flurandrenolide using pyridinium chlorochromate, after the method of Corey and Suggs (1975) (Scheme 5). This reagent was chosen in order to oxidize the C_{11} alcohol (a secondary alcohol) yet leave the C_{17} side-chain intact. Analysis of the product by NMR indicated the presence of an intact side-chain, and the mass spectrum showed a major peak at 375 mass units corresponding to the expected steroid backbone, less the C_{17} side-chain. Comparison with the mass of the steroid backbone fragment from flurandrenolide and the other products (377 mass units) was further evidence for the proposed structure.



Scheme 5. Reaction scheme for preparation of the C_{11} ketone VI.

Analysis and identification of degradation products

Fig. 2B depicts the chromatogram of the synthesized C_{20} acid IV using the ion pairing agent in the mobile phase. Using this HPLC system resulted in a longer retention time for IV than with the unmodified system. Fig. 2C is the chromatogram of an equal amount of the C_{20} acid IV and the C_{17} acid V showing that they are resolved in this system. A retention time of 2.3 min for V and 3 min for IV permitted the simultaneous analysis of the two acids in the presence of the extracted cream samples. Fig. 2A is the chromatogram of a cream extract in this modified HPLC system, showing a peak appearing at 2.3 min indicating the possible presence of the C_{17} acid.

Modifying the mobile phase to contain 55 parts methanol to 45 parts 0.005 M TBAH in pH 7 buffer increased the retention time of the C_{20} acid to 4.0 min and the retention time of the C_{17} acid to 3.1 min. When the extracts were analyzed using the 55 : 45 solvent ratio, the retention time of the suspected acid increased to 3.1 min also. This compound, the C_{17} acid V, was found to be the principal degradation product when the modified extraction procedure was employed on various aged cream samples. Small amounts of IV were observed in some lots; however, the predominant product was the acid V. These findings are consistent with the work recently reported by Hansen and Bundgaard (1979a and b; 1980a and b) in their studies on the degradation of hydrocortisone in aqueous media. These authors found that a major degradation product was the analogous C_{17} acid of hydrocortisone.

The relatively constant concentration of aldehyde observed in aged cream samples (about 5%) occurred because it was further oxidizing to one of the acidic degradation products.

The peak appearing with a retention time of 4.2 min was the last remaining

degradation product to be identified. It was postulated that this material may possibly be the C_{11} ketone of flurandrenolide (VI) or perhaps the desacetonide (i.e. flurandrenolone). An authentic sample of flurandrenolone was obtained and shown to have a retention time of 4.6 min in the 60 : 40 methanol–water system, thus excluding flurandrenolone as a degradation product.

The characterized sample of VI was analyzed by HPLC and showed a retention time of 4.2 min; however, this proved to be a coincidence, since manipulation of the mobile phase led to the separation of this material and the unknown compound.

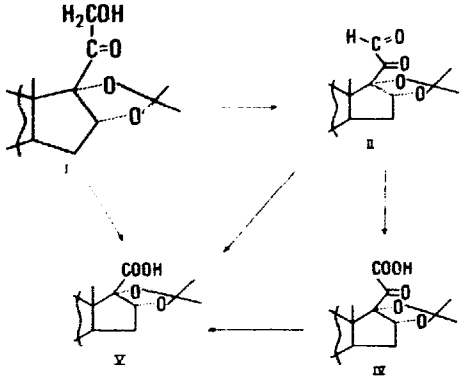
Preparative HPLC of aged cream extracts was then tried, collecting the material eluting at 4.2 min. The solvent was evaporated and the resulting material subjected to NMR and mass spectral analysis. The mass spectrum indicated an absence of a steroidal compound in the spectrum. The NMR spectrum was of very poor resolution; however, there was a lack of the familiar steroidal signal. Thus the nature of this material is as yet undetermined.

Conclusions

The isolation and identification of degradation products of flurandrenolide in its cream vehicle were studied. A major degradation product was the C_{20} aldehyde II, isolated as the glyoxal III. This product was shown to react positively in the blue tetrazolium assay leading to possible errors as an analytical method for flurandrenolide.

The aldehyde degraded further to form two acidic products, the C_{20} acid IV, in small amounts and the C_{17} acid V. Since the aldehyde is subject to further reactions, it appeared in an approximately steady-state concentration in aged cream samples.

The cream samples were extracted prior to analysis by a modified extraction scheme. The modification involved acidifying the aqueous phase in the last extraction step, suppressing ionization of the acidic products and ensuring their transfer to the organic analyte phase.



Scheme 6. Possible degradation pathway for flurandrenolide based upon the degradation products found in the cream formulation, and the work of Görög and Szász (1978).

These results are consistent with other in vitro studies of corticosteroid oxidative degradation (Görög and Szász, 1978; Hansen and Bundgaard, 1979, 1980), and a

probable pathway is depicted in Scheme 6. The inclusion of anti-oxidant and metal chelator compounds may reduce the degree of oxidative degradation and trial formulations are currently being studied.

Acknowledgements

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