Quingestrone—Determination of Minute Quantities of Decomposition Products by Paper Chromatography

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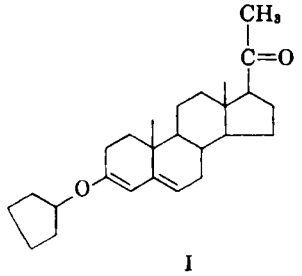
A comprehensive paper chromatographic assay procedure has been developed for quingestrone and its decomposition products. The complete assay procedure consists essentially of three steps: (*a*) separation and identification of the decomposition products, (*b*) quantitative determination of the decomposition products, and (*c*) quantitative assay of the parent compound. As little as 1 per cent of decomposition products can be determined with an accuracy of ±5 per cent. Stability data is presented for quingestrone in crystalline form, in solutions, and in the pharmaceutical dosage form.

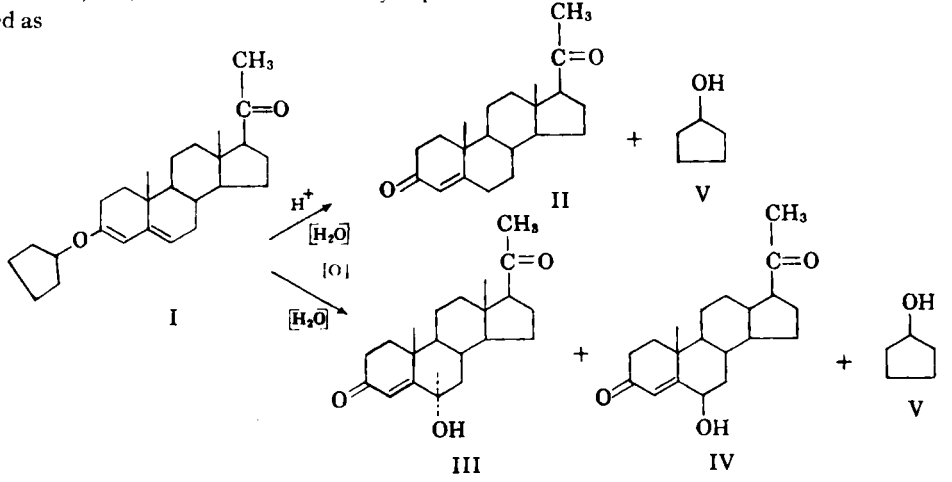
QUINGESTRONE[1] was supplied by Ercoli (1, 2), *et al.* It has the structural formula



I

To investigate the stability of quingestrone (I), a rapid, accurate analytical method was required which would differentiate quingestrone (I) from its decomposition products. Ercoli (3) reported the identification of progesterone (II), 6-α-hydroxyprogesterone (III), and 6-β-hydroxyprogesterone (IV) in addition to cyclopentanol (V) as the most likely decomposition products.

The apparent mechanism for the hydrolytic conversion of I in the presence of acids and/or oxygen to II, III, and IV is schematically represented as



These decomposition products could be identified using the E4 paper chromatographic system of Eberlein and Bongiovanni (4). However, this system was too time consuming and cumbersome for routine analytical work.

Several functional group procedures were investigated in an attempt to differentiate the 3-carbonyl group characteristic of each of the decomposition products from the 3-cyclopentyl enol ether of the parent compound. Reaction with 2,4-dinitrophenylhydrazine (5), or isonicotinic acid hydrazide (6) proved to be unsuitable when the reaction conditions gave quantitative conversion to progesterone. Reaction with 2,6-di-*tert*-butyl-*p*-cresol (7) was found to be unsuitable because of decomposition of quingestrone under the reaction conditions. Attention was then focussed upon developing procedures involving mild conditions to avoid *in situ* hydrolysis of quingestrone.

Ultraviolet spectroscopy as an analytical tool was unsuitable because of the proximity of absorption maxima and absorptivity of the decomposition products to that of quingestrone. Infrared evidence for the presence of nonetherified progesterone can be confirmed by the presence of the characteristic Δ^4-3-ketone band at 1680

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[1] The cyclopentyl enol ether of progesterone. Marketed as Enol Luteovis by Vister Laboratories, Milan, Italy.

TABLE I.—DETERMINATION OF THE ABSORPTIVITY OF THE PRINCIPLE DECOMPOSITION PRODUCTS

Progesterone Standard			6-β-Hydroxyprogesterone		
mg./10 ml.	A at 415 mμ	a	mg./10 ml.	A at 415 mμ	a
0.025	0.050	20.0	0.0235	0.026	11.1
0.050	0.106	21.2	0.0470	0.051	10.8
0.100	0.209	20.9	0.0940	0.109	11.5
0.200	0.428	21.4	0.1880	0.211	11.3
	Mean	20.9 ± 0.4		Mean	11.2 ± 0.2

cm.$^{-1}$ Quantitation based uponthis band was unsuccessful when applied to the dosage form containing quingestrone in sesame oil because of interference from the latter and poor resolution of the 1680 cm.$^{-1}$ band.

Several investigators (8–12) have reported the use of Zaffaroni type paper chromatographic solvent systems for the quantitative analysis of steroids in pharmaceutical dosage forms. Two previously reported (13) Zaffaroni systems herein described provided the nucleus for the development of a rapid, reproducible, precise, and sensitive tool for the stability investigation of quingestrone. The method is capable of separating and quantitatively measuring as little as 1% of the known decomposition products in the parent compound.

EXPERIMENTAL

Preparation of Standards

Solutions of progesterone, 6-α-hydroxyprogesterone, and 6-β-hydroxyprogesterone are prepared in absolute methanol at a concentration of 2 mg./ml. of solution. A standard solution of quingestrone is prepared in reagent grade heptane at a concentration of 2 mg./ml.

Preparation of Samples

Samples under investigation contained quingestrone in sesame oil, packaged in sealed ampuls and soft gelatin, one-piece capsules at concentrations of 50, 40, and 25 mg./ml. Aliquots of these oil solutions are diluted to a quingestrone concentration of 20 mg./ml. with reagent grade heptane just prior to application to the paper. For stability studies in various solvents, solutions of quingestrone are prepared at a concentration of 20 mg./ml.

Isoniazid Eluting Solvent

Dissolve 1 Gm. of U.S.P. isonicotinic acid hydrazide in 1 L. of absolute methanol containing 1.25 ml. of concentrated hydrochloric acid.

Method A

This is used for the separation and identification of decomposition products.

Filter Paper.—Whatman No. 1 is cut into 6 × 18-in. strips. A horizontal pencil line is drawn 3 in. from one end and five equidistant points are marked off. The paper is impregnated by dipping into a solution of 35–65 propylene glycol-methanol and blotted between paper towels.

Developing Solvent.—Toluene saturated with propylene glycol.

Chromatographic Chamber.—Any conventional chamber used for descending paper chromatography.

Paper Chromatographic Procedure.—To the five designated points, 0.010 ml. of each standard solution and 0.025 ml. of the sample solution is spotted using micro volumetric transfer pipets. The paper is then placed in the chromatographic chamber, the spots resting on the antisiphon rod, and the tab end placed into the trough which contains the developing solvent. When the solvent front is within 1 in. of the bottom of the paper (about 3 hours), the strips are removed from the chamber and air dried for 30 minutes.

The positions of the steroids on the chromatogram are located by examination through a fluorescent screen over a short wavelength ultraviolet light source (14). The presence or absence of decomposition products is confirmed by comparing the sample spots with the standard spots. 6-α-Hydroxyprogesterone, 6-β-hydroxyprogesterone, and progesterone can be seen as separate, dark spots having R_f values of 0.27, 0.38, and 0.80, respectively, while quingestrone is at the solvent front.

Method B

This is used for the quantitative determination of the decomposition products.

Filter Paper.—Whatman No. 3 MM is cut into 3 × 18-in. strips and a horizontal pencil line is drawn 3 in. from one end. The paper is impregnated by dipping into ethylene glycol monomethyl ether and blotted between paper towels.

Developing Solvent.—Heptane saturated with ethylene glycol monomethyl ether.

Paper Chromatographic Procedure.—To each of two paper strips, 0.250 ml. of sample solution is applied by streaking across the penciled line from a micro volumetric transfer pipet, leaving a 1/4-in. margin on either side. A third strip serves as a blank. The papers are then placed in the chromatographic chamber, the streaked sample located just below the antisiphon rod, and the tab end placed

TABLE II.—DETERMINATION OF PER CENT RECOVERY

mg. Applied	mg. I in Sesame Oil Applied	mg. Found	Theory, %
0.025 (II)	0	0.023 (II)	92
0.050 (II)	0	0.047 (II)	94
0.100 (II)	0	0.100 (II)	100
0.025 (II)	5.0	0.024 (II)	96
0.050 (II)	5.0	0.048 (II)	96
0.100 (II)	5.0	0.095 (II)	95
0.250 (II)	5.0	0.247 (II)	98.8
0.0235 (IV)	0	0.0268 (IV)	114
0.047 (IV)	0	0.0474 (IV)	101
0.094 (IV)	0	0.097 (IV)	103
0.186 (IV)	0	0.180 (IV)	97
0.0235 (IV)	5.0	0.025 (IV)	106
0.047 (IV)	5.0	0.050 (IV)	106
0.094 (IV)	5.0	0.100 (IV)	106
0.186 (IV)	5.0	0.178 (IV)	96

TABLE III.—STABILITY OF CRYSTALLINE QUINGESTERONE

Storage Conditions	—1 Gm. of Sample Contains— Quingesterone, mg.	Progesterone, mg.	6-β-Hydroxyprogesterone, mg.
4°C. under nitrogen	995	3	2
4°C. under air	930	24	50
25°C. under air	40	140	653

into the trough which contains the developing solvent. When the solvent front is within 1 in. of the bottom of the paper (about 3 hours), the strips are removed from the chamber and air dried for 30 minutes.

The positions of the decomposition products on the chromatograph are located by observing in a darkened room over an ultraviolet light source. The progesterone and 6-β-hydroxyprogesterone can be seen as darkened zones having R_f's of 0.30 and 0.10, respectively, while the quingestrone and sesame oil can be seen as a darkened zone from R_f 0.80 to the solvent front.

The decomposition product zones are cut out, cut into $^1/_2 \times {}^1/_2$-in. pieces and placed in a glass-stoppered 125-ml. Erlenmeyer flask. Appropriate zones of the blank chromatogram are treated as the corresponding sample zones. Ten milliliters of the isoniazid eluting solvent are pipeted into each flask, stoppered, and shaken mechanically for 1 hour.

The solution is then drawn into a pipet through cotton wrapped around the tip, to filter out paper fibers, and transferred to a 1-cm. corex cell. The absorption of the eluted steroid is determined at 415 mμ using the blank solution in the reference cell on a Beckman DU spectrophotometer.

In a similar manner, a series of standards, both with and without quingestrone in sesame oil, were analyzed to establish the accuracy and precision of the method.

Method C

This is used for the quantitative determination of quingestrone.

Filter Paper.—Same as *Method B*, except the paper is impregnated by dipping the paper into ethylene glycol monomethyl ether containing 0.1% α-tocopherol. The α-tocopherol is needed to prevent decomposition of the quingestrone during the drying of the chromatogram.

Developing Solvent.—This is the same as *Method B*.

Paper Chromatographic Procedure.—To each of two paper strips, 0.100 ml. of a $^1/_{10}$ heptane dilution of the sample solution is streaked. To a third strip, 0.100 ml. of a standard quingestrone solution is streaked, while a fourth strip serves as a blank. The chromatographs are developed, dried, the quingestrone detected, cut out, eluted, and measured as the decomposition products in *Method B*.

RESULTS AND DISCUSSION

In all the samples examined, only trace amounts of 6-α-hydroxyprogesterone can be detected, while in some samples substantial amounts of 6 β-hydroxyprogesterone and progesterone are found. The separation of progesterone and quingestrone in the sesame oil was not always satisfactory in the toluene–propylene glycol solvent system of *Method A* because the sesame oil distributed itself between the two zones. For this reason, quantitative analysis was done by *Methods B* and *C*.

TABLE IV.—STABILITY OF QUINGESTRONE IN SOLVENTS

Solvent	Conditions	Dec., % Progesterone	Dec., % 6-β-Hydroxyprogesterone
Chloroform	$^1/_2$ hr., RT	0.5	...
Chloroform	7 hr., RT	50	3
Chloroform	2 wks., RT	55	18
Methanol	7 hr., RT	trace	...
Methanol	2 wks., RT	24	49
Heptane	2 wks., RT	...	15
Sesame oil	4 mo., RT	2.2	5.7
Sesame oil	3 wks., 45°C.	15	36

Since the $\Delta^{5,6}$ analog of progesterone is a possible hydrolytic cleavage product which would not be detected by the indicated ultraviolet method, several chromatograms of decomposed samples were stained with 2,4-dinitrophenylhydrazine with the result that no non-ultraviolet absorbing 3-ketones were detected. This indicated that this compound is not among the decomposition products.

The precision and adherence to the Beer-Lambert law was confirmed by reacting varying amounts of progesterone and 6-β-hydroxyprogesterone with 10 ml. of the isoniazid solution. The absorbance of the resulting chromaphore was then determined at 415 mμ on a Beckman DU spectrophotometer. The results of this study are tabulated in Table I.

The described method is capable of detecting and quantitating 0.025 mg. of progesterone and 6-β-hydroxyprogesterone in the presence of 5 mg. of the parent compound. The precision and accuracy were established by the standard addition method. The standard quingestrone sample used in this study showed no evidence of decomposition.

The amount of progesterone or 6-β-hydroxyprogesterone present was calculated by the simple formula: mg. present $= (A/a) \times 10^{-3}$, where A = absorbance at λ 415 mμ found. The results of this investigation are summarized in Table II.

The accuracy of determining the indicated levels of decomposition products can be calculated to be ±5% from data in Table II.

Crystalline quingestrone is stable only if stored under an inert atmosphere such as nitrogen and preferably at low temperatures. If allowed to remain at room temperature under atmospheric conditions, decomposition can be detected after only 3 days, the major decomposition product being 6-β-hydroxyprogesterone together with some progesterone.

TABLE V.—STABILITY OF QUINGESTRONE IN THE PHARMACEUTICAL DOSAGE FORM

Condition	Quingestrone Analysis	Dec., % Progesterone	Dec., % 6-β-Hydroxyprogesterone
2 mo., RT	99.1	trace	0.9
6 mo., RT	100.0	...	0.1
2 yrs. RT and 4°C.	98.7	...	1.0
6 mo., 37°C.	101.0	...	1.5
1 mo., 45°C.	98.5	trace	1.0
4.5 mo., 45°C.	95.5	4.5	1.3
67 hr., 105°C.	98.5	0.3	0.9

If stored in the refrigerator under nitrogen and opened only occasionally to remove samples and subsequently blanketed with nitrogen, no decomposition is observed after several months.

Typical analyses of solid crystalline quingestrone after 5 months' storage are tabulated in Table III.

In solution, quingestrone is stable in the absence of acids, bases, and oxidizing agents, including atmospheric oxygen. It is less stable in chloroform and methanol than in heptane and exhibits best stability in a good grade vegetable oil such as sesame oil. In Table IV, stability data in solvents stored under the indicated conditions are presented. No precaution was taken to exclude air in these studies.

The pharmaceutical dosage form consists of quingestrone dissolved in sesame oil at concentrations of 50, 40, and 25 mg./ml. contained in sealed glass ampuls and/or in sealed, soft gelatin capsules. It is of the utmost importance that the peroxide content of the sesame oil be kept minimal, *i.e.*, less than 50 mcq per ml. as determined by the method of the American Oil Chemists Society (15). As a further precaution, an antioxidant, namely α-tocopherol is added at a concentration of 0.1% of the weight of sesame oil used.

In Table V, stability data are presented for this final dosage form.

SUMMARY

A quantitative paper chromatographic method has been developed which not only permits the determination of quingestrone, but also simultaneously provides a precise method for assaying as little as 1% of the known decomposition products, progesterone and 6-β-hydroxyprogesterone.

Data have been presented to indicate that a solution of the cyclopentyl enol ether of progesterone (quingestrone) in sesame oil of low peroxide content with addition of α-tocopherol and exclusion of atmospheric oxygen represents the best means for preserving the product in a pharmaceutical dosage form.

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Effect of Deuterium Oxide on the Growth of Peppermint (*Mentha piperita* L.) I

Morphological Study

By M. I. BLAKE, F. A. CRANE, R. A. UPHAUS, and J. J. KATZ

The effect of varying concentrations of D_2O on the growth of peppermint plants was studied over a period of at least 50 days. The extent of deuterium uptake is reported. This appears to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant.

THE EFFECTS of deuterium on living organisms have attracted the attention of many investigators since this heavy, stable isotope of hydrogen was first discovered in 1933. Early work has been reviewed by Morowitz and Brown (1). Current developments are described by Katz (2) and are the subject of a recent monograph (3). Interest in this general area has been stimulated by the discovery that a considerable variety of organisms can be grown in 99.8% D_2O (4, 5). Since it has proved possible to replace essentially all of the hydrogen in a variety of algae, bacteria, and molds, it becomes of interest to examine the behavior of higher plants in response to isotopic substitution. The effects of deuterium on mice (6), rats (7), and dogs (8) have been reported, but the present paper presents what we believe to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant.

Since deuterium oxide is now available in adequate quantities and at a moderate cost, it is possible to study the effect of high concentrations of this solvent on the growth of higher plants over an extended period of time. Peppermint plants are ideal for such an investigation since they can

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