DETERMINATION OF PURITY OF SOME TRITIATED STEROIDS.

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SUMMARY

When radiolabeled steroids at high specific activities are required for biological studies, the particular requirements of purity must be determined. The analyses to which the compound is subjected is based on these requirements. Analytical methods are complicated because of the radiochemical instability and the lack of sufficient sample. Methods have been developed which will allow IR and UV spectra to be determined with subsequent recovery of the sample. A prime requisite for this is specially purified solvents. The acceptability of certain solvents for this use can be determined by concentrating an aliquot of the solvent and then examining the residue by TLC. Spraying the developed chromatogram with a 5% solution of phosphomolybdic acid in ethanol, followed by heating will reveal the presence of impurities in the solvents. These impurities appear as spots and can readily be misinterpreted as contaminants in the sample if unpurified solvents are used to manipulate a pure steroid. Problems associated with the determination of radiochemical purity via GLC and TLC will be discussed. The synthesis of tritiated ethynyl estradiol its cyclopentyl ether and the cyclopentyl ether of norethindrone acetate will illustrate the above problems.

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DISCUSSION

Since many steroids require only microgram quantities to exhibit biological effects, the use of radiolabeled steroids having very high specific activities is mandatory if physiological doses are to be administered. The commercially available tritium and ^{14}C labeled steroids are supplied at, or very near, the maximum theoretical amounts of radioactivity of 29 Ci/milliatom of ^{3}H and 62 mCi/milliatom of ^{14}C, respectively.

In any discussion of purity, the important question for the user to consider is not how pure the material is but whether the substance is sufficiently pure for the intended purpose. It, therefore, follows that the requirements of purity will vary with each investigator. At Warner-Lambert where these radiochemicals are used in various tracer and metabolic studies, in order for a steroid to be considered "pure," it must assay between 98-102%. This includes both chemical and radiochemical purity and applies to both synthesized and purchased radiolabeled steroids.

The determination of the chemical purity of most steroids at these high specific activities is difficult because of the small sample size and the inherent radiochemical instability. The sample size is usually milligrams or less. Obviously, one cannot use the usual assays where no thought is given to sample recovery.

The thought of sample recovery is important and this problem must be discussed. These radiolabeled steroids are usually handled in solution and, therefore, the solvents become extremely important. Geller and Silvermann (1) have reported that certain impurities in solvents can increase the rate of decomposition of radiolabeled steroids. Frankel and Nalbandov (2) also noted this decomposition by impurities in solvents. It is absolutely essential that purified solvents be used when handling these small quantities of materials. Decomposition will occur just in the process of removing the solvent at room temperature. The methods which are used to purify our solvents are as referenced: methanol (3), ethylacetate (4), acetone (5), benzene (6), and cyclohexane (7).

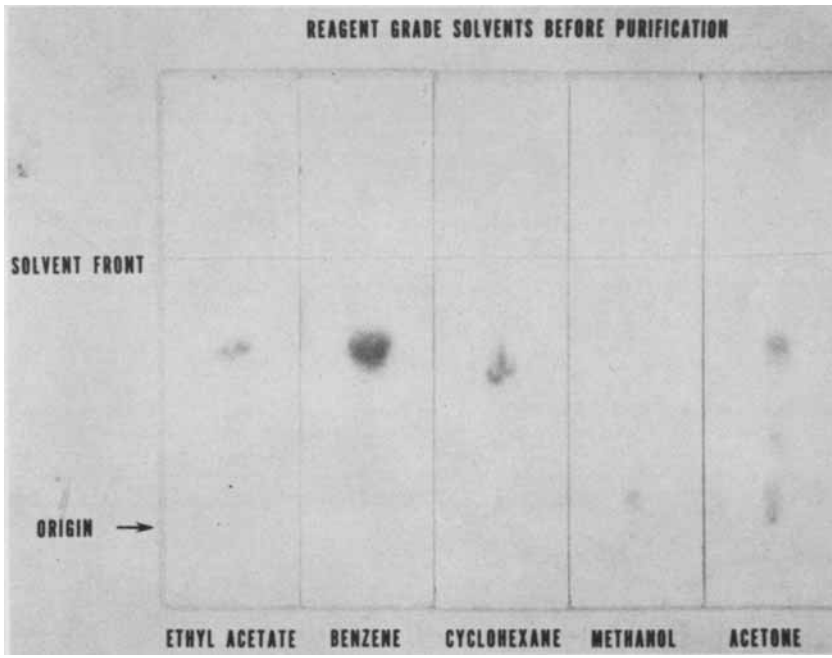
The empirical test that we use to check the solvents is as follows. Using ultrasonically cleaned glassware, 50 ml of the solvent in a flask is placed on a rotating evaporator and the solvent removed. Any residue in the flask is quantitatively transferred into an elongated test tube (8) with 10 ml of the same solvent. The solvent is again removed under a stream of purified oil pump nitrogen. Twenty microliters of the solvent were added, swirled to dissolve any residue, and 10 µl applied to a silica gel G or GF plate, previously washed with methanol, reactivated by heating, and developed in a mixture of 4 parts of cyclohexene and 1 part of acetone until the solvent front has risen to about 10 cm above the origin. The solvents are allowed to evaporate from the plate which is then sprayed with a 5% phosphomolybdic acid solution in ethanol. On heating at 120°C for 5-15 minutes, a visible blue spot or spots will appear at different R_f values if the solvents are impure, as shown in Fig. 1. Similar, but less sensitive, results were obtained with impure solvents after spraying with a 2% sulfuric acid solution in 50% aqueous methanol and heating. When about 3-4 liters of solvent are purified by the previously mentioned procedures, they will not exhibit spots by the above method. Scale-up of these above purification methods were unsuccessful for some unknown reason. I might add that nothing was detected in these solvents on several GLC columns nor by various spectral methods. Using these purified solvents, we were able to manipulate many steroids without decomposition.

Chemical Purity

The procedures usually used to determine the chemical purity of radiolabeled steroids include an examination of the ultraviolet (UV) spectrum, the infrared (IR) spectrum, a quantitative thin-layer chromatogram (TLC) or gas-liquid chromatography (GLC). For radiochemical stability reasons, the weighed steroid is usually kept in solution, in either benzene, hexane, cyclohexane, or in combination with another solvent, to reduce the radiochemical decomposition.

To determine the UV spectrum, an aliquot of this solution corresponding to about 50-100 µg depending on the chromaphore, is pipetted into a 5-ml volumetric flask, the solvent is removed and the sample dried in vacuo. The solvent in which the UV is to be determined, usually methanol, ethanol, or ethyl acetate, is then added to volume. The spectrum is determined in the usual manner using the universally available 1-cm cell and compared with a spectrum of an authentic sample similarly prepared. The entire sample can be recovered by again removing the solvent and reconstituting with whatever solvent is the most suitable for storage. Before this recovery step, the total amount of radioactivity can be determined on the solution used for the UV via liquid scintillation spectrometry. If it is necessary to dilute this solution to facilitate counting, nonradiolabeled

FIG. 1 TLC SPRAYED WITH PHOSPHOMOLYBDIC ACID



steroid or "carrier" must be added. A rule of thumb is to maintain a concentration of at least 1 mg of material per 100 ml of solution. If this is not maintained, erratic results will usually result, as previously reported by several workers (9).

Various methods were tried in order to obtain a satisfactory IR spectrum and the following method found to be the most reliable. An aliquot of the stock solution representing 20-100 µg of steroid is pipetted into a small test tube, and the solvent removed under a stream of nitrogen. The residue is dried in vacuo and transferred with chloroform or methylene chloride onto 20 mg of KBr. An excellent mix can be obtained on grinding, and the IR determined as a 0.1-0.5% KBr micropellet using a spectrophotometer with scale expansion and a beam condensing unit such as a Perkin-Elmer 621. It should be kept in mind that the IR of a high specific activity (SA) ^{3}H or ^{14}C labeled compound may differ from its nonradioactive counterpart, depending on the location of the label. The sample can be recovered by extraction if necessary.

A quantitative TLC system specifically designed to allow separation of the potential impurities can also be used to determine the chemical purity. Generally, when 25-100 µg of steroid are applied to the plate, and the developed chromatogram is visualized by any of the usual methods, impurities at the 0.1% level can be detected. Sometimes this same TLC can be scanned to establish the radiochemical purity.

If a GLC method is available, it also can be used to determine the chemical purity. The amount of sample consumed for the GLC is small, on the order of 10 µg with a flame ionization detector. However, the preparation of a 1% solution usually used in GLC work may be a problem. Normally, the equivalent of about 100 µg of the steroid is transferred into a test tube which, on removal of the solvent, concentrates the solids in a small area. Then the sample is reconstituted with 10 µl of an appropriate solvent.

Radiochemical Purity

Generally, the determination of the radiochemical purity is established by some form of chromatography coupled with radiodetection. With steroids of high specific activities, it is mandatory that they be applied to the chromatogram by superimposing them over a spot of the nonradioactive material. Decomposition may occur which can be mistaken for a radio contaminant as shown in Fig. 2.

To illustrate some of the previous statements, the actual analyses performed on two series of steroids, which were labeled by synthesis in our laboratories, will be discussed. Their purification was most easily accomplished by preparative TLC. The usual 250 micron thick 20 x 20 cm plate proved to be the most convenient. However, it is absolutely essential to prewash the plate by ascending chromatography in purified methanol to remove certain contaminants which exhibit end absorption in the UV. The plate must be reactivated by heating in an oven at 105°C for 15 minutes and then stored in a closed box to prevent reabsorption of any laboratory chemicals. Incidentally, silicone grease is never used because it readily dissolves in solvents and interferes with most analytical determinations.

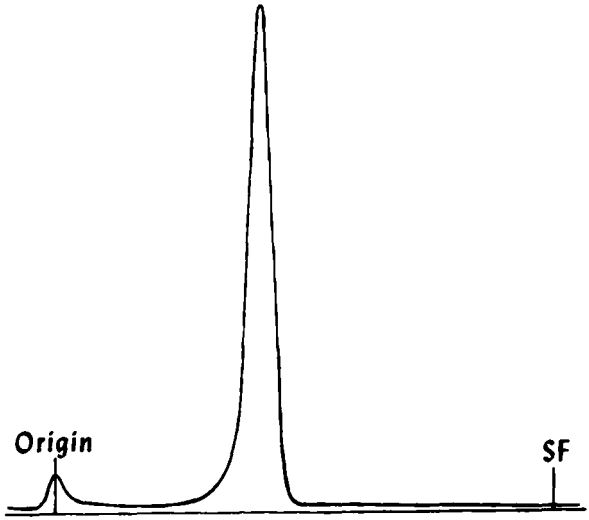


FIG. 2 RADIOSCAN OF ESTRONE

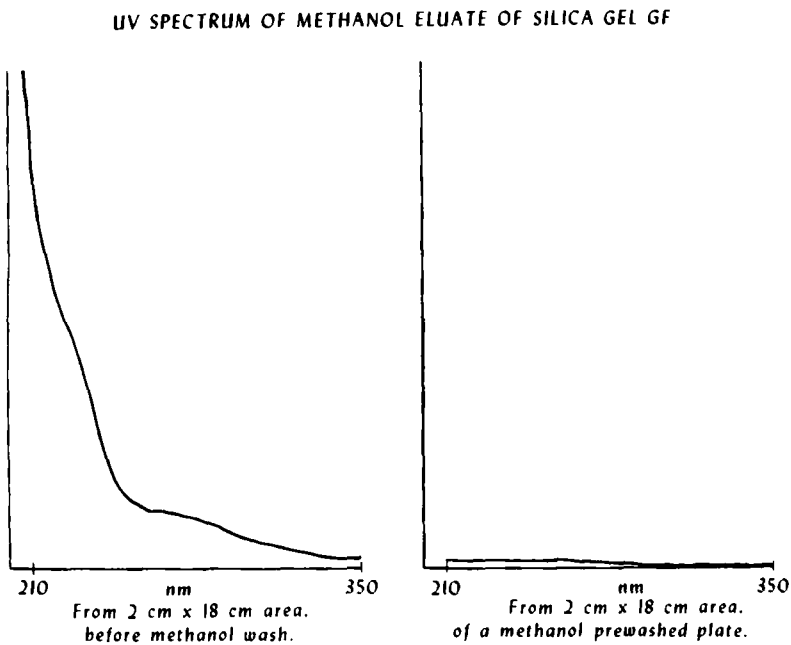


FIG. 3 UV SPECTRUM OF METHANOL ELUATE OF SILICA GEL PLATE

When a 2 cm x 18 cm area from an unwashed plate was scraped from the plate, packed into a small liquid chromatography column and eluted with 10 ml of methanol, the UV spectrum shown in Fig. 3 was obtained. A washed plate, similarly scraped, in which purified solvents have been used is shown also. The eluate was filtered through a fine fritted glass funnel to remove fine particles of the adsorbent.

Using the method shown in Fig. 4, ethynylestradiol-6,7-^{3}H (EE-^{3}H) and 3-cyclopentyloxy-17α-ethynylestradiol-6,7-^{3}H,[1] (quinestrol-6,7-^{3}H) were synthesized and purified (10). The UV and IR of each were identical with authentic samples. The EE-6,7-^{3}H was radiochemically unstable at the high specific activity of 54.2 Ci/mM in either ethyl acetate, benzene, or cyclohexane, and it was only after sequential reduction of the specific activity to 1.82 Ci/mM in benzene that the compound stabilized. Now, the rate of decomposition is about 3-4% per year.

By converting the EE-6,7-^{3}H having a specific activity of 54.2 Ci/mM immediately into its cyclopentyl ether and purifying by TLC, the product obtained was chemically and radiochemically pure by UV, IR, and TLC. The TLC solvent of cyclohexane/acetone (4:1) using silica gel GF coated plates (11) will separate

[1]Quinestrol is the accepted nonproprietary name.

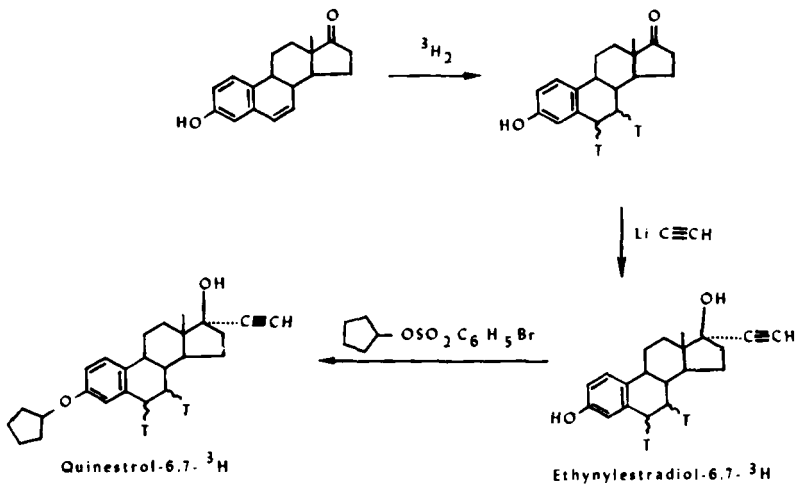


FIG. 4 SYNTHESIS OF QUINESTROL-6,7-^{3}H

the intermediates and is an excellent system for the determination of contaminants. The specific activity was 54.8 Ci/mM and had decomposed by 5% after 3 months, when stored in 9 pts. benzene and 1 pt. methanol at 5°C under N_2. When tritium was incorporated into the cyclopentyl ring at the 1^1, 2^1, and 3^1 positions at a specific activity of 42.7 Ci/mM, the compound exhibited about 3% decomposition after 3 months when stored under the same conditions. When the cyclopentyl ring was labeled with ^{14}C in the 1^1 position at a specific activity of 14.3 mCi/mM, no decomposition was noted after more than two years.

The other example of the synthesis and purification of several tritiated steroids (12) also began with 6-dehydroestrone as shown in Fig. 5. Again, because of the high SA's of these steroids, purifications were all accomplished by column or TLC procedures. The unusual feature of 3-cyclopentyloxy-19-nor-17α-pregna-3,5-dien-20-yn-17-ol-7-^{3}H 17-acetate (quingestanol-7-^{3}H acetate)[1] is that it is an enol ether which as a class are usually not stable at a pH below 7.

The ethyl enol ether did not separate on TLC from the cyclopentyl enol ether (quingestanol-7-^{3}H acetate). However, GLC proved very helpful. Using a 6 ft. x 0.1 in. glass column packed with 60-100 mesh Gas Chrom Q coated with 2% SE-30 using a helium flow of 50 ml/min and a temperature no higher than 213°C, the ethyl enol ether and the cyclopentyl enol ether had retention times (R_t) of about 6 and 18 min., respectively. Unexpectedly, the analytical GLC of the high specific activity quingestanol-7-^{3}H acetate exhibited an additional band. It was still present after repurification. Eventually, it was discovered that the injection temperature was critical. Any sample injected above 213°C

[1]Quingestanol acetate is listed in United States Accepted Names (USAN).

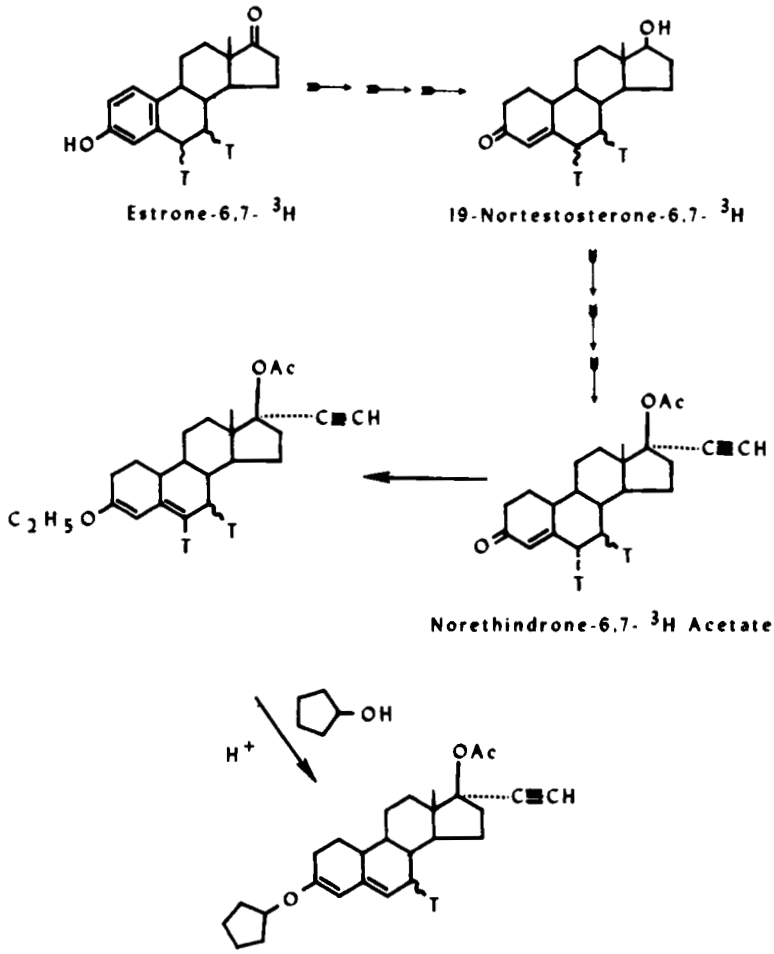


FIG. 5 SYNTHESIS OF QUINGESTANOL-7- ^{3}H ACETATE

would produce this band due to decomposition of the quingestanol-7-^{3}H acetate on the column.

The ease of hydrolysis of this compound should also be mentioned. A silica gel plate was sufficiently acid to completely hydrolyze the sample when spotted.

This steroid could be manipulated by incorporating 0.05% pyridine or piperidine into all the solvents which contacted the sample and by using alumina plates. Glassware was always prerinsed with these solvents to keep the solution on the basic side.

This paper has attempted to demonstrate that despite the instabilities usually encountered with radiolabeled high specific activity steroids, the usual analyses can be performed with recovery of the limited, expensive sample possible in most cases.

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