PURIFICATION OF LABELLED RETINOIC ACID AND CHARACTERIZATION OF A MAJOR IMPURITY

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SUMMARY

All-trans-[15-^{14}C]retinoic acid and all-trans-[10,11-$^{3}H_2$]retinoic acid were purified by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). A major impurity, present in both ^{14}C- and ^{3}H-labelled retinoic acid, was isolated, characterized and tentatively identified as an epoxide of retinoic acid.

INTRODUCTION

The importance of radiochemical purity in tracer analyses should be universally recognized. Yet, many researchers fail to appreciate the necessity for the routine monitoring of radiochemical purity. This is particularly important when working with relatively unstable compounds, where chemical decomposition per se is a potentially significant problem.

Thin-layer radiochromatography has become a primary means, frequently the only means, by which such purity analysis is attempted. The subjects of radiochromatography and purity analysis have been reviewed (1, 2). One of the ever present problems in radiochromatographic analysis is the possibility that the chosen solvent systems and/or supports may not effect the separation of all of the contaminants that might be present in the compound of interest. This problem seems to take on particular significance when working with lipids.

In the course of studies with labelled retinoic acid, a contaminant has

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been found which was not separated from the authentic compound by most of the TLC solvent systems tested. One effective solvent system was found, but the separation was highly dependent upon the condition of the support. This communication is concerned with the application of both TLC and HPLC in the purification and purity analysis of labelled retinoic acid.

MATERIALS AND METHODS

All-trans-[15-^{14}C]retinoic acid (approx. 19 mCi/mM) and all-trans-[10,11-$^{3}H_2$]retinoic acid (approx. 105 mCi/mM) were obtained as gifts from Dr. W. E. Scott, Hoffmann-La Roche Inc., Nutley, N. J. The radiochemicals, dissolved in toluene, were stored in the dark at 4°C. The approximate storage concentrations were 865 μCi/ml for the ^{3}H- and 104 μCi/ml for the ^{14}C-labelled retinoic acid.

Thin-layer chromatography was performed on both silica gel and cellulose supports. Plastic sheets coated with silica gel (P. No. 13179, Eastman Kodak Co., Rochester, N. Y.) MN Silica Gel G, MN Cellulose 300 (Brinkman Instruments, Inc., Westbury, N. Y.) and glass supported Q-1 silica gel plates (Quantum Ind., Fairfield, N. J.) were used. Many solvent systems were utilized. The most notable of these were: A, heptane:chloroform:methanol (8:1:1); B, benzene:chloroform:methanol (4:1:1); C, benzene:acetone:methanol:acetic acid (14:1:2:1); D, diethylether:isopropanol (9:1) and E, petroleum ether (36-37°C fraction):2-octanone (11:2). Distilled in glass, residue free solvents (Burdick and Jackson Laboratories, Inc., Muskegon, Mich.) or redistilled reagent grade solvents were used throughout. Water was deionized, charcoal filtered and glass distilled prior to use.

All procedures were carried out in near or total darkness. An effort was made to maintain a nitrogen atmosphere during sample handling. All handling procedures (excluding one to be discussed) were found to have no deleterious effects on authentic all-trans-retinoic acid.

High performance liquid chromatography was performed using 0.46 x 25 cm, bonded, reverse phase, ODS (octadecylsilane) columns (Partisil-10 ODS, Whatman Laboratory Products, Clifton, N. J.) and a methanol/water solvent system. Fraction collection was done under a foil light shield in vials which were continuously flushed with a gentle flow of nitrogen. Mass spectrometry was performed using the direct probe inlet on a low resolution mass spectrometer previously described (3). Autoradiography was performed using 8" x 10" no screen X-ray film (GAF Corp., New York, N. Y.) which was processed with the appropriate chemicals, as recommended by the manufacturer (Eastman Kodak Co., Rochester, N. Y.).

RESULTS AND DISCUSSION

An attempt to cocrystallize ^{3}H- and ^{14}C-labelled retinoic acid with unlabelled carrier revealed the presence of significant levels of contamination in both of the radiochemical stocks. A TLC and autoradiographic analysis of the crystalline material and the mother liquor confirmed the presence of several easily separated contaminants. However, in solvent system A, the presence of a contaminant which was not fully resolved from retinoic acid was indicated. Careful reanalysis of these samples and the original radiochemical stocks not only confirmed this finding but showed this contaminant to be the major radiochemical material present.

The level of contamination present in the stocks precluded the use of recrystallization procedures. Thus, TLC and then HPLC were utilized to establish viable procedures for both their repurification and effective quality control.

Since HPLC proved so successful, the TLC procedures were never fully pursued. However, the incomplete TLC data have been presented as a guide to those who either have no access to HPLC instrumentation or who have a desire to utilize TLC in addition to HPLC.

Radiochemical Purity Analysis by TLC — An autoradiogram obtained from a two-way chromatogram utilizing solvents A and B on a Q-1 plate is represented in Figure 1. Essentially identical results were obtained with both the ^{14}C- and ^{3}H-stocks. The separation obtained with solvent system A was routinely successful on this style of plate. However, acquisition of a quantitative purity estimate in this format was tedious. Thus, a change was made to plastic supported sheets which (being easily cut into strips) afforded a more efficient and accurate system for quantative analysis using liquid scintillation counting techniques.

Solvents B, C and D were all ineffective in separating the major contaminant from retinoic acid, regardless of the support used. For example, a comparison of the radiochemical purity estimates provided by solvents A and C revealed that A yielded an apparent purity estimate of 25% retinoic acid (R_f 0.25) while 35% of the label was found in the major contaminant spot (R_f 0.20) and the remainder was distributed among slower or nonmigrating contaminants. Simultaneously, solvent C yielded an apparent purity estimate of 82% as the major contaminant and several of the minor ones cochromatographed with the retinoic acid.

Solvent systems C and D were recommended by the supplier as being useful in monitoring the radiochemical purity of retinoic acid on silica gel. In our hands, and for this separations problem, these solvent systems proved to be ineffective. However, it should be pointed out that the separation systems used by suppliers of radiochemicals for purity analysis of their products probably have been developed to satisfy the demands of specific and frequently highly restricted separations problems which are dictated, in general, by the methods of preparation and handling. Although these systems may be satisfactory for their intended uses, they cannot be expected to routinely accomplish the separations of all of the possible decomposition products that might be generated by various treatments and/or abuses of these products.

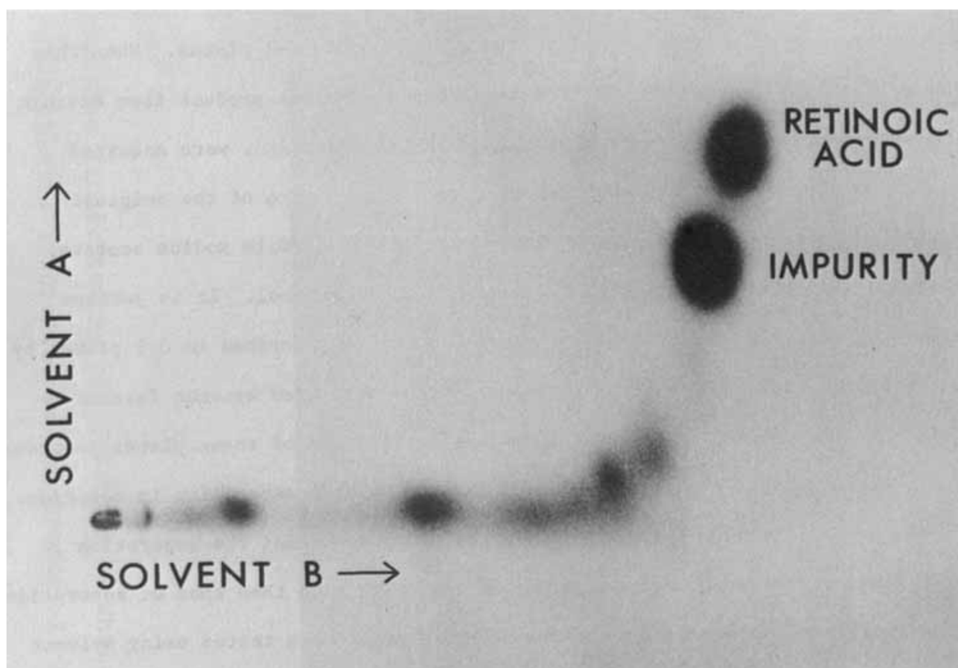

Figure 1. An autoradiogram of a two-way TLC of impure [15-^{14}C]retinoic acid using solvent systems A (heptane:chloroform:methanol, 8:1:1) and then B (benzene:chloroform:methanol, 4:1:1). The R_f values for retinoic acid were 0.28 and 0.37 in solvent systems A and B, respectively.

Another major TLC problem encountered in these studies involved the adequate definition of the support, in particular, of silica gel. The initial supply of plastic supported silica gel sheets used in these studies were an Eastman Kodak product. They were several years old and had been treated with 0.1M sodium acetate in 90% aqueous methanol. Before use, in this work, they were soaked in deionized water (approximately 1 liter per plate for about 30 minutes), drained, rinsed with methanol to quickly remove the excess water, air dried and used without further treatment. The results obtained with these plates were essentially identical to those obtained on Q-1 plates. When this supply of silica gel sheets was exhausted, the equivalent product from Eastman Kodak Co. and a similar one from Brinkmann Instruments, Inc. were acquired. Neither product yielded the expected results. A semblance of the original results was obtained when the new plates were soaked in 0.1M sodium acetate, dried and then predeveloped with 10% acetic acid in ethanol. It is perhaps noteworthy that, in addition to the special qualities ascribed to Q-1 plates by the supplier, these plates were also aged. They were used without further treatment. No effort was made to acquire a fresh stock of these plates in order to ascertain whether their age had any influence on the separation in question.

As these data were being acquired, they suggested that the separation might be more dependent upon the phenomenon of partition than that of adsorption. Thus, a plastic supported thin-layer cellulose medium was tested using solvent A. Separation was observed (retinoic acid R_f 0.87, major contaminant R_f 0.80). Spot size was reduced and the apparent resolution improved by progressive increases in heptane content of the system (eg. 64:1:1 yielded R_f's of 0.68 and 0.57, respectively. No resolution was observed with solvents B, C and D using this media.

Radiochemical purity analysis using TLC was not developed further due to the successful introduction of HPLC. The simplest and most dependable procedure extant at that point was chromatography with solvent A on a Q-1 plate followed

by autoradiography. The quantitative procedures based on the use of appropriately pretreated, plastic supported silica seemed to be useful, but this was not exhaustively established.

It is worthy of note that none of the above described procedures were found to be of use in establishing the isomeric purity of retinoic acid. Although the system was never applied to labelled retinoic acid, solvent E on Q-1 plates was capable of partial resolution of some of the geometric isomers of retinoic acid. This solvent system was never optimized to that purpose. It was an effort to simulate the solvent system reported by Planta et al. (4). Modifications of solvent E in combination with solvent A should prove most useful in the purity analysis of retinoic acid by TLC.

Purification by TLC — The above TLC procedures for radiochemical purity analysis of retinoic acid were never converted to a viable means of purification. The product recovered from Q-1 plates routinely yielded purity estimates of only 75-85%.

Dilution of the samples or prespotting the plates with non-labelled carrier, and predevelopment of the plates did not alleviate the problem. Samples were applied to the plates in a gas box under a flow of lamp grade nitrogen. Spots were dried by a gentle stream of nitrogen. Pure, unlabelled retinoic acid could be applied to the plates under these conditions without detectable deterioration.

Two-way chromatograms were developed utilizing solvent system A in both directions. The plates were dried under nitrogen between developments. Autoradiography of such plates proved that the retinoic acid was pure in situ following the first development and that no detectable deterioration had occurred in the process of drying and redeveloping the plate. Since all of the other handling procedures involved had proven viable, it seemed that the critical step involved the removal of the silica from the plate and the recovery of the retinoic acid from the silica gel. The reasons for the failure of this step have not been elucidated.

The apparent problem of recovering retinoic acid from silica gel made some other means of purification desirable. The TLC findings suggested that HPLC on reverse phase columns as a possible solution. As a result of the instant success of HPLC, the recovery of retinoic acid from cellulose supports was not investigated. This might prove a viable alternative to users of these compounds who are not equipped for HPLC.

Purification by HPLC — As would be predicted from the findings in normal phase systems, all of the radiochemical contaminants in the stock label were found to elute prior to retinoic acid in the reverse phase system (Figures 2 and 3). The major contaminant was found to be easily separated and thus, the purification was made surprisingly simple.

The retinoic acid, as eluted, was both radiochemically and isomerically pure and ready for use without further handling. If not used immediately, the purified material can be tightly sealed and stored in the dark, at minus 20°C (as eluted). Purified samples shown to have a minimum apparent purity of 97% immediately following their acquisition have been found to maintain this level of purity for periods of 2-3 weeks. Storage and stability studies, and the determination of which, if any, of the contaminants might represent products of autoradiolysis have not been pursued further. It is unlikely that this one step purification can be readily performed on adsorption or normal phase columns. It should also be pointed out that the reverse phase system described becomes unwieldy if the elution of compounds significantly less polar than methyl retinoate or retinyl acetate is required.

Characterization of the Major Contaminant — During purification of the retinoic acids, the contaminants were also collected. The major contaminant from the ^{14}C-retinoic acid was methylated and repurified by HPLC. The methyl ester of this compound was then analyzed by mass spectrometry. The mass spectrum contained all of the fragments found in methyl-5,6- and methyl-5,8-monoepoxy-retinoic acid (5).

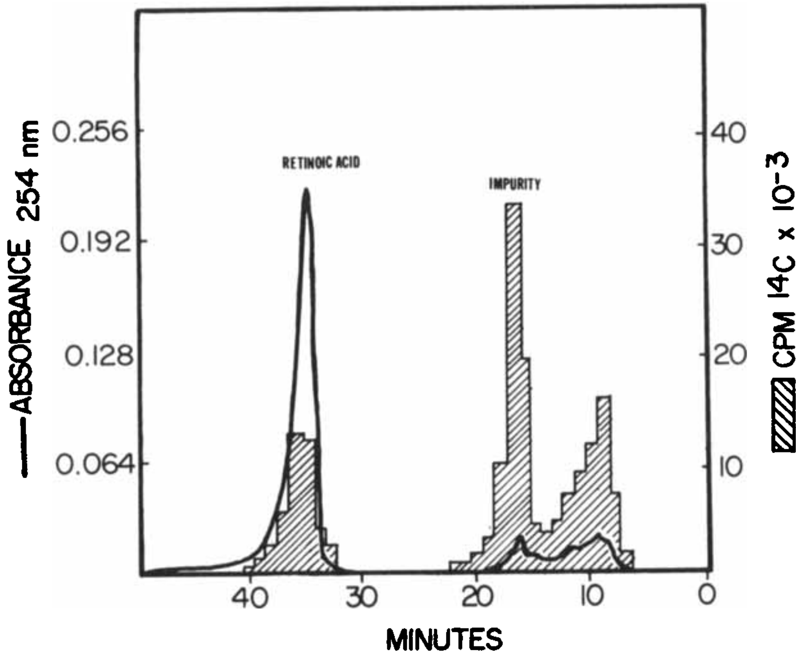

Figure 2. HPLC of impure [15-^{14}C]retinoic acid which had been spiked with all-<u>trans</u>-retinoic acid. The solvent was 71% methanol/water, flow rate = 0.4 ml/min at 20°C.

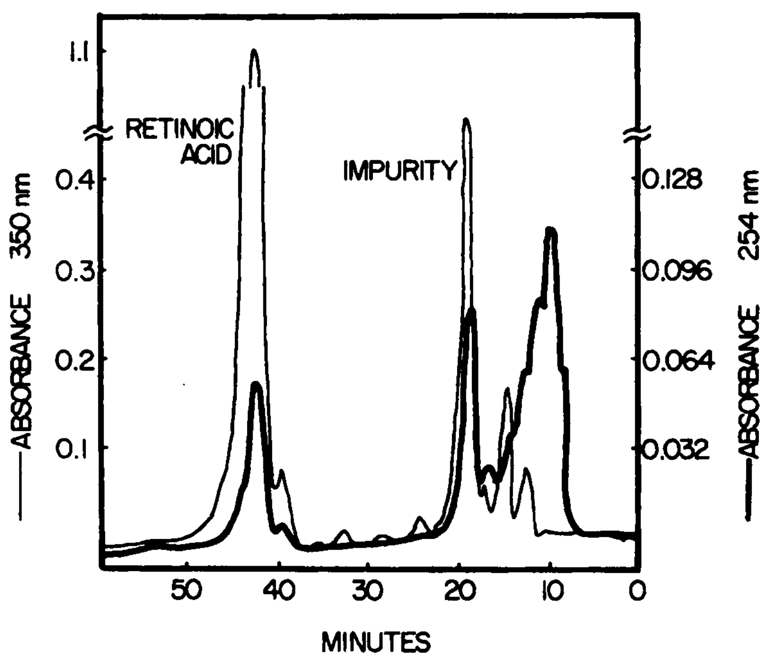

Figure 3. HPLC of impure [10,11-$^{3}H_2$]retinoic acid. The solvent was 70% methanol/water, flow rate = 0.4 ml/min and 20°C. Differences in retention times with respect to Figure 2 are attributable to differences in the eluting solvents and the strength and volume of the injected sample solvent.

The mass spectrum of this product also permitted the confirmation of both the specific activity and specificity of labelling in the original retinoic acid. In Figure 4a the high molecular weight segment of the mass spectrum of the major contaminant is shown. The mass ion at 330 is in keeping with that of a methyl epoxyretinoate but is accompanied by a disproportionately large $(M+2)^+$ ion at 332 which is attributable to the ^{14}C-atoms in the sample. The relative peak values from m/e 329 to 334 were determined for this spectrum and that of the equivalent unlabelled compound (Figure 4b). Thus, the isotopic abundance could be calculated. Using 62.4 mCi/mM as the equivalent of 100% isotopic abundance with single carbon labelling, the calculated specific activity of the oxidation product was found to be 18.65 mCi/mM. The specific activity of the parent acid was reported as being 18.95 mCi/mM.

If one considers the fragmentation pattern presented in Figure 4a, the classic fragments of a methyl ester are evident. In the cases where the fragments contained the carbonyl carbon of the original acid, they are accompanied by a correspondingly strong $(\underline{m}/\underline{e}+2)^+$ ion as was the case with the parent ion. These fragments are $(M-15)^+$ at $\underline{m}/\underline{e}$ 315 and $\underline{m}/\underline{e}$ 317 representing the characteristic loss of a methyl group from retinoic acid and related compounds (5, 6) and $(M-31)^+$ at $\underline{m}/\underline{e}$ 299 and $\underline{m}/\underline{e}$ 301 representing the loss of the methoxy group of the ester. However, with the loss of the carbonyl carbon there is a simultaneous loss of the disproportionately large $(\underline{m}/\underline{e}+2)^+$ ion. Thus, the $(M-59)^+$ ion, representing the loss of the $-COOCH_3$ function, displays the normal pattern found in the equivalent unlabelled compound (Figure 4b) and confirms the specificity of the labelling in the original acid.

The character of the faster eluting products has not been studied. They are almost certainly oxidation products of the parent acid in as much as their detection is dependent upon the presence of the ^{14}C-label. Some of these show little absorbance in the visible region of the spectrum suggesting that the conjugate system of double bonds has been interrupted or significantly shortened.

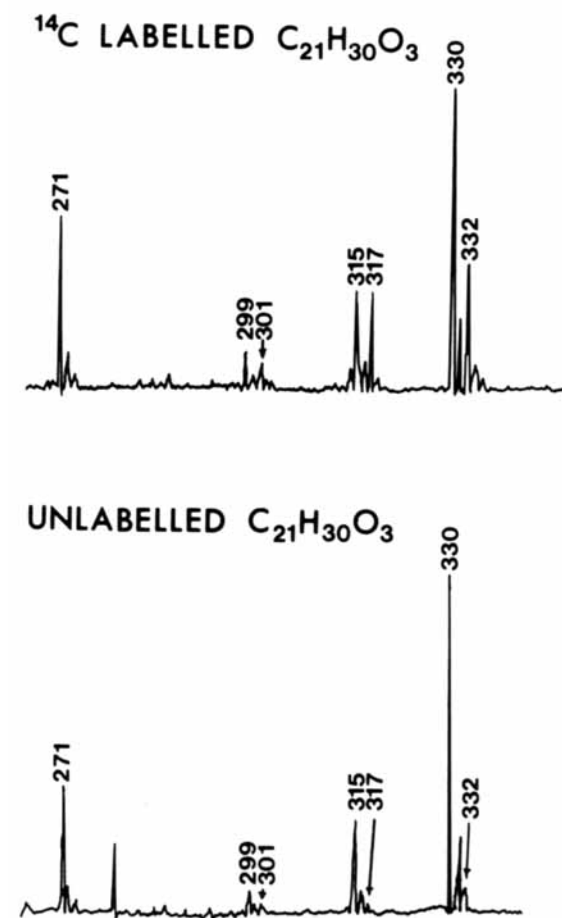

Figure 4. The high molecular weight region of the mass spectra of the methyl esters of a) the major impurity observed in [15-^{14}C]retinoic acid and b) the equivalent unlabelled compound.

Such an effect could be accomplished through single or multiple oxidation and might even represent oxidative cleavage of the compound to lower molecular weight fragments with only those bearing the label being immediately recognizable as such. The presumption that the contaminants found in the ^{3}H-stock might be the same as those observed in the ^{14}C-stock is based entirely upon their similar chromatographic behavior.

Possible Source of the Contamination — It must be presumed that the radiochemical stocks in question were pure at the outset. Although they had been on hand for about two years, it seemed highly unlikely that their extensive deterioration was the result of autoradiolysis since the two stocks of ^{14}C- and of ^{3}H-labelled retinoic acid were almost identical in radiochemical purity and relative contaminant distribution. Light was probably not involved since the samples showed no evidence of light induced isomerization. Possibly their degradation was due to air exposure, perhaps, as a result of the samples being permitted to dry as thin layers. There is no record to show that either of these stocks was ever so treated. However, the feasibility of this explanation was supported by the difficulties which were encountered while attempting to purify these stocks by TLC on silica gel plates. In one extreme instance, for example, a sample of 85% pure ^{14}C-retinoic acid was rechromatographed in an effort to further purify it. When it was recovered from the second purification and reanalyzed, it was found to be about 30% pure and was essentially indistinguishable from the original stock material.

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