Detection of 7-ketocholesterol in oxidized sterol preparations

Among the most frequently encountered autoxidation products of cholesterol (cholest-5-en-3β-ol) is 7-ketocholesterol (3β-hydroxycholest-5-en-7-one), which has been detected as such or as its dehydration product cholesta-3,5-dien-7-one in atherosclerotic aortal sterols[1,2], in swine testis sterols[3], in cholesterol irradiated[4] or aged[5] in air, in sheep wool sterols[6–8], in urine[9], and in colloidally dispersed cholesterol autoxidation products[10–12]. Whereas isolation of 7-ketocholesterol from such sterol preparations establishes its presence in the extracts as examined, the role of the sterol as an artifact or as a normal constituent of the sample is not necessarily established.

A specific means of detection of 7-ketocholesterol in complex sterol mixtures would have value in examination of such preparations suspected of containing autoxidation products but which also contain sought 7-hydroxylated products, as in bile acid biosynthesis for example. Resolution of 7-ketocholesterol from cholesterol and from other well-recognized autoxidation products of cholesterol is possible with paper chromatography[13,14], and with thin-layer chromatography[15]. Nevertheless specific detection of 7-ketocholesterol on such chromatograms is not readily accomplished. 7-Ketocholesterol may be detected by means of its ultraviolet light absorption properties, and in our experience it can be detected on silica gel HF_{254} chromatoplates to the 0.025–0.05 μg level. Charring with sulfuric acid permits detection to 0.01–0.025 μg. Though adequately sensitive neither of these procedures affords the specificity required for confident recognition of 7-ketocholesterol among other oxidized sterols in complex mixtures.

We have examined a number of acidic reagents for color development with 7-ketocholesterol on chromatoplates. Among these are 50 % aqueous sulfuric acid, concentrated sulfuric acid, 50 % aqueous sulfuric acid containing ceric sulfate, 25 % aqueous *p*-toluenesulfonic acid, 25 % aqueous perchloric acid, chlorosulfonic acid–acetic acid (1:2), 10 % ethanolic phosphomolybdic acid, saturated solutions of antimony trichloride and pentachloride, the Liebermann-Burchard reagent, 1 % vanillin in 50 % aqueous sulfuric acid, aqueous phosphoric acid solutions, etc., as well as ketone reagents 2,4-dinitrophenylhydrazine and isonicotinic acid hydrazide. None of these reagents afforded the sensitivity, specificity, or simplicity of use which was required.

It occurred to us that the known sensitive color visualization of the 7-hydroxycholesterols (cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol) with sulfuric acid could be applied to the detection of 7-ketocholesterol were the 7-ketone group reduced prior to visualization with the acidic reagent. The known lithium aluminum hydride reduction of 7-ketocholesterol to give as a major product 7β-hydroxycholesterol (with minor amounts of the 7α-epimer)[16] suggested that the more readily handled sodium borohydride would accomplish the task. Accordingly we investigated the use of sodium borohydride solutions as preliminary sprays for the reduction of the 7-ketone group on chromatographed samples of 7-ketocholesterol.

A 5-min exposure to a 1 % sodium borohydride solution in methanol sufficed to reduce 7-ketocholesterol deposited on a chromatoplate, as evidenced by total loss of quenching of fluorescence on silica gel HF_{254} plates and the concomitant appearance of the characteristic intense sky blue coloration associated with the 7-hydroxycholesterols after acid treatment.

Isolation of the sterol reduction product from methanolic sodium borohydride solutions of 7-ketocholesterol established that the 7β-epimer was the predominant reaction product. Additionally by spotting 7-ketocholesterol on the start line of a chromatogram and spotting over the sterol a solution of sodium borohydride followed by chromatographic irrigation it was possible to establish that the major reduction product of the reaction, whether in solution or on the chromatogram surface, was 7β-hydroxycholesterol with small amounts of the 7α-epimer (estimated 5–10 %).

We accordingly relate the sky blue coloration produced by the combined reduction/acid treatment procedure with the formation of 7β-hydroxycholesterol on the developed chromatoplate. The combined procedure thus affords a specific and sensitive means of detection of 7-ketocholesterol.

The complete reduction of 7-ketocholesterol to the chromogenic 7-hydroxycholesterol epimers should yield a procedure as sensitive as that used for detection of the 7-hydroxycholesterols *per se*. The sensitivity of detection of either 7-hydroxycholesterol with 50 % sulfuric acid ranges between 0.01–0.025 μg of sterol on irrigated chromatoplates. The sensitivity achieved by the combined procedure for 7-ketocholesterol extends to the 0.025–0.05 μg range, thus slightly less sensitive. The combined procedure is also about as sensitive as the non-specific detection of 7-ketocholesterol by means of its ultraviolet light absorption properties.

Association of an acid-induced intense sky blue coloration with steroids bearing the Δ^5-7-hydroxy feature is an old one which initiated in the very early work with the ill-defined "oxycholesterol". Numerous investigators have remarked on the characteristic blue colors obtained with these sterols[17–26]. So broadly encountered are these blue colors that a certain reliance on them as tentative identification for the class of Δ^5-7-hydroxysteroids appears warranted. It is known that $\Delta^{5,7}$-3-hydroxysteroids give blue colors; however, these blue colors are readily differentiated from those of the Δ^5-7-hydroxysteroid type.

We have employed the procedure herein described for the detection of 7-ketocholesterol in several commercial samples of cholesterol as well as in autoxidized cholesterol preparations.

Experimental

Thin-layer chromatographic resolution of 7-ketocholesterol from cholesterol and from other autoxidized sterol products was accomplished with one- and two-dimensional irrigation using silica gel HF_{254} (Brinkmann Instruments Inc., distributor of products of E. Merck, Darmstadt) chromatoplates 250 μm thick with the solvent systems ethyl acetate–heptane (1:1) and acetone–heptane (1:1). 7-Ketocholesterol was detected after irrigation by its quenching of fluorescence on the silica gel HF_{254} plates excited by a 254 nm mercury lamp or by charring at 110° after spraying with 50 % aqueous sulfuric acid. Added details of these procedures are being incorporated into another manuscript in preparation.

Irrigated chromatoplates were air dried and viewed under the 254 nm mercury lamp, and 7-ketocholesterol zones were marked. The chromatoplate was then sprayed with a freshly prepared 1 % (w/v) solution of sodium borohydride in methanol to the first appearance of saturation on the chromatoplate. After 5 min the plate was passed briefly over a hot plate for final drying and sprayed with 50 % aqueous sulfuric acid. Blue colored zones ordinarily appeared after a few minutes of air drying or more

rapidly by drying with a stream of warm air. The color display is noted during the drying. Rapid development of an intense sky blue color in exact coincidence with the previously located ultraviolet light absorbing zone constitutes a positive test for the presence of 7-ketocholesterol. By running a reference sample on the same chromatoplate both the location of the zone and the nature of the color display can be used for positive identification.

A solution of 40 mg of 7-ketocholesterol in 5 ml of freshly prepared cold 1 % (w/v) sodium borohydride solution in methanol was held at 0° for 5 h. Ether (20 ml) was added to precipitate inorganic material and the filtered solution was evaporated under vacuum, redissolved in ether and refiltered several times. On evaporation 32.8 mg of 7β-hydroxycholesterol, m.p. 172–175° (Kofler block), ν_{max}^{KBr} 1665, 3320 cm^{-1}, containing minor traces of 7α-hydroxycholesterol, was obtained.

For reduction of the sample at the start line prior to chromatographic irrigation, 20 μg of 7-ketocholesterol was applied to the chromatoplate as a small spot in the usual manner. Freshly prepared 1 % sodium borohydride solution in methanol (total 40 μl) was spotted carefully around the periphery of the sterol spot so as not to cause the sterol spot to spread. After drying in air for a minute the plate was irrigated in the usual two-dimensional fashion to resolve the reduced products. No 7-ketocholesterol was detected by ultraviolet light absorption methods and the only sterol zones detected with 50 % aqueous sulfuric acid were the major 7β-hydroxycholesterol component, with traces of the 7α-epimer.

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