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The High-Performance Liquid-Chromatographic Analysis of Ficaprenol and Dolichol

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A high-performance liquid-chromatographic method was devised which is capable of resolving the *p*-nitrobenzoyl derivatives of polyprenols containing 35–110 or more carbon atoms. This procedure was used for the determination of ficaprenol and pig liver dolichol composition and can be applied to mixtures of polyprenols as an analytical or preparative technique.

It is now well established that at least one function of the long-chain polyprenols is to serve as intermediates in glycoprotein synthesis [see reviews by Hemming (1974) and Waechter & Lennarz (1976)]. The metabolism of these compounds has therefore become a subject of increasing interest. The analysis of polyprenol mixtures is difficult and imprecise owing to the fact that these compounds possess no unique structural feature other than size, and no chromophoric or functional group which lends itself to easy analysis. The high molecular weight of dolichol (a group of related polyprenols C₈₀ to C₁₁₀) has precluded its analysis by g.l.c., and the only means for resolving these compounds is by reversed-phase chromatography on kieselguhr plates impregnated with paraffin oil (Dunphy *et al.*, 1967), although partial resolution of the dolichols was also obtained by chromatography on Lipidex-5000 by Mańkowski *et al.* (1976). Donnahey & Hemming (1975) have reported that they used high-performance liquid chromatography to separate ubiquinones, they were less successful with ficaprenol, and failed to resolve dolichol into its component isoprenologues.

In the present communication we describe the successful separation of these compounds, ficaprenol and dolichol, which were converted into their *p*-nitrobenzoyl derivatives. The method is sensitive and quantitative over a range of polyprenol concentrations likely to occur in biological samples, and should prove useful for studying the metabolism of the polyprenols as well as providing a simple preparative method for the isolation of individual polyprenols.

Materials and Methods

Materials

Dolichol was isolated from pig liver essentially as described by Richards & Hemming (1972), except

that a column-chromatographic procedure on Sephadex LH-20 was substituted for the final thin-layer separation used by these workers. Old leaves from *Ficus elastica* plants, provided by Calvin Lyons, Jr. (Agricultural Extension Service of Texas A & M University), were the source of ficaprenol, which was isolated as described by Stone *et al.* (1967). These samples were judged to be greater than 95% pure by t.l.c. and i.r. analysis. Propan-1-ol and benzene were purified by passage through columns of alumina (Brockmann grade 1). Pyridine was refluxed and distilled over BaO; all other solvents were reagent grade. Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) supplied *p*-nitrobenzoyl chloride. Silica-gel t.l.c. plates were purchased from Analtech Apparatus, Wilmington, DE, U.S.A.

Chromatography analysis was carried out on either a Varian model 1400 high-performance liquid-chromatography apparatus equipped with a Glenco high-pressure sample-injection valve, or a Waters model 6000A instrument. Both instruments had 254nm detectors. The analytical column used in these studies was a Bondapak C₁₈ reverse-phase column (30cm×4mm internal diam.), manufactured by Waters Associates. With time, as the back pressure increased and the resolution of the peaks became poorer, it was necessary to flush the column with dimethyl sulphoxide followed by methanol. This restored the resolving power and decreased the back pressure.

Preparation of *p*-nitrobenzoyl derivatives

Stock solutions of the polyprenols containing approx. 2.5mg (2μmol) of polyprenol/ml were prepared in benzene. Samples of the stock solutions containing between 0.06 and 1mg (approx. 0.05–0.8μmol) of polyprenol were mixed with 100μl of pyridine containing 3mg (16μmol) of *p*-nitrobenzoyl chloride. The solution was usually allowed to react at

60°C in a capped 1.5 ml tube for 45 min. The progress of the derivative-forming reaction could be followed by chromatographing small portions of the reaction mixture on silica-gel G thin-layer plates. The plates were developed with light petroleum/diethyl ether/acetic acid (90:10:1, by vol.), and the disappearance of the polyprenol spot was monitored.

At the end of the reaction, the tubes were allowed to cool and 0.8 ml of hexane was added. The hexane layer was washed successively twice with 0.4 ml of 0.1M-HCl and once with 0.4 ml of saturated NaHCO₃. The washed hexane phase was evaporated to dryness under N₂ and the residue was dissolved in a suitable amount (200 μ l) of propanol for injection. Blanks containing only benzene were also carried through this procedure.

In experiments in which dolichol and ficaprenol were hydrogenated before derivative formation, a procedure similar to that of Applequist (1972) was used. The polyprenol sample (0.5–1.0 mg) was transferred to a 13 mm $\times$ 100 mm 2-dram vial fitted with a silicone rubber septum. After the vial had been purged with H₂ gas, a slurry of about 1 mg of platinum oxide in 0.5 ml of methanol was injected into the vial. After incubation for 30 min at 50°C, the sample was transferred to a 1.5 ml centrifuge tube with chloroform and the vial was rinsed with chloroform. The catalyst was sedimented, washed with chloroform and the combined extracts were dried under N₂. The hydrogenated polyprenols modified as described above showed no indication of the unchanged starting materials.

Results and Discussion

Chromatographic separation of ficaprenol and dolichol p-nitrobenzoates

The chromatographic separations shown in Fig. 1 are typical of those obtained in a large number of runs. Despite the fact that the solvent and samples were purified, a sizable peak of u.v.-absorbing material was obtained which was not retained by the column. This material does not interfere with the analysis, except for shorter polyprenols such as betulaprenol. Increasing the polarity of the solvent would probably resolve these compounds, but this has not been tested as yet. Other high-molecular-weight alcohols such as cholesterol are also eluted much earlier than the long-chain polyprenols. The best separations of dolichol were obtained with 95% (v/v) propan-1-ol as a solvent, which was used in all the work reported here. The *p*-nitrobenzoates are particularly good derivatives to use for high-performance liquid-chromatographic separations, since the λ_{max} , 256 nm reported by Burgos *et al.* (1963) is very close to maximum sensitivity of the commonly used 254 nm detectors. The *p*-nitrobenzoyl derivatives have

a molar extinction coefficient of approx. 14000 litre·mol⁻¹·cm⁻¹ (Burgos *et al.*, 1963), which permits detection of very low concentrations. The free polyprenols are readily prepared from the benzoates by alkaline hydrolysis, so that the method could be used for the isolation of samples of individual isoprenologues as well as the determination of the composition of a mixture of polyprenols. The method should prove particularly useful in biosynthetic studies in which the isotopic labelling of polyprenols is measured. We modified [³H]dolichol prepared as previously described (Keenan & Kruczek, 1975) and found that the radioactivity corresponded to the principal dolichol peaks.

Composition of ficaprenol and dolichol

The percentages of each major component in the ficaprenol and dolichol samples were determined from these relative peak areas. Ficaprenol was shown to consist of 2.3% C₄₅, 23.8% C₅₀, 58.3% C₅₅ and 15.6% C₆₀ plus traces of other components. These results are more precise than, but in agreement with, those reported by Stone *et al.* (1967), who estimated weights of the polyprenols eluted from reversed-phase thin-layer chromatograms.

The pig liver dolichol sample contains 1.8% C₈₅, 17.6% C₉₀, 46.3% C₉₅, 27.7% C₁₀₀, 6.5% C₁₀₅ and traces of other components. These results are also in agreement with those reported by Butterworth & Hemming (1968). It is expected that there may be variations in polyprenol content due to age, diet etc. It will also be of interest to apply the method to a wide variety of biological materials to determine the relative make-up of their polyprenol mixtures.

Quantitative determination of polyprenols

Samples of a solution of pig liver dolichol which contained 60–500 μ g of polyprenols were carried through the modification procedure described in the Materials and Methods section. Since only one-quarter of the final extract was injected, the total quantity of material modified could be decreased if desired. The results given in Fig. 2, which are expressed as a function of the peak height of the C₉₅ dolichol, show that the assay is linear over a convenient range of sample sizes. Since C₉₅ dolichol accounted for only 46% of the total polyprenol mixture, it is clear that individual isoprenologues were detected at levels of 3–5 μ g without resorting to the highest sensitivity of the detector. If reasonable care is taken, it should not be difficult to apply the method to samples containing as little as 1 μ g of the individual polyprenols, although in theory much greater sensitivity is possible. The major difficulty in quantifying the dolichols lies in the preparation and clean-up of the *p*-nitrobenzoates, which involves reproducible

Fig. 1. High-performance liquid-chromatographic separation of ficaprenol and pig liver dolichol *p*-nitrobenzoates. Chromatographic conditions were: elution solvent 95% (v/v) propan-1-ol; flow rate 0.5 ml/min. The numbers on the major peaks refer to the total isoprene units. Detection was at 254 nm. (a) 38 μ g of ficaprenol; full-scale deflexion 1.0 A unit; chart speed 0.5 cm/min (0.2 in/min). (b) 188 μ g of pig liver dolichol; full-scale deflexion 0.5 A unit; chart speed 0.25 cm/min (0.1 in/min).

Fig. 2. Detection response versus dolichol concentration. Samples of pig liver dolichol were modified and chromatographed as described in the text. The results are expressed in terms of the peak height of C_{95} dolichol, the largest component in the mixture.

losses. This problem can be easily avoided by using a suitable internal standard, such as [3H]dolichol, and correcting for losses.

Relationship of structure to elution time

This method would be particularly useful if it could be used to characterize unknown polyprenols by their relative retention times. The known polyprenols differ in the degree of unsaturation and the isomeric nature of the double bonds as well as in the length of the carbon chain. Fig. 3(a), which is a plot of $\log(\text{retention time})$ versus number of isoprene units, is linear for each type of polyprenol, but it is clear that ficaprenol and dolichol do not fall on the same or parallel lines. To explore the reason for this further, the polyprenol mixtures were hydrogenated before modification, thus eliminating differences due to factors other than chain length. When these compounds were subjected to high-performance liquid chromatography the result was that the re-

Fig. 3. Retention times of ficaprenol and dolichol *p*-nitrobenzoates and their hydrogenation products versus number of isoprene units
(a) Ficaprenol (●) and dolichol derivatives (■); (b) hydrogenated polyprenol derivatives.

tention times were increased severalfold. The peaks for the hydrogenated dolichol derivatives were broad, but easily discernible. The results plotted in Fig. 3(b) show that the elution time is not linear with respect to chain length when the sizes of the molecules are very different. The reason is undoubtedly that the effect of the *p*-nitrobenzoyl group is to retard elution, but this becomes increasingly less important as the chain length increases. Unfortunately we do not have available medium-chain-length polyprenols, such as those found in yeast, to prove that this is the case. There would be no difficulty in determining chain length within a given class of compounds, however. As far as the influence of structure on the rate of elution is concerned, it is very unlikely that subtle differences in unsaturation or the nature of the geometric isomers could be detected in the long-chain polyprenols by this method, but these factors should produce changes in the elution of the smaller polyprenols. Indeed, we have observed a small peak in pig liver dolichol which is eluted just slightly ahead of C₃₅ ficaprenol. This may represent the C₃₅ dolichol-type polyprenol reported by Mańkowski *et al.* (1976). In any event, there would be no problem in distinguishing between various polyprenols with respect to their relative chain length by hydrogenating them before chromatography. This represents a simple method of separating polyprenol fractions, which could be further characterized by means such as mass spectra.

In the present communication we describe the successful separation of ficaprenol and dolichol by converting them into their *p*-nitrobenzoyl derivatives.

This has the added advantage of greatly increasing the sensitivity of the assay. This method yields quantitative results and should prove useful for studying the metabolism of the polyprenols.

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