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Note

Separation of ubiquinone homologues by reversed-phase high-performance liquid chromatography

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Ubiquinone is a prominent member of the polyisoprenoid family. In addition to its well known role in oxidative phosphorylation[1], a potential role in non-mitochondrial oxidoreductive reaction has also been proposed[2]. Recently, the biosynthesis of ubiquinone and its regulation have received renewed attention due to the realization that the relative flux of mevalonate, a common intermediate in polyisoprenoid biosyntheses, to the individual branch pathways of polyisoprenoids is tightly regulated[3].

Several ubiquinones which differ in the length of isoprene side chain have been found in nature[1]. Thin-layer chromatography (TLC) and gas chromatography[4,5] are often employed for the analysis and the identification of individual ubiquinone species, high-performance liquid chromatography (HPLC) is highly desirable (see also refs. 6–9). To investigate ubiquinone species in small amounts of biological specimen, a sensitive HPLC system capable of quantifying individual ubiquinone species was developed.

EXPERIMENTAL

A high-performance liquid chromatograph (6000A, Waters Assoc.) equipped with a variable-wavelength detector (450, Waters Assoc.) or an electrochemical detector (LC-3A, BioAnalytical Systems) with a glassy carbon electrode (TL-5, BioAnalytical Systems) was used. The instrument was connected to a computing integrator (3390A, Hewlett Packard).

A reversed-phase column (C_{18}, 600 RP, 25 cm × 4.6 mm I.D., Alltech) was used. The mobile phase was pumped at a flow-rate of 1.0 ml/min (1500 p.s.i.). The column was operated at room temperature (20°C).

Different ubiquinone species were obtained from Sigma and dissolved in chloroform–methanol (2:1) to a concentration of 10 mg/ml. Their purity was ascertained by TLC[4] and by spectrophotometric analysis[5]. The concentrations were determined by the absorbance at 275 nm using the established extinction coefficiencies[5]. The attenuation of the detector was 0.4 a.u.f.s.

Sodium borohydride (Sigma, St. Louis, U.S.A.) was used to reduce the ubiquinone homologues. The sodium borohydride was dissolved in methanol (2 mg/ml).

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Equal volumes of the ubiquinone homologues and sodium borohydride were added together and the reaction allowed to proceed for five minutes. The reaction mixture was diluted with chloroform–methanol (2:1) to the appropriate volume prior to HPLC analysis.

RESULTS

The chromatographic behavior of ubiquinone-6, -7, -9 and -10 was investigated to define conditions suitable for routine HPLC. The results are summarized in Fig. 1. As can be seen, reversed-phase HPLC can be employed to resolve ubiquinone homologues according to the numbers of isoprenyl units. The effect of methanol contents prolonged elution time but resulted in better separation. Since ubiquinone-8 was not commercially available, the results were replotted to obtain information about the effect of the side chain on chromatographic behavior of each ubiquinone

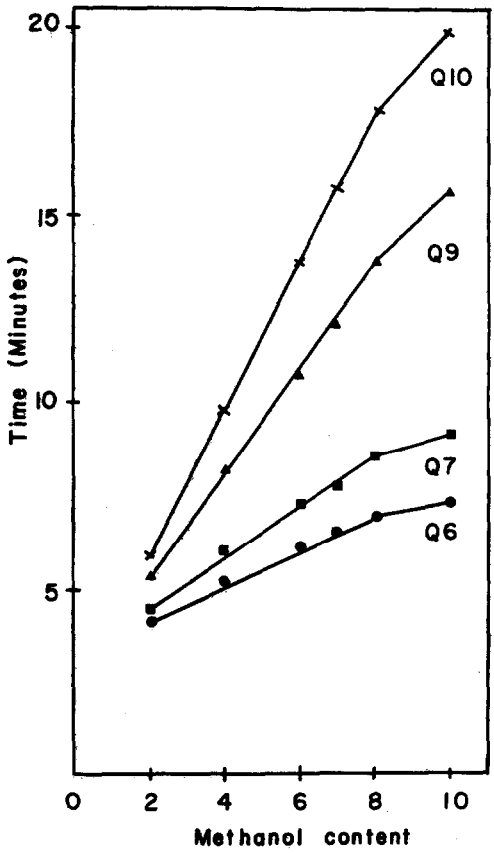

Fig. 1. Effect of methanol content on the chromatographic behavior of ubiquinone homologues. In this experiment hexane and isopropanol were kept at one unit. Thus, methanol of 2 represented the following solvent system: hexane–isopropanol–methanol (1:1:2). Q = ubiquinone.

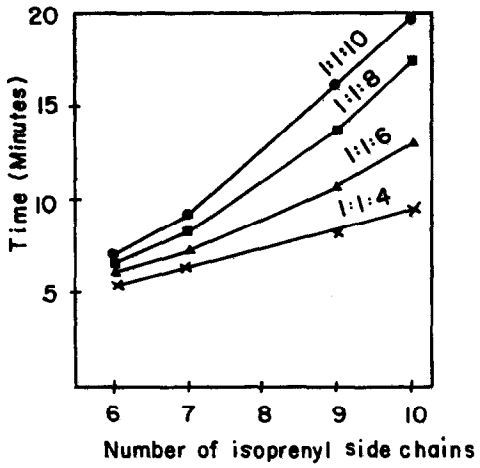

Fig. 2. Effect of isoprenyl side chain length on the chromatographic behavior of ubiquinones. Solvent system = hexane–isopropanol–methanol.

species (Fig. 2). According to this figure, ubiquinone-8 could probably be separated from other species.

To avoid any mistake in identifying ubiquinone in a complex biological specimen, a different solvent system was desirable. Isopropanol was eliminated from the above system. The results are expressed in Fig. 3. A typical run is shown in Fig. 4.

The HPLC assay was reproducible (± 3%). As low as 5 ng of ubiquinone could be easily detected by this method.

Biological samples may contain a great number of unknown interfering substances. Although a variety of solvent systems outlined above can help to eliminate any mistaken identification, chemical properties of ubiquinone presented another avenue to study this problem. Ubiquinones could be quantitatively reduced to dihydroquinones. These reduced products can then be analysed by reversed-phase HPLC. A summary of reduced forms of ubiquinones in different solvent systems was listed (Table I). Due to the low extinction coefficient of dihydroubiquinone this method was only approximately one-fourth as sensitive as that for ubiquinone. The reduced form of each ubiquinone homologue eluted at a slightly faster rate in all the solvent systems tested. This property allowed for the determination of the efficiency of the reduction by using UV detection. As can be seen in Fig. 5, the reduction appears to be 100%.

One requirement for electrochemical detection (ED) is that the compound of interest must be able to be oxidized or reduced. The ubiquinone homologues fall into this category. Reductive ED requires that *all* oxygen be removed from the HPLC system. Although quite feasible, the necessary arrangement may be cumbersome for many laboratories.

For oxidative ED, testing at several voltage potentials indicated the range of 0.6 V to 0.8 V to be very suitable, with 0.7 V to be the optimum.

The chromatography was reproducible to within ± 3%. As low as 120 pg could be quantitated (Fig. 6). It is possible that by using a more efficient column, an even higher sensitivity can be achieved.

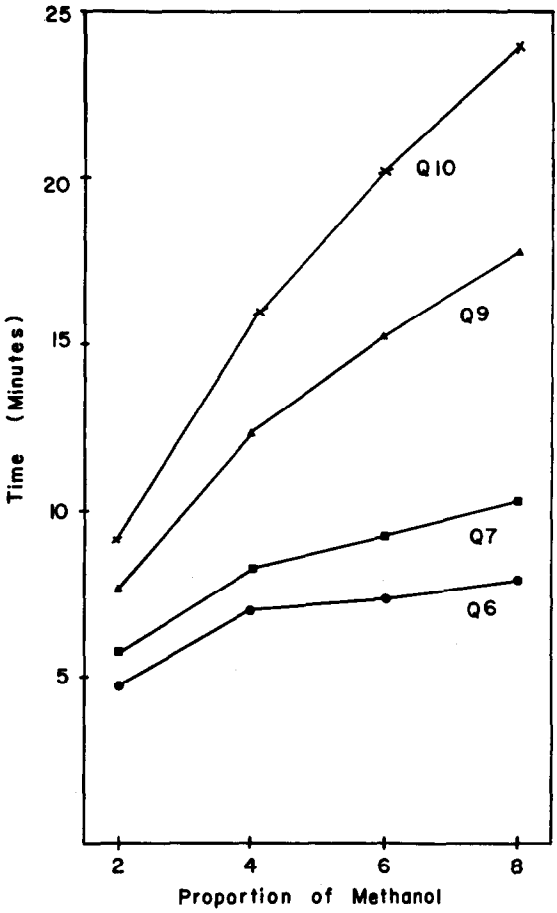

Fig. 3. Effect of methanol content on chromatographic behavior of ubiquinone homologues. The experiment was identical to that in Fig. 1 except isopropanol was eliminated. Q = ubiquinone.

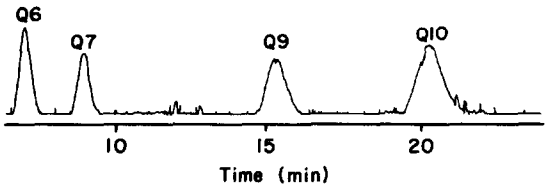

Fig. 4. Resolution of ubiquinone homologues by reversed-phase HPLC. Q = ubiquinone.

By this method it has been shown that the ubiquinone homologues can be conveniently reduced and when combined with HPLC–ED, the quantitative limits of detectability increases approximately forty times over UV detection. The increase in sensitivity afforded by ED will allow for the quantitation of ubiquinone homologues in small amounts of biological specimens.

TABLE I

RETENTION TIMES (min) OF THE REDUCED UBIQUINONE HOMOLOGUES IN TWO SOLVENT SYSTEMS

Q = ubiquinone.

Solvent system (hexane–methanol)	*Q6*	*Q7*	*Q9*	*Q10*
1:6	4.8	5.7	8.4	10.2
1:2	3.3	3.6	4.6	5.1

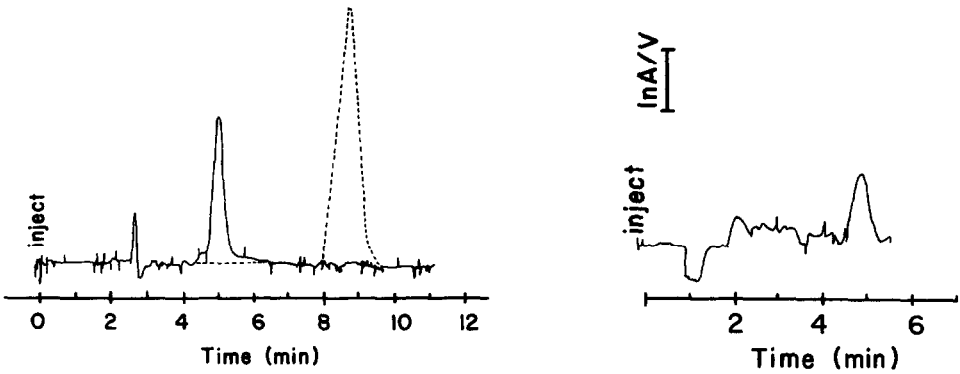

Fig. 5. HPLC chromatograms (superimposed) of ubiquinone-10 before (-----) and after (——) reduction. Mobile phase: hexane–methanol (1:2); flow-rate 1 ml/min, UV at 290 nm with attenuation 0.4 a.u.f.s.

Fig. 6. HPLC chromatogram of reduced ubiquinone-6. Mobile phase: hexane–methanol (1:6); flow-rate 1 ml/min; potential = 0.7 V. Amount of compound injected is 120 pg.

CONCLUSIONS

Reversed-phase HPLC was shown to be a useful technique in separating ubiquinone species different in the length of the isoprenyl side chain. The complete resolution of homologues in the reversed-phase chromatography in several solvent systems outlined here is also helpful for positive identification of ubiquinone in complex biological samples. The chemical selectivity offered by the reported electrochemical detection method can further eliminate any coincidential peaks in biological specimens. Finally, the sensitivity of this method demonstrates this method may be applicable to quantify biological samples in which only a small amount of ubiquinone is obtainable.

In two meeting abstracts, a method for assaying isoprenoids including ubiquinone was presented. However, a gradient solvent was necessary for the separation of ubiquinone homologues[6,7]. More recently, the determination of oxidation–reduction levels of ubiquinone by HPLC was reported[8,9]. Our system offers the following improvements over the existing methods: (i) isocratic separation of ubiquinone homologues in several solvent systems and (ii) easy reduction of ubiquinone to ubiquinol for HPLC–ED using a more convenient oxidation mode.

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