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ESTIMATION OF INDIVIDUAL COENZYME Q HOMOLOGUES IN A COENZYME Q MIXTURE BY DENSITOMETRY

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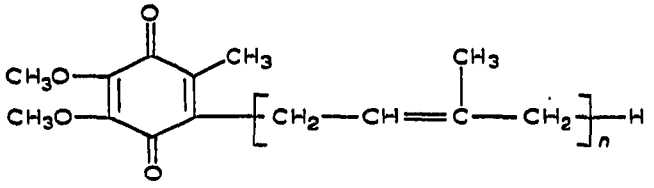
SUMMARY

A method for the densitometric determination of coenzymes Q individual homologues after separation by reversed-phase paper chromatography is described. The parameters associated with the reproducibility and accuracy of the method are discussed. Comparison is made between the total coenzyme Q concentration in liver and spleen of mice obtained by densitometry and by the modified Craven test.

INTRODUCTION

The biosynthetic isoprenylation of the coenzymes Q (co Qs) (I) leads to a series of co Qs with different number of isoprene units in the side chain[1]. Each species biosynthesizes predominantly one co Q, but other homologues with shorter and longer side chains are also formed. When determining the total co Q content of tissues and blood of experimental animals, it is usually necessary to remove interfering substances prior to the actual assay of the coenzymes. This is accomplished by saponification and extraction methods followed by one or several chromatographic steps[2]. The co Q fraction obtained in this manner contains the mixture of all the homologues present which is then quantitatively assayed by means of ultraviolet (UV) spectrophotometry (characteristically by differential UV absorption between the quinone and hydroquinone forms)[2] or by a modified Craven test[3,4]. The results are then usually expressed as the concentration of the predominant co Q. However, the individual homologues present in the extract can be detected by one of several excellent paper (PC) and thin-layer chromatographic (TLC) techniques available, all based on reversed-phase chromatography[2].

In studying the total co Q concentration in tissues or blood, it is at times desirable or even necessary to determine the concentration of the individual homologues in the mixture in a quantitative manner. Although this can be accomplished by extraction of each spot obtained from a reversed-phase chromatogram followed by quantitation by spectrophotometry[5], such elution procedures require, in general, larger amounts of material than is usually available from a single spot. Moreover, in many cases it is necessary to remove, by chromatographic means, the supporting



material used to impregnate the paper, as its presence usually interferes with the spectrophotometric determination of the coenzymes[6]. Therefore, we have developed a procedure for the direct estimation of the individual spots in the paper chromatogram by means of densitometry. The values of total coenzyme Q concentration obtained by this densitometric technique were compared with those obtained using the modified Craven test of REDALIEU *et al.*[3,4]. Mouse livers and spleens were used for testing and developing the method.

EXPERIMENTAL

Standards

Coenzyme Q_{10} was obtained from Cudahy Laboratories, Omaha, Nebr., U.S.A.; coenzyme Q_9 was isolated from rat liver by the methods described below; coenzymes Q_8, Q_7 and Q_4 were kindly supplied by Dr. KARL FOLKERS, Institute for Biomedical Research, Austin, Texas, U.S.A.; coenzyme Q_6 was obtained from Sigma Chemical Company, Saint Louis, Mo., U.S.A.

All solvents were purified prior to use.

Extraction of the coenzymes Q from tissue

The saponification method of SUGIMURA *et al.*[7] was used to prepare a purified co Q fraction from mouse tissues (liver or spleen). For duplicate determinations 10 g of wet tissue sufficed. The unsaponifiable fraction was purified by two successive thin-layer chromatographies. For development of the first plate (20 × 20 cm, Silica Gel G, 1 mm thick, prewashed in chloroform–methanol (4:1)) hexane–diethyl ether (60:40) was used as solvent. The portion of the silica gel corresponding to a comparison sample of pure "standard" co Q was extracted with diethyl ether. The residue on evaporation of the ether extract was then further purified by TLC using petroleum ether (b.p. 42–60°)–diethyl ether (90:10) as solvent. The residue of eluting with diethyl ether the co Q band was next taken up in ethanol and made up to volume. An aliquot was then used to assay the total co Q content in the tissue by a modified Craven test. The remainder of this residue after evaporation of the ethanol was then taken up in hexane and subjected to PC.

Estimation of the total coenzyme Q content in purified tissue extracts by the modified Craven test

The method described by REDALIEU *et al.* was used[3]. The accuracy of this procedure has been reported to be 97% and its standard error 3.8%[4]. The absorbance measurements were carried out in a Beckman DB spectrophotometer.

The saponification method of SUGIMURA *et al.*[7] was selected for this work because methanol rather than ethanol is used in the saponification medium. It has

been shown that ethoxy derivatives of the coenzymes Q are formed as artifacts during hot alkaline saponification in ethanolic solution[8,9]. When methanol is used as the medium, such alkoxy derivatives are not formed.

This modified Craven test was selected as our standard method for determining the total coenzyme Q concentration on the purified tissue extracts because of its reliability and high specificity. No other methoxy derivative existing in tissue is known which might interfere with this assay[3].

Paper chromatography of the coenzyme Q mixture

The method of LINN *et al.* was used[10]. Whatman No. 1 sheets (24 × 56 cm) were impregnated with petroleum jelly (5%, w/v in petroleum ether) and developed with N,N-dimethylformamide–water (97:3) saturated with petroleum jelly. The ascending technique was used.

After application of the sample, the paper was equilibrated in the vapor phase of the solvent for 0.5 h prior to development. Development time was 42 h. At the end of the chromatography the paper was air-dried in the dark for 6 h at room temperature. The solvent front had advanced approximately 30 cm from the origin.

The R_F values of the coenzymes Q in this system are approximately 0.71, 0.60, 0.49, 0.35, 0.26 and 0.17 for co Q_4, co Q_6, co Q_7, co Q_8, co Q_9 and co Q_{10}, respectively.

The chromatogram was then immersed for 30 sec in a 0.2% solution of $KMnO_4$. Excess permanganate was removed by washing three times with distilled water and the chromatogram then air-dried overnight before using it for densitometry. This treatment gives brown spots against an off-white background.

Densitometric assay

All densitometric measurements were made by transmission, using a Photovolt Densitometer Model 530, equipped with a Varicord variable-response recorder Model 42 with automatic scanning. The light source was an incandescent bulb. The scanning of the spot was carried out perpendicularly to the line of development of the chromatogram and through the center, darker portion of the spot. The recorder response was set to that which corresponds approximately to the logarithm of the light absorption. The densitometric traces obtained were integrated with a planimeter.

In preliminary work it was established that impregnated paper, treated with the $KMnO_4$ solution as described, produced an almost straight base line on the densitometric readings. It was also found that the brown color developed by the spots on permanganate treatment was stable for up to four days, giving reproducible densitometric readings. This is useful for cases where it is not convenient to run the densitometric measurements immediately after treatment.

Only five initial spots were applied on each chromatographic sheet, 4 cm apart. This distance allowed enough separation between adjacent spots in the developed chromatogram to enable the base-line between adjacent densitometric curves to be reestablished.

In the preliminary studies, solutions containing different concentrations of the co Qs were prepared and various volumes of these solutions were spotted on the paper, in order to determine the optimal amount of co Q per spot for densitometry and the optimal volume of solution for each application. Very uniform, small spots were produced by application with a micropipette of between 25–75 λ of solution. After

development of the chromatogram, those spots containing between 5 aud 35 μg of co Q were round and well defined. As will be discussed later, these amounts were the lower and upper limits of accurate detection by densitometry.

RESULTS AND DISCUSSION

Typical calibration curves for some coenzymes Q are shown in Fig. 1. The integrated area under a densitometric peak was plotted against the amount of co Q present on the spot. Spots containing more than 35 μg of co Q reacted incompletely with the $KMnO_4$ solution under the conditions described in EXPERIMENTAL, leading to

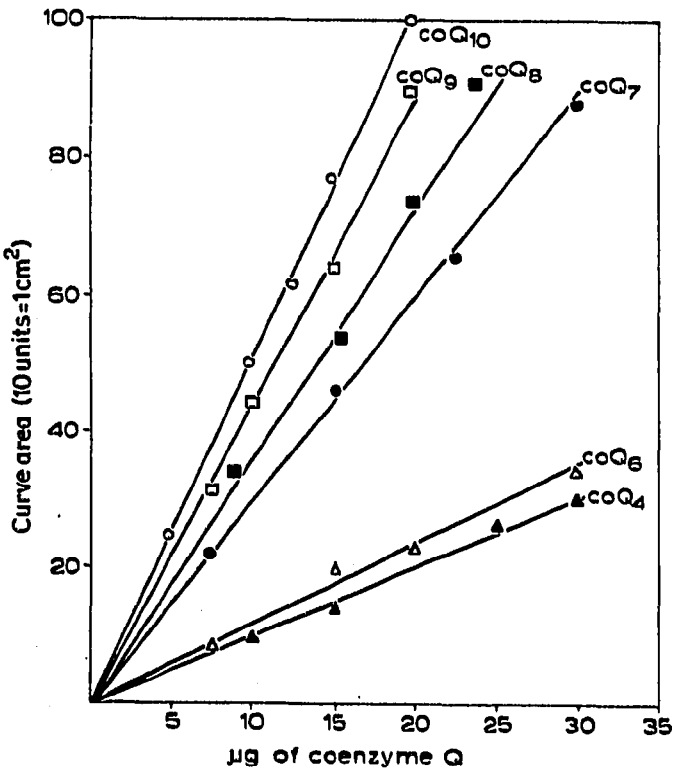


Fig. 1. Calibration curves for a series of coenzymes Q.

deviation of linearity in the densitometric response. In contrast, spots containing less than 5 μg did not develop a sufficiently intense color to be determined with accuracy. Thus the limits of detection were established as between 5 and 35 μg of co Q. It is of interest to note that as the length of the side chain increases in the co Q family, the slope of the response curve increases. This behavior is to be expected since the deposit of MnO_2 should increase as the number of double bonds per molecule increases.

The precision of the densitometric readings (by planimeter) was tested on the same and among different chromatographic sheets by applying a series of five replicates of a coenzymes Q mixture to impregnated paper. Each initial spot was produced from 10 μl of a hexane solution containing 5 μg of co Q_{10}, 5 μg of co Q_9, 12 μg of co Q_7 and 16 μg of co Q_6. Four such sheets were prepared, run chromatographically and stained with the $KMnO_4$ solution. The area under each densitometric peak was integrated and the results analyzed (*cf.* Table I).

TABLE I

REPRODUCIBILITY OF THE ANALYSIS OF A MIXTURE OF COENZYMES Q

Sheet	*Planimeter readings*[a] ± *% coefficient of variation*			
	co Q_{10}	co Q_9	co Q_7	co Q_6
1	26.4 ± 5.7	20.0 ± 6.1	23.8 ± 5.0	29.2 ± 3.6
2	32.0 ± 3.8	25.2 ± 2.4	35.5 ± 0.9	29.8 ± 8.7
3	32.4 ± 6.2	26.8 ± 3.5	30.2 ± 3.1	32.3 ± 5.6
4	27.6 ± 4.7	20.2 ± 5.4	30.8 ± 3.1	26.3 ± 2.9

[a] 10 units = sq. cm.

The fact that area readings for a constant amount of material will vary from sheet to sheet is a well known problem in quantitative paper chromatography[11]. Therefore, in all cases where unknowns were involved a standard solution in two different concentrations was also applied on the same sheet beside the sample and worked up to a calibration curve. The treatment of sample and standards was then identical throughout the chromatography.

A further mixture of co Qs of known concentration was then analyzed for these control purposes. Three aliquots (25, 50 and 75 μl) of a hexane solution containing 156.4 μg of co Q_{10}, 145.6 μg of co Q_9 and 188 μg of co Q_6 per 1 ml of solution were applied to paper, along with two different concentrations of a standard solution. From the calibration curves the amount of co Q in each spot was determined, the concentration of each co Q in the original solution calculated and the values of the three determinations then averaged. Table II shows a comparison between the composition values by known weight and that measured by densitometry, thus giving a measure of the percentage of recovery.

TABLE II

ANALYSIS OF A SYNTHETIC MIXTURE OF THREE COENZYMES Q BY DENSITOMETRY

Coenzyme	*Theoretical values (μg/ml)*	*Experimental values*[a] *(μg/ml)*	*Recovery (%)*
Q_{10}	156.4	149.5 ± 0.3%	95.6
Q_9	145.6	151.3 ± 3.5%	103.8
Q_6	188.0	186.8 ± 6.0%	97.6

[a] Mean ± coefficient of variation of three determinations.

Next the densitometric and the modified Craven tests were compared as to their accuracy for the determination of the total coenzyme Q concentration in a mixture of known composition containing four homologues. Triplicate aliquots (10 μl) of a hexane solution containing 0.5 mg of Q_{10}, 0.5 mg of co Q_9, 1.2 mg of co Q_7 and 1.6 mg of co Q_6 per ml of solution were applied to paper along with two different concentrations of a standard solution. The concentration of each spot was estimated from the standard curves and the mean values and standard deviations calculated. Total co-

TABLE III

COMPARATIVE ANALYSIS OF TOTAL COENZYME Q CONCENTRATION BY DENSITOMETER AND MODIFIED CRAVEN TEST IN A MIXTURE OF FOUR HOMOLOGUES

	Total coenzyme Q content (μmole/ml)	*Individual homologues (μmole (mg)/ml)*			
		co Q_{10}	*co* Q_9	*co* Q_7	*co* Q_6
Theoretical	5.731	0.579 (0.500)	0.628 (0.500)	1.821 (1.200)	2.703 (1.600)
Modified Craven test	5.564 ± 0.1[a]	—	—	—	—
Densitometer	5.827[b]	0.671 (0.580 ± 0.036)[c]	0.6702 (0.533 ± 0.046)[c]	1.997 (1.316 ± 0.035)[c]	2.489 (1.473 ± 0.040)[c]

[a] Mean ± standard deviation of three determinations.
[b] Calculated by adding the results for the individual homologues.
[c] Mean ± standard deviation of three determinations.

enzyme Q concentrations were also determined by the modified Craven test on three aliquots of the solution and mean values and standard deviations calculated again. A comparison of the results obtained by both methods is shown in Table III. It should be noted that the results obtained with the modified Craven test have been expressed as μmole per ml instead of as mg per ml. The molar extinction coefficients of the colored species formed from the reaction of ethyl cyanoacetate and the coenzyme Q individual homologues in basic medium are very similar (*i.e.*, ε = 1.3, 1.6, 1.2, 1.2, 1.3 × 10^3 for co Q_{10}, co Q_9, co Q_8, co Q_7, co Q_6, respectively, at 630 mμ). In expressing the results as μmole per ml rather than as mg per ml the comparison of the errors is thus standardized.

TABLE IV

COENZYME Q CONCENTRATION IN SPLEEN AND LIVER OF MICE[a]

Tissue	*Total coenzyme Q (μg/g wet tissue)*		*Individual homologues (by densitometry) (μg/g wet tissue)*		
	Modified Craven test[b]	*Densitometer*	*co* Q_8	*co* Q_9	*co* Q_{10}
Liver	51.8 ± 3.4	53.8 ± 5.0	5.2 ± 1.0	48.5 ± 4.3	—
Spleen	12.5 ± 4.9	13.6 ± 4.8	—	10.2 ± 2.3	3.4 ± 0.6

[a] Data are expressed as the mean ± standard error of six determinations.
[b] Expressed as co Q_9.

A comparative study was made to discern whether the concentration of total coenzyme Q determined by densitometry was in agreement with that obtained by the modified Craven test in a natural mixture of individual homologues. Spleen and liver of mice were used in these experiments. These tissues are known to biosynthesize predominantly co Q_9. The results are shown in Table IV.

It can be seen from the figures in the table that a good correlation exists between the two methods. Moreover, the total values found are in excellent agreement with those reported by other workers for these tissues[5,12].

In general tissues do not contain appreciable amounts of more than three or four co Q homologues, with one of them usually being highly predominant. A preliminary qualitative study of the purified coenzyme Q fraction by paper chromatography would be recommended to adjust the amounts to be employed in the present quantitative assay when the nature and relative concentration of the individual homologues is not known.

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