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LIQUID CHROMATOGRAPHIC SEPARATION AND DETECTION OF NANOGRAM QUANTITIES OF BIOLOGICALLY IMPORTANT DICARBOXYLIC ACIDS

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SUMMARY

Phenacyl and benzyl derivatives of some biologically significant dicarboxylic acids have been prepared in a quantitative manner using a crown ether catalyst. They were separated using reversed-phase chromatography. The column used was packed with Corasil II to which a C_9 phase (nonyl) was bonded. Water–methanol (68:32) was used as the mobile phase. Detection limits in the range of 5–15 ng have been obtained for a non-optimized system. The ease of preparation of these derivatives, coupled with their excellent chromatographic properties, makes this a very attractive procedure for the investigation of mixtures containing biologically significant acids. The implications and future of this technique are discussed.

INTRODUCTION

In a previous paper, Durst *et al.*[1] have shown that by using a crown ether catalyst, *p*-bromophenacyl esters of monofunctional carboxylic acids can be prepared, in a quantitative manner, in virtually any aprotic solvent. These esters were separated by high-pressure liquid chromatography with detection limits in the nanogram range using a UV detector at 254 nm. The ease and rapidity of the reaction, coupled with the fact that total exclusion of water from the reaction mixture was not necessary, makes this a very attractive technique.

Some of the dicarboxylic acids, especially the C_4 acids, are of biological importance and it is, therefore, of interest to determine if the technique described previously is useful for the analysis of this class of compounds. Of particular importance are succinic, fumaric and malic acids (which take part in the citric acid cycle), methylmalonic and malonic acids (which are indicative of certain metabolic disorders such as vitamin B_{12} difficiency), and glutaric and adipic acids (which are involved in metabolism of some lower forms of life). In addition, some of these acids are precursors in synthetic organic chemistry. Dicarboxylic acids have been separated by a variety of chromatographic techniques (see refs. 2–17). In general, however, either the analysis times were too long, or the detection limits were too high —typically in the microgram range. These papers by no means give a total review of the literature;

they rather point out some of the techniques used and the inherent difficulties in analyzing dibasic acids.

The present paper reports the extention of the crown ether catalyzed phenacyl derivatization technique to dicarboxylic acids. The separation of nanogram quantities of esters of several biologically important dicarboxylic acids on a reversed-phase liquid chromatography system is described.

EXPERIMENTAL

Liquid chromatography

The liquid chromatograph used in this study consisted of the following equipment. The mobile phase was delivered with a Tracer Model 5000 chromatographic pump. Pressures were monitored with an Acco Helicoid Gage, 0–1500 p.s.i. The detector was a 254-nm LDC Model 1285 UV monitor with a cell volume of 8 μl and a 1-cm pathlength. The column used throughout the study was 25 cm $\times$ 0.4 cm I.D., packed by the usual technique with Corasil II to which C_9 (nonyl) was bonded. The surface coverage of the C_9 was 22% of the active Si–OH sites available. The column was thermostated as described previously[1]. The temperature used in this study was 40° $\pm$ 0.02°. The mobile phase consisted of 32% methanol and 68% water. The water was distilled and passed through an Illinois Water Treatment Co. (Rockford, Ill., U.S.A.) ion-exchange column. The methanol, certified ACS reagent grade obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.), was used without further treatment.

UV spectra

UV spectra were run in ACS reagent grade methanol in a 1-cm pathlength cell on a Cary Model 14 spectrophotometer.

Reagents for the derivatization

The alkylating reagents α-bromoacetophenone, α,*p*-dibromoacetophenone, and *p*-nitrobenzyl bromide were obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Matheson, Coleman & Bell (East Rutherford, N.J., U.S.A.). Both α-bromoacetophenone and α,*p*-dibromoacetophenone were recrystallized from ethanol before use. The *p*-nitrobenzyl bromide was used without recrystallization. The catalysts 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) and dicyclohexyl-18-Crown-6 (2,3,11,12-dicyclohexyl-1,4,7,10,13,16-hexaoxacyclooctadecane) were obtained from Aldrich or P.C.R. (Gainesville, Fla., U.S.A.). Dicyclohexyl-18-Crown-6 is brownish in color and may be purified by column chromatography on alumina, but in most cases this impurity does not interfere with the analysis and the crude crown can be used as is.

The standard alkylation solutions were made up so that 1 ml of acetonitrile contained 0.2 m*M* of alkylating reagent and 0.15 n*M* of crown ether. Alkylating reagent A contained 0.2 m*M* α-bromoacetophenone along with 0.15 n*M* of 18-Crown-6 per ml of acetonitrile. Alkylating reagent B contained 0.2 m*M* of α-bromoacetophenone along with 0.15 n*M* of dicyclohexyl-18-Crown-6 per ml of acetonitrile. Alkylating reagent C contained 0.2 m*M* of *p*-nitrobenzyl bromide along with 0.15 n*M* of dicyclohexyl-18-Crown-6 per ml of acetonitrile.

Alkylation procedure to form phenacyl ester derivatives

Methanol or water solutions of the organic diacid were neutralized to a phenolphthalein end point with a KOH–methanol solution. The solvent was removed under aspirator vacuum to give the potassium salts of the diacids. If the salts looked wet, several milliliters of benzene were added to the salts and then removed under vacuum. This usually dries the salts sufficiently to perform the alkylation. Small amounts of water or methanol do not seem to affect the subsequent alkylation so complete drying of the salt is not necessary. The salts are usually white or slightly pink solids. If the KOH–methanol solution is standardized, the total number of milliequivalents of acids can be determined (in most cases this is not necessary because the alkylating reagents are used in excess). For approximately every 0.1 m*M* of the acid salt, 1.1 ml of alkylating reagent solution A or B is added and the mixture refluxed for 30 min. The solution is then ready for chromatographic analysis. The succinic acid diphenacyl ester derivative is sparingly soluble in acetonitrile at 30° while the fumaric acid diphenacyl derivative is totally insoluble in 30° acetonitrile and only sparingly soluble in warm acetonitrile. Both of these acids should be analyzed for using the *p*-nitrobenzyl esters.

Alkylation procedure to form p-nitrobenzyl ester derivatives

The potassium salts of the acids were generated as discussed previously. For approximately every 0.1 m*M* of acid, 1.2 ml of alkylating reagent C is added, and the mixture refluxed for 1.5 h. The solution is then ready for chromatography. Succinic acid di-*p*-nitrobenzyl ester is very soluble in acetonitrile at 30°. Fumaric acid di-*p*-nitrobenzyl ester is only sparingly soluble in acetonitrile at 30° but is soluble in hot acetonitrile.

RESULTS AND DISCUSSION

Three different classes of derivatives were prepared for the diacids in this study. Initially, and as in the previous paper[1], *p*-bromophenacyl derivatives were attempted. However, the *p*-bromophenacyl derivatives of some diacids, especially fumaric and succinic, are insoluble in almost all solvents. It was decided to try different derivatizing agents in order to increase the solubility of the products. In all the reactions acetonitrile

TABLE I

ISOLATED YIELDS OF DERIVATIVES USED IN THIS STUDY

Acid	*Reagent used*	*Isolated yield (%)**
Malic	A, B and C	97
Succinic	A, B and C	>98
Fumaric	B and C	80
Glutaric	A and B	97
Adipic	A and B	95
Malonic	A and B	94
Methylmalonic	B	95

* Yield of the most soluble derivative in acetonitrile.

was used as the solvent because of solubility problems of the derivatives. The alkylating agents tried were phenacyl and *p*-nitrobenzyl bromide. The phenacyl derivative had adequate solubility properties for all except the fumaric derivative. Adequate solubilities in the mobile phase were obtained for all compounds using the *p*-nitrobenzyl derivatives. In the alkylation of C_4 diacids with *p*-nitrobenzyl bromide, dicyclohexyl-18-Crown-6 is the catalyst of choice. When 18-Crown-6 and *p*-nitrobenzyl chloride are used, slightly lower yields result and some byproducts are formed. It should be noted that the α-bromoacetophenone is a much more reactive alkylating agent than *p*-nitrobenzyl bromide under all conditions. The only advantage in using the nitrobenzyl ester lies in the increased solubility, especially in the case of the fumaric acid derivative. Table I shows the acids used, the types of derivative made, and isolated yields for the soluble esters only. The high yields indicate that the reactions are quantitative for all practical purposes.

Fig. 1 shows the UV spectra of the phenacyl derivative, $\lambda_{max.} = 243.4$ nm, and the *p*-nitrobenzyl derivative, $\lambda_{max.} = 266.1$ nm, of succinic acid in methanol. It is interesting to note that the spectra crossed at 252 nm, very near the wavelength of the detector (254 nm). From Fig. 1 it can be seen that in terms of sensitivity both derivatives are about the same. At a detector attenuation of 0.02 a.u.f.s. the limits of detect-

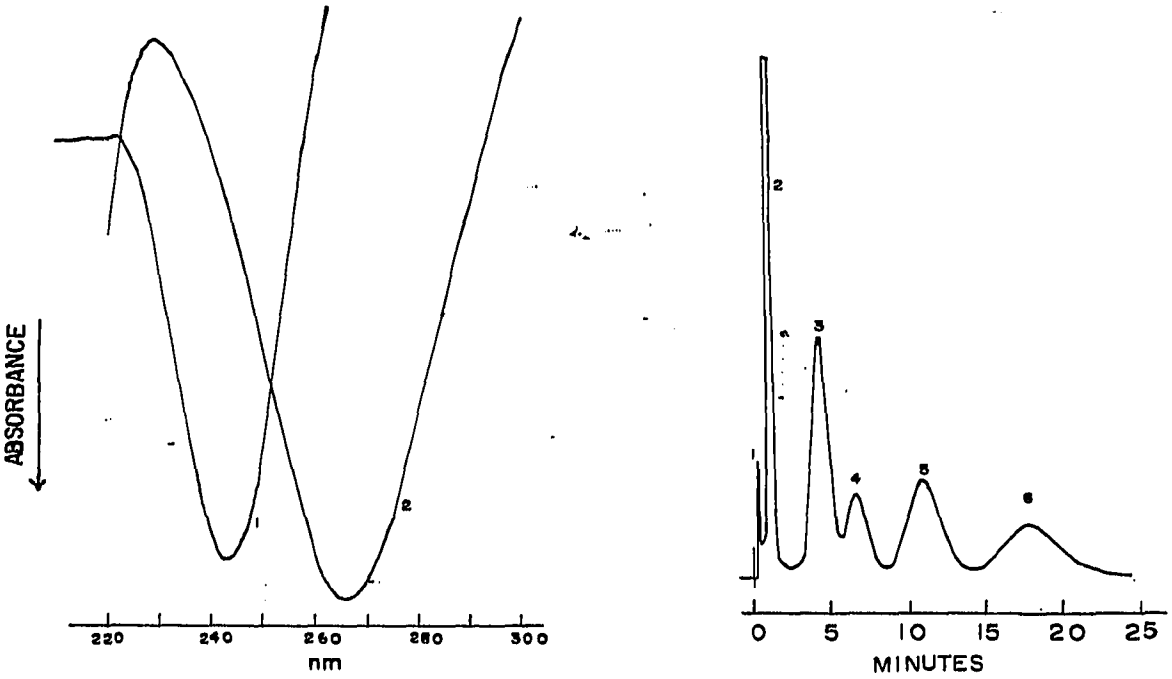
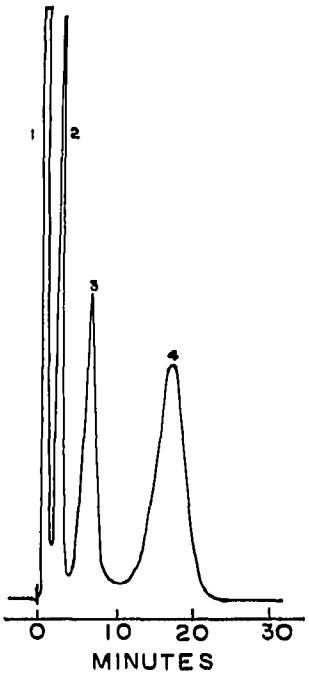


Fig. 1. UV absorption spectra of succinic acid derivatives in methanol. 1 = Phenacyl; 2 = *p*-nitrobenzyl.

Fig. 2. Separation of phenacyl derivatives of C_3–C_6 dibasic acids. Column, 25 cm × 4 mm I.D., C_9 Corasil II; temperature, 40°; mobile phase, water–methanol (68:32); flow-rate, 3.6 ml/min; attenuation, 0.08 a.u.f.s. 1 = Impurity; 2 = α-bromoacetophenone; 3 = malonic acid derivative; 4 = succinic acid derivative; 5 = glutaric acid derivative; 6 = adipic acid derivative.

ability were found to be 5 ng for the malonic derivative and 15 ng for the adipic derivative. These two acids represent the two extremes in retention times (see the figures). The other dibasic acids had similar detection limits with the present system. Only the fumaric acid derivative was difficult to characterize due to incomplete solubility in the mobile phase. Although solubility studies were not performed on this derivative, it seems reasonable to assume that the detection limits are similar to those of the other acids. It is believed that detection limits an order of magnitude smaller could be obtained by expanding the scale by a factor of 10 as was done in the previous paper[1]. In addition, better sensitivity can be realized when monitoring the esters at $\lambda_{max.}$ for the various derivatives. The molar absorptivities of the phenacyl derivatives are estimated to be around 35,000 while those of the nitrobenzyl derivatives are about 20,000 at $\lambda_{max.}$. No attempts were made to further lower the detection limits.

Fig. 2 shows the separation of phenacyl derivatives of C_3–C_6 dibasic acids in just over 20 min. This is a typical reversed-phase separation with the more hydrocarbon-like adipic (C_6) acid derivative eluting last. Fig. 3 shows the separation of some *p*-nitrobenzyl derivatives of C_4 dicarboxylic acids. Again, reversed-phase order is shown: the most polar (malic acid) derivative is eluted first while the least soluble (fumaric acid) derivative is eluted last. Finally, Fig. 4 shows the separation of phenacyl derivatives of malonic and methylmalonic acids. It is interesting to note that



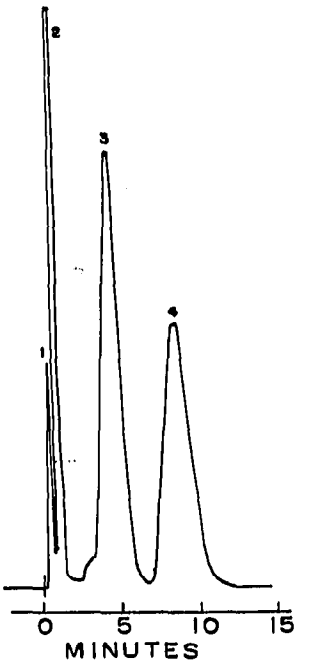


Fig. 3. Separation of some *p*-nitrobenzyl derivatives of C_4 dicarboxylic acids. Column, temperature mobile phase, and attenuation, same as in Fig. 2; flow-rate, 4.85 ml/min. 1 = *p*-Nitrobenzyl bromide; 2 = malic acid derivative; 3 = succinic acid derivative; 4 = fumaric acid derivative.

Fig. 4. Separation of malonic and methylmalonic acids. Column, temperature, mobile phase, and attenuation, same as in Fig. 2; flow-rate, 3.22 ml/min. 1 = Impurity; 2 = α-bromoacetophenone; 3 = malonic acid derivative; 4 = methylmalonic acid derivative.

propionic acid and methylmalonic acid appear in the urine of persons deficient in vitamin B_{12}. The previous paper[1] gave the condition for the separation of propionic acid. A gradient elution to a higher methanol concentration would be required in order to elute propionic acid derivatives off the column after the methylmalonate has been eluted. This was not attempted in the present study.

CONCLUSION

It has been shown that various phenacyl and benzyl derivatives of biologically important diacids can be easily prepared via a crown ether catalyst. It should be noted that other biologically important acids, such as triacids, can be analyzed by this method. In fact, a citric acid phenacyl derivative was easily prepared by this method. The ease and speed of preparation of these derivatives, coupled with excellent chromatographic separation and high sensitivities at the commonly used 254-nm detector, make this a very attractive method. It should be of great help in urine and blood analysis of persons suffering from various metabolic disorders.

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