THE SEPARATION AND IDENTIFICATION OF VANILMANDELIC ACID AND RELATED COMPOUNDS BY ELECTROPHORESIS ON CELLULOSE ACETATE

MARGARET M. KASER, WALDEMAR A. ROSENTHAL, KAREN NIQUETTE AND PATRICIA CULLEN

Clinical Chemistry Section, Laboratory Service, Veterans Administration Center, Wood, Wisc. (U.S.A.)

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INTRODUCTION

For a number of years the excretion of an increased amount of vanilmandelic acid (VMA) in the urine has been an indication of the presence of a pheochromocytoma. The presence of VMA and a method for its detection in urine by paper chromatography have been described by ARMSTRONG and his associates[1–3]. Paper chromatography was also used by ELLMAN[4] and GITLOW and co-workers[5]. Paper electrophoresis as a means of separation of VMA was employed by WOLF *et al.*[6], EPSTEIN, SCHIEVER AND GAMBINO[7], and KLEIN AND CHERNAIK[8]. A series of articles by VON STUDNITZ and others have described attempts to isolate VMA and related compounds by paper chromatography and paper electrophoresis with both low and high voltages[9–13].

In recent years cellulose acetate as a supporting medium has been found to be superior to paper for electrophoresis for a variety of purposes because of its lack of interaction with substances such as protein, and the rapid migration and clear resolution obtained. Also, various methods of quantitation are possible or can be developed. This paper reports the results of an investigation of cellulose acetate electrophoresis and an evaluation of the optimum conditions for the separation of VMA and related compounds.

EXPERIMENTAL

Reagents

Formate solution, 0.2 N, pH 3.0. To 575 ml of 0.2 *N* formic acid are added 100 ml of 0.2 *N* ammonium hydroxide. The pH is adjusted to pH 3.0 by the addition of formic acid or ammonium hydroxide.

Acetate buffer, 0.2 N, pH 3.6. 15 ml of 0.2 *N* sodium acetate are added to 185 ml of 0.2 *N* acetic acid. The pH is adjusted to pH 3.6 with acetic acid or sodium hydroxide.

Diazo reagent. A mixture of 10 ml of 2.5 % sodium nitrite and 7.5 ml of 0.5 % *p*-nitroaniline is freshly prepared from stock solutions kept at 5°. It is essential that the mixture be colorless.

Potassium carbonate, 5 %. 5 g potassium carbonate are dissolved in 100 ml of distilled water.

Phenolic acids

The vanilmandelic acid and related compounds used were obtained from Calbiochem and the Aldrich Chemical Company. They were dissolved in the formate solvent in concentrations of 1.0 μg/μl.

Equipment

Electrophoresis was performed on Sepraphore cellulose acetate strips with the micro electrophoresis unit of the Gelman Instrument Company.

Procedure

The cellulose acetate strips were soaked in acetate buffer for a minimum of 45 min before being placed in the electrophoresis apparatus. An aliquot of 1.0 μg of VMA in 1.0 μl of formate solvent was applied in a band at the origin near the cathode end of a strip. With other compounds amounts varying from 1.0 to 3.0 μg were similarly used. The samples were allowed to dry, and the unit was covered. Then, a current of 1.25 mA per strip or 7.5 mA for a series of 6 strips was applied for one hour at room temperature. When electrophoresis was completed, the excess buffer solution was removed by pressing the strips between sheets of filter paper. Fresh diazo reagent was poured into a long, narrow glass tray, and the cellulose acetate strips were floated one at a time on the surface for 30 sec. With a pipet 10 ml of 5 % potassium carbonate were then allowed to flow over the surface of the strip. A strip with 1.0 μg of VMA was included in each run, and the color development was continued until the VMA was clearly visible. The strips could be further dried by pressing them between two sheets of Whatman No. 1 chromatography paper at room temperature overnight or at 37° for one hour.

RESULTS

Because of our primary interest in VMA, the first experiments were designed to determine the optimum pH of a formate solution to be used as a solvent and also the pH of acetate buffer to provide the maximum rate of migration in electrophoresis on cellulose acetate. In each case the sample was 1.0 μg of VMA in 1.0 μl of formate solution. The results are given in Table I. The width of the band obtained after electrophoresis was 5 to 6 mm. The distances given are those to the center of the stained band. In a pH range of 3.0 to 4.0 the pH of the formate solvent had little influence on the

TABLE I

THE EFFECT OF VARIATIONS IN THE pH OF FORMATE SOLVENT AND ACETATE BUFFER ON THE MIGRATION OF VMA WITH CELLULOSE ACETATE ELECTROPHORESIS

pH of acetate buffer	*Distance of migration (mm) in formate solvent*		
	pH 4.0	*pH 3.6*	*pH 3.0*
4.0	32	42	38
3.6	52	51	55
3.0	14	15	15

TABLE II

ELECTROPHORETIC MIGRATION AND COLORS OBTAINED WITH DIAZOTIZED *p*-NITROANILINE WITH VMA AND RELATED COMPOUNDS

No.	*Compound*	*Migration (mm)*	*Band width (mm)*	*Color*
1	Tyrosine	0	—	Pink
2	Phenylalanine	0	—	Purple
3	Ferulic acid	0–3	3	Blue-green
4	3-Methoxy-4-hydroxyphenethylene glycol	0–5	5	Purple
5	5-Hydroxyindole-acetic acid	1–5	4	Pink
6	3,4-Dihydroxyphenylalanine	1–6	5	Yellow
7	Protocatechuic acid	3–7	4	Purple
8	Vanillic acid	3–8	5	Purple
9	Homovanillic acid	6–12	6	Brown
10	3,4-Dihydroxyphenylacetic acid	15–18	3	Olive
11	*p*-Hydroxyphenylpyruvic acid	15–18	3	Purple
12	Homogentisic acid	19–23	4	Yellow-green
13	Gentisic acid	48–51	3	Yellow-green
14	3-Methoxy-4-hydroxymandelic acid (VMA)	56–61	5	Purple
15	3,4-Dihydroxymandelic acid	60–68	8	Yellow
16	DL-*p*-Hydroxymandelic acid	64–72	8	Pink

electrophoretic migration, but the pH of the acetate buffer in the same range had a marked effect on the distance of migration. It is apparent that solution of the compounds in 0.2 *N* formate, pH 3.0, and the use of an acetate buffer, pH 3.6, for electrophoresis produced the maximum migration. Moderate variation in the concentration of these two solutions was without effect. Therefore, the indicated combination was used in all succeeding experiments.

VMA and a series of related compounds were investigated under the conditions established above. The results are shown in Table II. Samples of 1.0 to 3.0 μg were used. Good separation of these compounds was obtained, and they could be readily distinguished from VMA and each other by their rates of migration and their staining characteristics.

SUMMARY

A method has been developed for the resolution and identification of mixtures of vanilmandelic acid (VMA) and a series of related compounds by means of electrophoresis on cellulose acetate followed by staining with diazotized *p*-nitroaniline. It was established that the maximum distance of migration was obtained if the compounds were dissolved in 0.2 *N* formate solution, pH 3.0, and a 0.2 *N* acetate buffer, pH 3.6, was used for electrophoresis. The various compounds could be identified by their rates of migration and their staining characteristics. The method allows the detection of as little as 1.0 μg of each of the compounds studied.

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