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Janice Franz
State University of Iowa

Dorothy Schottelius
State University of Iowa

Emilio Arredondo
State University of Iowa

W. D. Paul
State University of Iowa

J. I. Routh
State University of Iowa

The Separation of Salicylate Metabolites By Paper Electrophoresis

By Janice Franz, Dorothy Schottelius, Emilio Arredondo, W. D. Paul and J. I. Routh

Abstract. The technique of paper electrophoresis was employed to develop a method for the separation of the endproducts of salicylate metabolism. A phthalate buffer with a pH of 3.2 and an ionic strength of 0.0125 to 0.05 produced the most satisfactory separation of the metabolites. It was found that the endproducts, i.e., salicylic, acetyl salicylic, salicyluric, and gentisic acids, could best be detected by the fluorescent spots they exhibited when the paper strips were viewed under ultraviolet light. The sensitivity of the method was such that as little as one microgram of salicylic acid could be detected as a fluorescent spot after electrophoretic separation.

Although salicylates have been used therapeutically for many years, their metabolism was not extensively studied until 1942. These studies were handicapped by the lack of a suitable procedure for their estimation. A method for the determination of salicylates in plasma was developed by Brodie, Udenfriend and Coburn (1944). This was followed by a procedure for the quantitative estimation of salicylic acid and its metabolites in urine by Smith, *et al.* (1946). The purplish-blue fluorescence exhibited by salicylic acid when irradiated with ultraviolet light was used as the basis for its determination by Saltzman (1948). More recently Truitt, *et al.* (1955) have applied the fluorimetric technique to the quantitation of salicylic acid and salicyluric acid in plasma and urine. Schachter and Manis (1958) reported a sensitive fluorimetric method for the determination of salicylate metabolites including the glucuronides.

The separation and qualitative detection of the salicylates by the technique of paper chromatography has been the subject of a few investigations. Bray, *et al.* (1950) separated salicylate and related compounds by this technique. A study of solvent systems and detection agents was carried out in our laboratory which resulted in a satisfactory separation of salicylic, acetyl salicylic, salicyluric, and gentisic acids (Paul, *et al.*, 1951). A salicyl acyl glucuronide derivative has been quantitated by paper chromatography by Schachter (1957).

The present paper represents an attempt to separate salicylate metabolites by the technique of paper electrophoresis. Further plans include the application of the increased sensitivity of this method to the search for salicylate metabolites in the plasma as well as in the urine.

Experimental

Preliminary studies involved the choice of the proper detecting agent. Aliquots of solutions of salicylic, acetyl salicylic, salicyluric, and gentisic acids were spotted on Whatman 3 mm. filter paper. These solutions were prepared by dissolving the sodium salts of each compound. The salicyluric acid was synthesized while the other three compounds were used as purchased. The strips were dried and sprayed with various detecting agents known to give a color with salicylates. The two most satisfactory agents were ferric chloride and a mixture of copper sulfate, pyridine, and water.

The paper electrophoretic technique of Kunkel and Tiselius (1951) was applied to an investigation of the conditions for satisfactory separation of salicylate metabolites. Strips of Whatman 3 mm. filter paper (either 10 x 30 cm. or 10 x 56 cm.) were wet with buffer and the paper blotted to remove excess liquid. Aliquots (5 to 50 microliters) of the various salicylate solutions were spotted on the midline of the paper. The strips were then clamped between siliconed glass plates which were wider and shorter than the strips to permit sealing the sides with silicone grease and to allow the ends of the strip to extend into the buffer compartments of the electrode vessels. The glass plates rested on the edges of the two lucite electrode vessels (1000 ml. capacity) fitted with platinum electrodes. The system was allowed to equilibrate for 30 to 60 minutes before the current was applied. A direct current power source capable of delivering 5-25 milliamperes at 50-500 volts was used as the driving force. At the end of the run (1 to 8 hours) the glass plates were carefully separated, the paper was removed and dried in a horizontal position in an oven at 110° C. The paper was then sprayed with appropriate agents or viewed under ultraviolet light to detect the separated spots. Hydrolysis of the acetyl salicylate spot was carried out by suspending the strip in an atmosphere of ammonia gas for one hour followed by a 10-minute exposure to the vapors of glacial acetic acid. Viewing the strips under ultraviolet light was found to be the most sensitive of all detection methods.

Table 1
Mobilities of Salicylate Metabolites in Buffers of Varying pH

pH	Time hrs.	Salicylate cm.	Gentisate cm.	Acetyl Salicylate cm.	Salicylurate cm.
3	6.5	9.4	7.3	4.3	3.6
4	4.0	9.6	8.2	8.0	7.0
5	4.5	11.0	9.7	9.7	8.6
6	4.5	10.7	9.7	9.7	8.5
7	4.5	10.2	8.7	9.0	7.9
8	5.0	12.7	10.7	11.1	10.5
9	4.5	11.1	9.5	9.2	9.6
10	3.5	9.9	8.6	8.2	12.4
11	2.0	6.6	5.9	5.6	8.6

The effect of the pH of the buffer on the migration rate of the metabolites was first studied. A universal type stock buffer solution containing disodium hydrogen phosphate, citric acid, boric acid, and sodium hydroxide was prepared. Buffers varying in pH from 2 to 11.5 and ionic strength 0.07 to 0.1 could be prepared from the stock solution. The effects of varying the pH of this buffer on the electrophoresis of the metabolites is shown in Table 1.

Other buffers varying in pH from 3.0 to 11.2 were also investigated to determine the effect of buffer composition on mobility. The results of this study are shown in Table 2.

Table 2

Mobilities of Salicylate Metabolites in Buffers of Varying Composition

Buffer	pH	Time hrs.	SA cm.	ASA cm.	GA cm.	SUA cm.
HCl-KHPhthal.	3.0	5	12.8	6.8	10.5	5.9
NaOH-KHPhthal.	4.2	5	20.1	17.0	17.8	15.0
NaOH-KHPhthal.	5.6	4.3	18.4	16.2	15.3	14.2
NaOH-KH_2PO_4	6.9	4.5	15.5	14.0	13.5	12.7
NaOH-KH_2PO_4	7.4	4.3	12.7	11.2	10.4	11.0
NaOH-KH_2PO_4	8.2	4.7	12.6	10.5	10.2	14.9
NaOH-barbital	8.6	5	12.8	11.2	11.2	12.7
NaOH-barbital	9.0	5	12.4	10.1	10.7	13.1
NaOH-barbital	11.2	5	10.3	9.2	11.5	12.6

Since it was observed that the phthalate buffer of pH 3 gave the most clear cut separation of the metabolites, further experiments with this buffer were carried out. Even better separation was obtained when 7 milliamperes at 400 volts was applied for longer periods of time using a phthalate buffer with a pH of 3.2 and an ionic strength of 0.035 (Table 3).

Table 3

Mobilities of Salicylate Metabolites in Phthalate Buffer pH 3.2

Time hrs.	Salicylate cm.	Gentisate cm.	Acetyl Salicylate cm.	Salicylurate cm.
8	21.7	17.5	10.9	6.9
7.5	19.0	15.2	10.2	6.9

After hydrolysis of the acetyl salicylate spot, the strips were viewed under ultraviolet light and revealed four well separated fluorescent spots.

When satisfied with our ability to separate and detect the metabolites under the above conditions, we then investigated the separation in short term experiments. Separation from aqueous solutions and from serum and variations in ionic strength of the buffer were also studied. Typical results are shown in Table 4.

Table 4

Mobilities of Salicylate Metabolites in Phthalate Buffer pH 3.2 With Varying Ionic Strength

Serum μl.	Water μl.	Time hrs.	Current m.a.	Voltage v.	μ	SA cm.	ASA cm.	GA cm.	SUA cm.
	10	2.5	2.2	413	0.0125	7.5	5.5		
	10	1	7.3	305	0.025	4.1	2.3	3.5	2.2
	10	2	8	271	0.025	7.1	3.3	5.9	3.4
	3	2	8.3	280	0.025	7.3	3.7	6.0	3.2
	10	3	10	271	0.05	9.5	4.7	8.3	4.5
	10	2	9.9	263	0.05	8.0	4.1	6.9	
10		1	3	336	0.0125	4.6	2.8		
10		1	5	310	0.025	4.3	2.7		
10		2	5.5	308	0.025	9.4	5.3		
10		2.5	9.8	255	0.05	8.4	3.7	6.8	3.5

Discussion

When the four salicylate metabolites used in this investigation were subjected to the paper electrophoresis technique described earlier using a universal type buffer, the most satisfactory separation was obtained at pH 3 (Table 1). From the results in Table 2, it can be seen that the phthalate buffer pH 3 gave even better separation of the metabolites. This was also evident when electrophoresis was carried out for longer periods as shown in Table 3.

The results of experiments shown in Table 4 indicate that in runs of 1 to 2 hours duration applying 2 to 10 milliamperes at 308 to 413 volts and a variation of ionic strength of the buffer from 0.0125 to 0.05 produced satisfactory separation of three of the four metabolites. These short term experiments were more satisfactory for serial runs when the technique was applied to metabolic studies. When a mixture of the four metabolites was added to serum, the resulting paper strip showed three spots under ultraviolet light. After hydrolysis the acetyl salicylate spot appeared with its leading edge a few millimeters beyond the edge of the salicylurate spot. The two spots also exhibited a different colored fluorescense. By decreasing the amount of salicylate applied to the paper, it was found that as little as 1 microgram of salicylic acid could be detected as a fluorescent spot. This degree of sensitivity should be of assistance in the search for small concentrations of the various metabolites in the serum after the ingestion of salicylates.

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Departments of Biochemistry and Physical Medicine and Rehabilitation
State University of Iowa
Iowa City, Iowa