Thin-Layer Chromatographic Separation of Isomers of Ascorbic Acid and Dehydroascorbic Acid as Sodium Borate Complexes on Silica Gel and Cellulose Plates

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A thin-layer chromatography (TLC) method is described for isomers of ascorbic acid (AA) and their oxidation product, dehydroascorbic acid (DHAA), on sodium borate impregnated (B) silica gel and cellulose plates. Borate complexes of AA and DHAA could be separated from each other, with AA migrating further. For comparative purposes, TLC was also attempted using direct (D), reversed-phase (RP), and reversed-phase sodium borate (RP-B) TLC plates with and without metaphosphoric acid (MPA). A reasonable separation of the three AA isomers is achieved on D and RP cellulose plates. Using an RP−silica−MPA plate the three AA isomers as well as the three DHAA isomers are fairly separated from their own group members. The separation of six stereoisomers, three AA isomers, and three DHAA is achieved using a D-cellulose−MPA plate. This procedure has been adopted to separate and identify AA and its oxidation product DHAA in food products, pharmaceutical preparations, and biological tissues and fluids.

Keywords: *Ascorbic acid; borate complexes; metaphosphoric acid*

INTRODUCTION

L-Ascorbic acid (AA) is a multifunctional nutrient involving many physiological functions. AA is fairly stable in organic solvent, but it is extremely unstable in aqueous solution (Halliwell and Gutteridge, 1989; Davies et al., 1991). Some AA derivatives have been shown to be relatively stable against oxidation even after prolonged storage (Seib and Tolbert, 1982). Such AA derivatives have great potential for use in the preservation of food and in nonfood products. Boron complexes of AA constitute one such group of derivatives. In our laboratory, many stabilizing agents and solvent systems have been studied. We found that borate is an excellent stabilizing agent (Tsao and Young, 1996).

Many investigators have used thin-layer chromatography (TLC) for the separation of AA and its oxidation product, dehydroascorbic acid (DHAA). Its simplicity, quickness, and efficiency have led to widespread use of this technique. TLC has been used to determine AA concentrations in food (Beljaars et al., 1974), pharmaceutical preparations, and biological fluids (Chatterjee and Banerjee, 1979; DiMattio, 1989). Brenner et al. (1964) have separated AA on metaphosphoric acid (MPA) by TLC. Saari et al. (1967) used TLC for the separation of oxidation degradation products of [^{14}C]AA. Recently, Okamura (1994) used TLC to separate AA in 19 kinds of edible mushrooms. An excellent review on the separation of AA and related compounds has been written by Nguyen (1985). No prior attempts have been made to separate AA and DHAA isomers as boron complexes. In this paper we describe a TLC method for AA isomers and their oxidation product, DHAA, on sodium borate (B) silica gel and cellulose plates. The use of borate-impregnated plates for this purpose is new. This procedure has been adopted to separate and identify AA and its oxidation products, DHAA, in food products, pharmaceutical preparations, and biological tissues and fluids.

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EXPERIMENTAL PROCEDURES

Materials and Reagents. L-AA, D-isoascorbic acid (D-IAA), 2,4-dinitrophenylhydrazine (2,4-DNP), and Norit were purchased from Sigma (St. Louis, MO). DHAA was obtained from ICN Biomedical Inc. (Aurora, OH), and D-AA was obtained from Hoffmann-La Roche Inc. (Nutley, NJ).

Silica gel (13181) and cellulose (13254) TLC plates (20 × 20 cm) were purchased from Eastman Kodak Co. (Rochester, NY). Silica gel was coated on inert flexible poly(ethylene terephthalate) to a 100-μm thickness. Polyacrylic was used as a binder in these plates. Cellulose plates were coated to a 160-μm thickness without binder on inert flexible Estar base. Impregnation with sodium borate was accomplished by uniformly spraying a 3% sodium borate (B) solution on silica gel or cellulose plates (Morris, 1963; Roomi et al., 1966). The plates were air-dried and then heated to 100 °C for 20 min. For RP-TLC the dried silica gel, cellulose, or borate plates were uniformly impregnated with silicone oil (Dow Corning fluid 200) by allowing a 5% solution in ether to ascend the plate in the developing chamber (Roomi et al., 1964). MPA plates were prepared by uniformly spraying a 2% solution on silica or cellulose plates, which were then air-dried and heated at 100 °C for 20 min. MPA plates were either sprayed with B or made RP. All of the above plates were stored in a desiccator until required.

Solvents Used. Solvents used were of analytical grade quality from Aldrich (Milwaukee, WI). Several solvent systems were tried using acetonitrile, butylnitrile, acetone, methanol, acetic acid, and water in various proportions. Good separation was obtained with the following two solvent systems: (1) acetonitrile/acetone/water/acetic acid (80:5:15:2); (2) acetonitrile/butylnitrile/water/acetic acid (66:33:15:2).

Standards. The structural formulas of four stereoisomers of AA are shown in Figure 1, and the oxidation of L-AA to its

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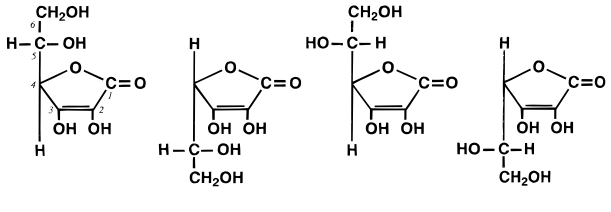

Figure 1. Structures of different stereoisomers of AA.

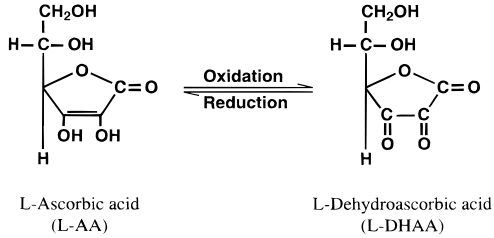

Figure 2. Structures of L-AA and its biological active oxidation product DHAA.

Table 1. TLC of Stereoisomers of AA and DHAA (R_f Values × 100)[a]

	silica				cellulose			
compound	D	B	RP	RB-B	D	B	RP	RP-B
L-AA	27	13	30	15	49	17	36	11
D-AA	28	13	32	16	43	16	31	11
D-IAA	28	13	35	16	54	17	42	11
L-DHAA	58	5	64	6	59	5	46	0
L-DHAA (Norit-treated L-AA)	58	5	64	6	59	5	47	0
D-DHAA (Norit-treated D-AA)	58	5	64	6	59	5	47	0
D-dehydroisoascorbic acid (Norit-treated D-IAA)	58	5	64	6	59	5	47	0

[a] D, direct; B, sodium borate; RP, reversed-phase; RP-B, reversed-phase sodium borate. Solvent: acetonitrile/acetone/water/acetic acid (80:5:15:2). R_f values are the means of three determinations. The coefficient of variation is 5%.

biological active oxidation product DHAA is shown in Figure 2. Only L-, D-, and D-IAA were used in the present study. Oxidation of AA stereoisomers to the corresponding DHAA was accomplished by addition of 200 mg of Norit to 1 mL of 4% trichloroacetic acid (TCA) containing 1 mg of AA isomers at room temperature. Fresh solutions of AA and DHAA (1 mg/mL) were prepared daily, and 10 μL of the solution was applied with a micropipet at a starting point 2−3 cm from the end of the plate. The plates were then developed inside the chamber saturated with solvent vapors. Usually 40−60 min was required for the solvent front to cover a distance of 15 cm. The plates were then removed, dried, and sprayed with 2% 2,4-DNP in ethanol containing 0.3 N HCl.

Sample Pretreatment. AA and its biologically active oxidation product DHAA were isolated from foodstuffs (fresh orange and lime juices), tissues from guinea pigs fed high levels (2.5%) of AA (liver, kidney, and eye lens) (Roomi and Tsao, 1995), biological fluid from guinea pig (Roomi and Tsao, 1996) (urine and plasma), and pharmaceutical preparations of ascorbic acid (Bronson Pharmaceutical, St. Louis, MO) according to the method of Schaffert and Kingsley (1955). The samples were applied to the D-, B-, RP-, and RP-B silica and cellulose plates and developed as described above. The plates were then sprayed with 2% 2,4-DNP in ethanol containing 0.3 M HCl and heated to 37 °C for 2 h.

RESULTS AND DISCUSSION

Tables 1 and 2 show the TLC of stereoisomers of AA and DHAA on B and MPA in terms of $R_f \times 100$ on silica gel and cellulose plates using acetonitrile/acetone/water/acetic acid (80:5:15:2). TLC was also carried out on

Table 2. TLC of Stereoisomers of AA and DHAA on Metaphosphoric Acid Impregnated Plates (R_f Values × 100)[a]

	silica				cellulose			
compound	D	B	RP	RB-B	D	B	RP	RP-B
L-AA	31	13	32	15	42	12	43	13
D-AA	34	13	34	16	40	11	37	12
D-IAA	37	13	36	17	50	13	43	13
L-DHAA	62	7	40	9	55	0	41	0
L-DHAA (Norit-treated L-AA)	62	7	40	9	55	0	41	0
D-DHAA (Norit-treated D-AA)	60	7	32	9	52	0	39	0
D-dehydroisoascorbic acid (Norit-treated D-IAA)	59	7	24	9	47	0	37	0

[a] D, direct; B, sodium borate; RP, reversed-phase; RP-B, reversed-phase sodium borate. Solvent: acetonitrile/acetone/water/acetic acid (80:5:15:2). R_f values are the means of three determinations. The coefficient of variation is 5%.

direct (D), reversed-phase (RP), and reversed-phase sodium borate (RP-B) plates. The results obtained are summarized below:

(a) Separation of L-*AA and* L-*DHAA and Their Isomers.* L-AA was separated from L-DHAA by D-TLC on silica gel plates; L-AA moved more slowly than L-DHAA. The various stereoisomers of AA had similar R_f values, as did the DHAA isomers. On B-TLC the mobility of all the compounds under study was reduced. The R_f value of L-AA was greater than that of L-DHAA. It is clear that boron complexes rendered all AA isomers less polar, such that they moved more rapidly than the DHAA isomers. Thus, the technique does not separate different isomers of AA or DHAA. The use of RP and RP-B gave similar results.

(b) Separation of Isomers within the AA and DHAA Group. Separation of the three AA isomers was achieved on D and RP cellulose plates, but the three DHAA isomers were not separated. On the RP silica−MPA plate the three AA isomers, as well as the three DHAA isomers, are separated from their own isomers. However, two isomers, one of each group, were not separated, that is, L-AA and D-DHAA (R_f values are both 32).

(c) Separation of All Isomers. The separation of all six stereoisomers was achieved using D-cellulose−MPA plates, although the separation is marginal; the R_f values of three stereoisomers of AA are 42, 40, and 50 and the R_f values of three DHAA are 55, 52, and 47.

Changing the solvent system to acetonitrilr/butylnitrile/water/acetic acid (66:33:15:2) did not provide any improvement in the separation of isomers of AA and DHAA on silica gel or cellulose plates with and without MPA by all four systems (data not shown). Tailing can occur on D and RP silica and cellulose plates. However, no tailing occurred with the boron system, but discrete spots were observed.

AA and DHAA can be separated as their boron complexes on silica gel and cellulose TLC plates. On D TLC, boron complexes render AA less polar than the corresponding DHAA; hence, they moved more rapidly. In addition, the boron complexes retard the mobility. This could be one of the reasons that isomers of AA and DHAA were not separated.

Sodium borate or boric acid is known to form complexes with glycols, probably by chelation in many types of organic compounds. Complex formation is proposed to take place more readily with threo glycols than with erythro isomers (Wittcof et al., 1948; Frah and Mills, 1959; Loomis and Durst, 1992). Thin-layer plates containing complexing agents have been used for the

Table 3. Separation of AA and Its Oxidation Product DHAA Derived from Biological Tissues and Fluids, Pharmaceutical Preparations, and Foodstuffs (R_f Values × 100)[a]

	silica				cellulose			
	D	B	RP	RP-B	D	B	RP	RP-B
L-AA	30	12	30	11	50	16	44	12
L-DHAA	57	4	60	5	62	5	50	0
biological tissues								
1. guinea pig liver								
a. TCA extract	30 (O); 15, 3 (B)	12 (O); 0 (B); 8, 4 (Y)	30 (O); 25, 5 (B)	11 (O); 5 (B)	52 (O); 20, 10 (B)	16 (O); 10, 5 (B)	44 (O); 15, 5 (B)	12 (O); 5 (B)
b. Norit treated	57 (O); 15,3 (B)	4 (O); 8, 0 (Y)	58 (O); 25, 5 (B)	5 (O); 0 (B)	62 (O); 20, 10 (B)	5 (O); 5 (B)	50 (O); 15, 5 (B)	0 (O); 5 (B)
2. guinea pig kidney								
a. TCA extract	30 (O); 15, 5 (B)	12 (O); 0 (B); 8, 4 (Y)	30 (O); 20, 5 (B)	11 (O); 5 (B)	52 (O); 20, 10 (B)	16 (O); 10, 5 (B)	44 (O); 15, 5 (B)	12 (O); 5 (B)
b. Norit treated	57 (O); 15, 5 (B)	4 (O); 8, 4 (Y)	58 (O); 20, 5 (B)	5 (O); 0 (B)	62 (O); 20, 10 (B)	5 (O); 5 (B)	5 (O); 5 (B)	0 (O); 5 (B)
3. guinea pig eye lens								
a. TCA extract	30 (O); 12, 3 (B)	12 (O); 4 (Y)	30 (O); 25, 5 (B)	11 (O); 5 (B)	52 (O); 10, 5 (B)	16 (O); 12 (B)	44 (O); 10, 5 (B)	12 (O); 5 (B)
b. Norit treated	57 (O); 15 (B)	4 (O); 0 (B)	58 (O); 25, 20, 5 (B)	5 (O); 0 (B)	62 (O); 15, 7 (B)	5 (O); 10 (B)	5 (O); 12, 5 (B)	0 (O); 5 (B)
biological fluids								
1. guinea pig plasma								
a. TCA extract	28 (O); 22, 15 (B)	13 (O); 4, 0 (B)	30 (O); 25, 20, 5 (B)	11 (O); 5 (Y)	52 (O); 15, 5 (Y)	16 (O); 10, 5 (B)	44 (O); 15, 5 (B)	12 (O); 8, 5 (B)
b. Norit treated	57 (O); 15, 3 (B)	4 (O); 0 (B)	60 (O); 25, 20, 5 (B)	5 (O); 0 (Y)	62 (O); 20, 5 (B)	15 (O); 10 (B)	50 (O); 15, 5 (B)	0 (O); 8 (B)
2. guinea pig urine								
a. TCA extract	30 (O); 15, 5 (B)	13 (O); 4, 0 (B)	30 (O); 25, 5 (B)	11 (O); 0 (B)	52 (O); 15, 10 (B)	16 (O); 10 (B)	44 (O); 15, 8 (B)	12 (O); 8 (B)
b. Norit treated	57 (O); 15, 3 (B)	4 (O); 0 (B)	60 (O); 25, 20, 5 (B)	5 (O); 0 (B)	62 (O); 15, 10 (B)	5 (O); 10 (B)	50 (O); 15, 0 (B)	0 (O); 8 (B)
pharmaceutical preparations								
AA from Bronson Pharmaceutical								
a. TCA extract	30 (O)	12 (O)	30 (O)	11 (O)	50 (O)	16 (O)	44 (O)	12 (O)
b. Norit treated	57, 30 (O)	0, 12 (O)	60, 30 (O)	11, 5 (O)	62, 50 (O)	16, 5 (O)	50, 44 (O)	12, 0 (O)
food products								
1. fresh orange juice								
a. TCA extract	30 (O); 5, 8, 50 (B)	12 (O); 50 (B)	30 (O); 25, 5 (B)	11 (O); 7 (B)	5 (O); 15, 5 (B)	16 (O); 10, 5, 3 (B)	44 (O); 10, 5 (B)	12 (O); 10, 5, 3 (B)
b. Norit treated	57 (O); 15, 8 (B)	0 (O); 8, 4 (B)	60 (O); 25, 15 (B)	5 (O); 0 (B)	62 (O); 15, 5 (B)	5 (O); 10, 5 ,3 (B)	50 (O); 10, 5 (B)	0 (O); 10, 5, 3 (B)
2. fresh lime juice								
a. TCA extract	30 (O); 15, 12 (B)	12 (O); 4 (Y); 0 (B)	30 (O); 25, 10 (B)	11 (O); 7 (B)	50 (O); 15 (B)	16 (O); 10, 5 (B)	44 (O); 15 (B)	12 (O); 10, 3 (B)
b. Norit treated	5 7(O); 15, 10, 3 (B)	0 (O); 4 (B)	60 (O); 25, 5 (B)	50 (O); 7 (B)	60 (O); 15 (B)	5 (O); 10, 5 (B)	50 (O); 15 (B)	0 (O); 10, 3 (B)

[a] D, direct; B, sodium borate; RP, reversed-phase; RP-B, reversed-phase sodium borate. In parentheses O = orange, B = brown, and Y = yellow. Solvent: acetonitrile/acetone/water/acetic acid (80:5:15:2).

separation of sugars (Pasturka, 1961) and hydroxy fatty acids (Morris, 1963; Roomi et al., 1966). This principle has been extended in the present investigation to the isomers of AA and DHAA.

The use of a complexing agent has been employed for the separation and identification of AA and its oxidation products from foodstuff, biological tissues and fluids, and pharmaceutical preparations. The samples were either homogenized or extracted in 10% TCA containing thiourea, which keeps AA in the reduced form, according to the method of Schaffert and Kingsley (1955). A portion of the extract was treated with Norit, which converted AA to DHAA. Both the extracted and oxidized samples were applied on TLC plates, developed and sprayed with 2,4-DNP. The results are shown in Table 3. Orange (O), brown (B), and yellow (Y) spots developed. Orange spots are those of AA and DHAA, which were not further identified. For example, guinea pig liver TCA extract on D-TLC gave three spots with R_f values of 30 (O) and 15, 3 (B). The orange spot for R_f value 30 is due to AA, and the brown spots with R_f values 15 and 3 are those of unknown compounds which were not identified. Similarly, on B TLC four spots were observed: 12 (O); 0 (B); and 8, 4 (Y). The orange spot with R_f 12 is due to AA, and the brown spot with R_f 0 and the yellow spots with R_f 8 and 4 are those of unidentified compounds. On Norit treatment the orange spots are due to DHAA.

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