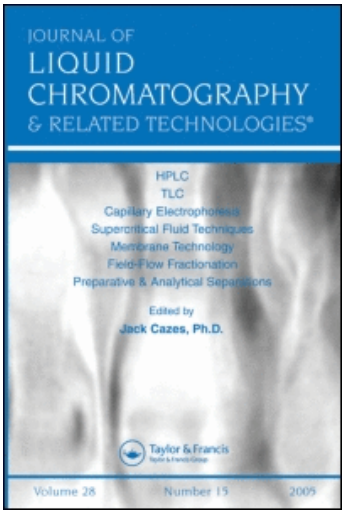

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Comparison of Amino Acid Separations on High Performance Silica Gel, Cellulose, and C-18 Reversed Phase Layers and Application of HPTLC to the Determination of Amino Acids in *Biomphalaria Glabrata* Snails

E. Norfolk[a]; S. H. Khan[a]; B. Fried[b]; J. Sherma[a]
[a] Department of Chemistry, Lafayette College, Easton, Pennsylvania [b] Department of Biology, Lafayette College, Easton, Pennsylvania

To cite this Article Norfolk, E. , Khan, S. H. , Fried, B. and Sherma, J.(1994) 'Comparison of Amino Acid Separations on High Performance Silica Gel, Cellulose, and C-18 Reversed Phase Layers and Application of HPTLC to the Determination of Amino Acids in *Biomphalaria Glabrata* Snails', Journal of Liquid Chromatography & Related Technologies, 17: 6, 1317 — 1326
To link to this Article: DOI: 10.1080/10826079408013765
URL: http://dx.doi.org/10.1080/10826079408013765

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(6), 1317-1326 (1994)

COMPARISON OF AMINO ACID SEPARATIONS ON HIGH PERFORMANCE SILICA GEL, CELLULOSE, AND C-18 REVERSED PHASE LAYERS AND APPLICATION OF HPTLC TO THE DETERMINATION OF AMINO ACIDS IN *BIOMPHALARIA GLABRATA* SNAILS

EVAN NORFOLK[1], SHAHREYAR H. KHAN[1], BERNARD FRIED[2], AND JOSEPH SHERMA[1]
[1]*Department of Chemistry*
[2]*Department of Biology*
Lafayette College
Easton, Pennsylvania 18042

ABSTRACT

The separation of 18 amino acids on high performance silica gel, cellulose, and reversed phase bonded silica gel plates has been compared. Zones were detected with ninhydrin reagent. The ability to identify amino acids in Biomphalaria glabrata snail hemolymph and digestive gland-gonad complex (DGG) by HPTLC was studied, and alanine and aspartic acid were quantified in hemolymph and DGG by scanning densitometry. Analytical results were compared to those reported earlier using column ion exchange and reversed phase liquid chromatography and paper chromatography.

INTRODUCTION

In earlier studies in our laboratories, separations of 18 amino acids on silica gel, cellulose, ion exchange,

and reversed phase thin layer chromatography (TLC) plates and sheets were compared (1,2), and amino acids were determined by TLC in adult Echinostoma revolutum (Trematoda) (3). We have now extended these studies to amino acid separations on cellulose, silica gel, and chemically bonded C-18 modern high performance thin layer chromatography (HPTLC) plates, all of which contained a preadsorbent zone except the cellulose.

Most analyses of amino acids in biological samples have been performed using techniques other than TLC, i.e., gas-liquid chromatography and column ion exchange chromatography, particularly with automated amino acid analyzers. Recent reviews (4,5) have suggested the efficacy of TLC as an alternative method for the analysis of amino acids, and in this study we have used HPTLC to examine amino acids in the hemolymph and digestive gland-gonad complex (DGG) of the medically important snail, Biomphalaria glabrata. Results are compared with those reported earlier for amino acids in the hemolymph and digestive gland of Biomphalaria glabrata and other snail species based on ion exchange (6-8) and C-18 (9) column chromatography and paper chromatography (10,11) analyses.

EXPERIMENTAL

Sample Preparation

Snails with shell diameters of 10-20 mm were maintained in glass vessels with artificial spring water and fed a boiled lettuce diet for 7 days as previously described (12).

Amino acids were extracted from the DGG and hemolymph of pooled snails using the same procedures described earlier for carbohydrates (12). Typical sample sizes were 400 mg of DGG and 500 ul of hemolymph from a

pool of 5-10 snails. Extract solutions were stored in a refrigerator and used within two days of preparation. The effect of desalting on the chromatograms was tested by treating evaporated extract with 1 ml of 0.5% HCl in 95% ethanol for 24 hr (3) prior to TLC.

TLC Analysis

Standards of the amino acids listed in Table 1 were obtained from Sigma (St. Louis, MO). Stock standard solutions of each acid were prepared in 70% ethanol at concentrations of 1.00 mg/ml. TLC standards were prepared at concentrations of 100 and 500 ng/μl by dilution with 70% ethanol. For quantitative analysis, standards of alanine and aspartic acid were further diluted to 10.0 ng/μl with 70% ethanol.

Initial zones were applied using a 10 μl Drummond (Broomall, PA) digital microdispenser. For determination of R_f values and comparison of systems, 1.00 μl of the 500 ng/μl standards were spotted. For qualitative analysis of snail extracts, 5.00 μl of the 100 ng/μl standards and extracts reconstituted in 200 μl of solvent were applied. For quantitative analysis, 2.00, 4.00, 8.00 and 10.0 μl of the standards (20.0 to 500 ng) and extracts reconstituted in 100 μl of solvent were spotted.

The following 20 x 10 cm precoated thin layers were used: Whatman (Clifton, NJ) high performance silica gel LHPKDF with channels and preadsorbent area, catalog no. 4806-711 and C-18 chemically bonded silica gel with preadsorbent area, catalog no. 4800-820; E. Merck (Gibbstown, NJ) cellulose F, no. 15036-6; Merck RP-18F, CZ reversed phase chemically bonded C-18 silica gel with concentrating zone, no. 15498-6; and Merck RP-18WF, CZ water stable C-18 bonded silica gel layer with concentrating zone, no. 14235-6. All plates were 20 x 10

TABLE 1

hR_f Values of Amino Acid Standards on Reversed Phase Layers

	TLC System*					
	1	2	3	4	5	6
Aspartic acid	30	59	72	60	83	73
Arginine	28	4	35	28	86	82
Serine	55	36	69	50	82	73
Glycine	50	38	62	45	69	54
Tyrosine	91	77	88	68	77	67
Alanine	78	59	71	63	71	54
Glutamic acid	82	70	86	67	83	69
Proline	56	69	64	40	65	46
Cystine	11	12	39	33	85	84
Methionine	90	74	82	60	65	42
Lysine	31	84	27	24	74	79
Tryptophan	90	77	85	63	72	63
Valine	90	74	75	59	75	61
Threonine	78	52	68	50	72	57
Histidine	21	3	29	23	77	68
Phenylalanine	90	76	83	65	72	61
Leucine	90	77	81	62	75	63
Isoleucine	91	77	81	62	74	61

*Layers: 1,2 = Whatman C-18; 3,5 = Merck RP-18; 4,6 = Merck RP-18W; mobile phases: 1,3,4 = n-butanol-glacial acetic acid water (3:1:1); 2,5,6 = n-propanol-water (7:3)

cm except Whatman C-18, which was 20 x 20 cm. n-Butanol or 2-butanol-glacial acetic acid-water (3:1:1) and 70% aqueous n-propanol were employed as mobile phases.

Plates were developed in a Camag (Wilmington, NC) paper-lined, solvent vapor equilibrated HPTLC twin-trough chamber for a distance of 7 cm beyond the origin (11 cm for Whatman C-18), which required 45-150 min depending on the layer and mobile phase. The chromatogram was air dried in a fumehood, sprayed with ninhydrin detection solution (0.3 g ninhydrin in 100 ml of n-butanol plus 3 ml of glacial acetic acid), air dried for 5 sec, resprayed, and heated in an oven at 110°C for 10 min.

Quantification was carried out by scanning standard and sample zones at 610 nm with a Shimadzu (Columbia, MD) CS-930 scanner in the single beam, reflectance mode. The absorption maximum for scanning was determined by measuring the in situ spectrum of a standard zone using the visible (400-700 nm) spectral mode of the densitometer. The concentration of amino acid was calculated as μmol/g for DGG and μmol/ml for hemolymph from the scan areas of the standard zones and a bracketed sample zone, the amount of sample taken, the reconstitution volume of the sample extract, and the volumes of standards and sample spotted.

RESULTS AND DISCUSSION

A large number of silica gel, cellulose, and reversed phase systems suggested by a search of the literature and our previous experience (1,2) were tested for resolution of the standards of 18 important essential and nonessential amino acids. Most of these compounds were likely to be found in B. glabrata snails based on the results of previously published studies. hR_f values (R_f x 100) for the amino acids in the best systems, as evidenced by their efficiency (compactness of zones) and selectivity (separation of zone centers), are shown in Tables 1 and 2. Different recipes of ninhydrin and fluorescamine (13) spray reagents found in the paper chromatography and TLC literature were also compared, and the butanol-acetic acid formulation of ninhydrin gave the best sensitivity, stability, and color differentiation. Depending on the layer, amino acid zones were various shades of purple, blue, orange, yellow, or red. In general, amino acids formed compact bands on silica gel, cellulose, and Merck C-18 plates and were somewhat more diffuse on Whatman C-18 plates.

TABLE 2

hR_f Values of Amino Acid Standards on Normal Phase Layers

	TLC System*			
	1	2	3	4
Aspartic acid	28	27	26	58
Arginine	18	18	17	2
Serine	26	30	27	40
Glycine	26	32	28	43
Tyrosine	46	58	53	78
Alanine	38	32	32	55
Glutamic acid	69	56	50	64
Proline	45	32	28	50
Cystine	10	11	9	30
Methionine	60	59	51	72
Lysine	15	13	10	4
Tryptophan	55	63	57	82
Valine	60	56	49	68
Threonine	34	32	32	53
Histidine	14	14	11	17
Phenylalanine	68	61	55	80
Leucine	79	61	55	78
Isoleucine	78	59	54	77

*Layers: 1 = cellulose; 2, 4 = Whatman silica gel; 3 = Merck silica gel; mobile phases: 1 = 2-butanol-glacial acetic acid water (3:1:1); 2, 3 = n-butanol-glacial acetic acid-water (3:1:1); 4 = n-propanol-water (7:3)

The migration sequences indicated by the data in Tables 1 and 2 do not clearly demonstrate that a different mechanism was operative for the normal phase (silica gel, cellulose) and reversed phase (C-18) plates, but suggest that the separations were most likely governed by a combination of adsorption, partition, and/or solubility (2). The possible variations among different brands of the same type of stationary phase is demonstrated in Table 1 for arginine and histidine, which have very low R_f values on Whatman C-18 plates but high

values on Merck RP-18 and RP-18W. The unique selectivities of the systems caused the amino acids to be resolved into several diverse groups with differing mobility on each plate, which would allow a large number of binary and larger amino acid mixtures to be separated based on the data in Tables 1 and 2. However, only the following compounds were completely resolved from all others on any single plate: histidine and cystine on Whatman C-18 developed with n-butanol-acetic acid-water (3:1:1); threonine, alanine, and cystine on cellulose developed with 2-butanol-acetic acid-water (3:1:1); arginine and cystine on Merck silica gel developed with n-butanol-acetic acid-water (3:1:1); methionine, aspartic acid, and histidine on Whatman silica gel developed with n-propanol-water (7:3); and arginine and cystine on Merck RP-18 developed with n-butanol-acetic acid-water (3:1:1).

Extracts of B. glabrata DGG and hemolymph were developed in all of the systems involving butanol-acetic acid water mobile phases and with n-propanol-water (7:3) on Whatman silica gel, and the resulting chromatograms were very complex. A desalting procedure (3) was applied to the extracts to determine if a small degree of trailing in some of the sample chromatograms could be decreased or eliminated. It was found that extract chromatograms were essentially identical with or without desalting.

Comparison of R_f values and zone colors and intensities between samples and standards in the various systems indicated that DGG and hemolymph extract chromatograms were similar and that most, if not all, of the 18 amino acids were probably present in both extracts. However, only the seven completely resolved compounds mentioned above, i.e., histidine, cystine, threonine, alanine, arginine, methionine, and aspartic acid, could be definitely identified. Based on zone

intensities, methionine and cystine were present in very low amounts, while the other five amino acids were major components. It was not possible to definitely confirm the presence or absence of the other acids, despite the use of a number of different types of high performance plates. These findings cast doubt on qualitative and quantitative results reported earlier (10,11) for amino acids in B. glabrata based on paper chromatography, which is considerably less efficient than HPTLC.

Earlier publications on the qualitative and quantitative analysis of amino acids in B. glabrata hemolymph also reported the presence of many amino acids, but results were not consistent. Stanislawski et al. (8) found 17 of the 18 amino acids we investigated present by ion exchange chromatography at levels ranging from 8.1 to 90.6 μmol/L; tryptophan was not reported. Schnell et al. (9) determined 15 of the 18 acids at concentrations greater than 13 μmol/L in hemolymph and 0.2 μmol/g in digestive gland using C-18 HPLC, along with traces of methionine and tryptophan; cystine, proline, and histidine were not found in either sample. Gilbertson et al. (7) found 15 of the 18 acids in hemolymph at levels between 1-57 μmol/L, plus a trace of tyrosine; neither tryptophan nor cystine were reported. Using paper chromatography, Dusanic and Lewert (11) identified lysine, glycine, proline, threonine, and leucine, and Targett (10) identified all of the 18 acids except tryptophan. In a study of the hemolymph of five species of freshwater snails not including B. glabrata, Gilbertson and Schmid (6) detected 18 different amino acids by ion exchange chromatography, among which serine, glutamic acid, aspartic acid, glycine, threonine, and alanine were the major compounds in all species.

To demonstrate the applicability of quantitative HPTLC to amino acid analysis in snails, DGG and hemolymph

extracts were analyzed for two of the completely resolved acids, i.e., alanine on cellulose developed with 2-butanol-acetic acid-water (3:1:1), and aspartic acid on Whatman silica gel developed with n-propanol-water (7:3). Calibration curves for the two acids had linearity coefficients of 0.99 over the range of standards spotted. The results for alanine were 106 μmol/L in hemolymph and 0.326 μmol/g in the DGG, while the corresponding values for aspartic acid were 30.3 μmol/L and 0.508 μmol/g. These values are in the range of those found earlier for B. glabrata by column LC: 27 (7), 98 (8), and 107 (9) μmol/L for alanine in hemolymph and 1.1 μmol/g (9) in the digestive gland, and 16 (9), 21 (7), and 58 (8) μmol/L for aspartic acid in hemolymph and 0.45 μmol/g (9) in the digestive gland.

The variations described above in reported qualitative and quantitative results for amino acids in B. glabrata snails are due not only to the different analytical methods employed, but also to differences in strain, development, nutrition, and maintenance of the snails.

Our present and earlier (1,2) studies demonstrate that HPTLC can be used for the separation, detection, identification, and quantification of amino acids in selected mixtures. However, the method has limited applicability to the analysis of amino acids in freshwater snails because of the complexity of the mixture present in aqueous extracts. Of the procedures proposed in the literature, column ion exchange chromatography (amino acid analyzer) appears to be the method of choice for optimal qualitative and quantitative analysis of snail amino acids.

REFERENCES

1. Sleckman, B.P. and Sherma, J., J. Liq. Chromatogr. 5, 1051 (1982)

2. Sherma, J., Sleckman, B.P., and Armstrong, D.W., J. Liq. Chromatogr. 6, 95 (1983)

3. Bailey, R.S., Jr. and Fried, B., Int. J. Parasitol. 7, 497 (1977)

4. Bhushan, R., in Handbook of Thin Layer Chromatography (Sherma, J. and Fried, B., editors), Marcel Dekker, Inc., New York, 1991, pp. 353-387

5. Blackburn, S. (editor), CRC Handbook of Chromatography: Amino Acids and Amines, vol. II (Zweig, G. and Sherma, J.,editors-in-chief), CRC Press, Boca Raton, FL, 1989

6. Gilbertson, D.E. and Schmid, L.S., Comp. Biochem. Physiol. 51B, 201 (1975)

7. Gilbertson, D.E., Etges, F.J., and Ogle, J.D., J. Parasitol. 53, 565 (1967)

8. Stanislawski, E., Becker, W., and Mueller, G., Comp. Biochem. Physiol. 63B, 477 (1979)

9. Schnell, S., Becker, W., and Winkler, A., Comp. Biochem. Physiol. 81B, 1001 (1985)

10. Targett, G.A.T., Ann. Trop. Med. Parasitol. 56, 61 (1962)

11. Dusanic, D.G. and Lewert, R.M., J. Inf. Dis. 112, 243 (1963)

12. Anderton, C.A., Fried, B., and Sherma, J., J. Planar Chromatogr.-Mod. TLC 6, 51 (1993)

13. Sherma, J. and Touchstone, J.C. Anal. Lett. 7, 279 (1974)

Received: September 3, 1993
Accepted: September 21, 1993