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SELECTIVE TLC SEPARATION OF LYSINE AND THREONINE IN PHARMACEUTICAL PREPARATIONS

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SUMMARY

Thin-layer chromatography of α-amino acids has been performed on layers prepared from a 1:4 stannic arsenate–cellulose mixture. R_F values of the amino acids were determined after development with a variety of mobile phases. Important synthetic ternary separations have been achieved on the basis of different R_F values. Lysine and threonine have been selectively separated and quantitatively determined from among the mixture of amino acids present in a commercially available drug.

INTRODUCTION

Amino acids are the basic components of proteins, the principal materials of skin, muscle, tendons, nerves, blood, enzymes, antibiotics, and many hormones. Chromatography has long been used as a separation technique, for amino acids. Although inorganic ion-exchange materials have frequently been used for separation of metal ions [1–3] they have seldom been used for the separation of organic compounds. Papers impregnated with titanium arsenate and zirconium phosphate have been used to separate amino acids [4–9] and alkaloids [10], and stannic tungstate cation exchangers have been used by Nabi et al. to separate amino acids [11]. Lepri et al. [12] used a mixture of ammonium tungstophosphate and silanized silica gel, and ammonium tungstophosphate molybdophosphate layers, for separation of amino acids. Sleckman et al. [13] studied the retention behaviour of nine essential amino acids on silica gel, cellulose, and cation exchanger (Fixion strong acid). Laskar and Basak [14] used silica gel G for identification of amino acids and Ruzhilo et al. [15]. separated amino acids on thin layers of chitin. Bhushan et al. [16] used silica gel impregnated with zinc, cadmium, and mercury with some new solvent systems.

This paper describes the potential of mixed stannic arsenate–cellulose layers for the separation of α-amino acids. α-Amino acids were studied because of their biomedical, physiological, and pharmaceutical importance. The use of stannic arsenate, a weak cation exchanger, is advantageous because it is easily buffered at the desired pH.

EXPERIMENTAL

Reagents and Chemicals

Stannic chloride pentahydrate was from Loba Chemie (India), sodium arsenate from E. Merck (India), amino acids from Loba Chemie, and cellulose powder from Merck. Other reagents were of AR grade.

Preparation of Ion-Exchange Materials and TLC Plates

Stannic arsenate was synthesized by slowly adding 0.1 M aqueous sodium arsenate to a 2:1 mixture of 0.1 M stannic chloride solution and 0.1 M HCl. The mixture was intermittently shaken during mixing and the resulting precipitate was finally left for 24 h at room temperature. The mixture was washed several times with distilled water, by decantation, and then filtered under vacuum. The product was then dried in an oven at 50 ± 2°C. The dried product was broken into small granules simply by immersing in distilled water. The granules were again washed several times with distilled water and finally dried at 50°C. The cation-exchange capacities of the ion exchange material for Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} were found to be 0.77, 1.04, 1.03, and 1.31 mmol g^{-1}, respectively.

Preparation of TLC Plates

For preparation of thin-layer plates, granules of stannic arsenate were well powdered in a mortar and mixed with cellulose powder in 1:4 ratio and 10% $CaSO_4$ was added as binder. A slurry of this mixed product was prepared in 50 mL water per 10 g material and spread over glass plates, by means of applicator, to form uniform thin layers 0.2 mm thick. The plates were then dried in an oven at 60°C.

Procedure

For quantitative work 1% solutions of the amino acids were applied to the plates by means of a fine glass capillary. After drying the spots, the plates were developed in different mobile phases (Table I) at

Table I

The mobile phases investigated

Code	Components	Composition
S_1	Butanol–acetic acid–water	5:4:1
S_2	Butanol–formic acid–water	7:2:1
S_3	Butanol–formic acid	7:3
S_4	Butanol–formic acid	6:4
S_5	Butanol–formic acid	5:5
S_6	Butanol–formic acid	4:6
S_7	Isopropanol–acetic acid–water	8:1:1
S_8	Isopropanol–formic acid–water	7:2:1
S_9	Ethyl methyl ketone–ethyl acetate–acetic acid–water	2:6:1:1
S_{10}	Ethyl methyl ketone–ethyl acetate–formic acid–water	2:6:1:1
S_{11}	Acetonitrile–formic acid–chloroform	1:3:3
S_{12}	Ethyl methyl ketone–acetic acid–water	2:5:1
S_{13}	Ethyl methyl ketone–formic acid:	5:5
S_{14}	Ethyl methyl ketone–acetone–formic acid–water	3:2:3:2

room temperature (20 ± 2°C). Ascending development was performed to a distance of 15 cm from the point of application. The developed plates were again dried at 60°C. The location of the amino acid spots were detected by treatment of the plate surface with a solution of ninhydrin in butanol (1%). hR_F values are listed in Table II.

Quantitative Separations

A mixture of amino acids (50 μL) was spotted on a plate by means of a syringe. The plates were developed with appropriate mobile phases in the usual way. The positions of spots were detected by use of ninhydrin reagent. For quantitative separations of α-amino acids the plates were developed under conditions identical with those described above. The regions of adsorbent containing the separated amino acids were scraped from the plates and extracted with small amounts of water (10 mL). The supernatant from the extract was then analysed spectrophotometrically (Spectronic 20-D Genesys spectrophotometer) for amino acids by use of hydrindantin–methyl cellosolve reagent [17].

Table II

hR_F Values of amino acids on 1:4 stannic arsenate–cellulose layers

Amino acid	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}	S_{14}
Serine	54	33	42	55	69	75	25	40	06	07	67	66	67	84
Threonine	62	49	50	65	73	85	62	54	08	14	76	65	71	86
Cysteine	71	76	56	69	87	87	65	81	16	17	82	90	90	87
Glycine	55	44	33	41	71	76	31	44	05	09	68	59	66	92
Lysine	37	24	4	32	46	67	12	19	0.0	10	77	52	62	75
Alanine	77	66	46	69	81	92	69	67	19	24	50	76	82	86
Valine	85	86	85	42	100	100	100	77	34	75	90	86	94	94
Leucine	91	94	97	96	100	100	100	96	69	91	95	94	95	89
Isoleucine	88	87	81	93	100	100	87	89	55	86	94	88	86	90
Tryptophan	82	75	72	94	100	100	46	66	54	80	87	70	83	87
Methionine	81	70	75	89	42	100	77	81	42	82	90	90	81	91
Aspartic acid	54	41	31	53	67	75	41	54	01	06	46	66	61	76
Glutamic acid	60	69	44	60	66	86	47	55	14	40	80	74	60	74
Arginine	41	30	10	27	51	70	09	15	0.0	36	75	64	57	71
Norleucine	94	96	91	94	100	100	100	94	66	59	94	90	91	89
Ornithine	35	15	64	23	39	57	07	07	0.0	15	55	49	56	62

RESULTS AND DISCUSSION

Results from this study reveal that stannic arsenate–cellulose layers can be used for separation of amino acids. Several important ternary separations of amino acids have been achieved (Table III). The number of such separations was larger than on layers prepared from cellulose alone (Table IV). Ion exchange and adsorption occur simultaneously, resulting in different rates of migration of amino acids; this enhances the possibility of separation. Among the mobile phases studied, *n*-butanol–formic acid–water, isopropanol–acetic acid–water, and isopropanol–formic acid–water resulted in the best resolution. It is clear from Table II that for butanol–formic acid, R_F values generally increase as the concentration of formic acid in the mixture is increased to the composition butanol–formic acid, 5:5. Increasing the formic acid concentration further does not affect the movement substantially except for the basic amino acids arginine and lysine.

Because of the different rates of migration of amino acids in these systems, several important separations were possible. Sharp and clear ternary separations of amino acids with compact spots were achieved because of the rapid migration of some amino acids, e.g. threonine, cysteine, alanine, valine, lysine, isoleucine, and methionine, compared with others. The average rate of migration of glycine, tryptophan, aspartic acid, and glutamic acid and the

Table III

Ternary separations of amino acids on 1:4 stannic arsenate–cellulose layers

Mobile phase[a]	Separations achieved and R_F values ($R_L - R_T$)[b]
S_3	Lysine–threonine–valine (0.20–0.0) (0.56–0.49) (0.90–0.79)
	Lysine–threonine–leucine (0.22–0.0) (0.57–0.48) (1.0–0.89)
	Lysine–threonine–isoleucine (0.21–0.0) (0.57–0.48) (0.88–0.75)
	Lysine–threonine–methionine (0.20–0.0) (0.58–0.47) (0.82–0.70)
	Ornithine–aspartic acid–valine (0.18–0.06) (0.74–0.65) (0.98–0.96)
S_7	Ornithine–aspartic acid–leucine (0.17–0.06) (0.77–0.65) (1–0.91)
	Ornithine–aspartic acid–isoleucine (0.19–0.07) (0.78–0.65) (0.99–0.92)
	Ornithine–glutamic acid–methionine (0.20–0.06) (0.75–0.64) (0.96–0.88)
	Ornithine–glutamic acid–valine (0.19–0.07) (0.84–0.74) (0.94–0.88)
	Ornithine–glutamic acid–leucine (0.20–0.07) (0.88–0.74) (0.99–0.92)
	Ornithine–glutamic acid–isoleucine (0.17–0.05) (0.85–0.74) (0.96–0.85)
	Ornithine–glutamic acid–methionine (0.15–0.02) (0.84–0.73) (0.97–0.85)
	Arginine–tryptophan–valine (0.28–0.12) (0.78–0.66) (0.93–0.83)
	Arginine–tryptophan–leucine (0.28–0.10) (0.80–0.68) (0.97–0.86)
	Arginine–tryptophan–isoleucine (0.26–0.10) (0.76–0.64) (0.97–0.84)
	Arginine–tryptophan–methionine (0.26–0.10) (0.71–0.60) (0.96–0.81)
	Arginine–glycine–valine (0.26–0.05) (0.44–0.32) (0.92–0.80)
	Arginine–glycine–leucine (0.26–0.06) (0.46–0.36) (0.96–0.82)
	Arginine–glycine–isoleucine (0.26–0.06) (0.50–0.37) (0.96–0.83)
	Arginine–glycine–methionine (0.28–0.08) (0.47–0.34) (0.92–0.80)
S_{10}	Serine–glutamic acid–valine (0.15–0.05) (0.27–0.18) (0.72–0.54)
	Serine–glutamic acid–leucine (0.13–0.06) (0.23–0.15) (0.84–0.69)
	Serine–glutamic acid–methionine (0.14–0.05) (0.24–0.16) (0.68–0.53)
	Serine–arginine–valine (0.04–0.0) (0.16–0.06) (0.66–0.50)
	Serine–arginine–leucine (0.02–0.0) (0.14–0.06) (0.88–0.74)
	Serine–arginine–methionine (0.03–0.0) (0.14–0.08) (0.68–0.56)
	Aspartic acid–glutamic acid–valine (0.19–0.12) (0.26–0.20) (0.66–0.48)
	Aspartic acid–glutamic acid–leucine (0.19–0.14) (0.25–0.20) (0.84–0.66)
	Aspartic acid–glutamic acid–methionine (0.18–0.13) (0.24–0.19) (0.64–0.48)
	Aspartic acid–arginine–valine (0.09–0.0) (0.23–0.14) (0.58–0.44)
	Aspartic acid–arginine–leucine (0.08–0.0) (0.22–0.12) (0.79–0.66)
	Aspartic acid–arginine–methionine (0.06–0.0) (0.21–0.11) (0.64–0.54)

[a]See Table I
[b]R_L and R_T are the R_F values of the leading and trailing extremities of the spots

slower rate of migration of other amino acids, viz. serine, lysine, arginine, and ornithine made other separations possible. It is apparent from the R_F

Table IV

Ternary separations of amino acids on thin layers of cellulose only

Mobile phase[a]	Separations achieved and R_F values ($R_L - R_T$)[b]
S_7	Arginine–tryptophan–valine (0.17–0.06) (0.56–0.21) (0.79–0.53)
	Arginine–tryptophan–leucine (0.15–0.07) (0.52–0.21) (0.96–0.78)
	Arginine–tryptophan–isoleucine (0.17–0.04) (0.54–0.21) (0.91–0.70)
	Arginine–tryptophan–methionine (0.15–0.04) (0.48–0.27) (0.70–0.50)
	Arginine–glycine–valine (0.18–0.0) (0.30–0.18) (0.80–0.58)
	Arginine–glycine–leucine (0.17–0.05) (0.31–0.17) (0.97–0.77)
S_{10}	Serine–arginine–valine (0.0–0.0) (0.05–0.0) (0.31–0.16)
	Serine–arginine–leucine (0.0–0.0) (0.0–0.0) (0.49–0.33)
	Serine–arginine–methionine (0.0–0.0) (0.04–0.0) (0.26–0.17)
	Aspartic acid–arginine–valine (0.0–0.0) (0.05–0.0) (0.25–0.12)
	Aspartic acid–arginine–leucine (0.0–0.0) (0.05–0.0) (0.46–0.28)
	Aspartic acid–arginine–methionine (0.0–0.0) (0.05–0.0) (0.26–0.17)

[a]See Table I
[b]R_L and R_T are the R_F values of the leading and trailing extremities of the spots

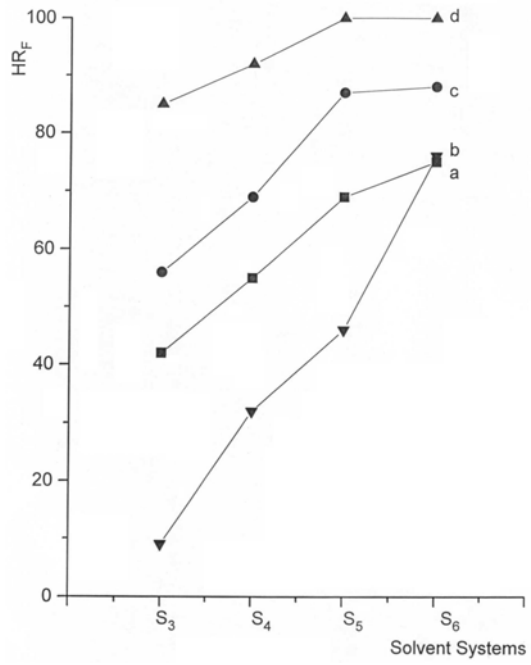

Fig. 1

Effect of formic acid concentration on the R_F values of the amino acids: a. lysine, b. serine, c. cysteine, d. valine

data in Table II and Figs 1–4 that propanol–formic acid–water, ethyl methyl ketone–ethyl acetate–acetic acid–water, and ethyl methyl ketone–ethyl acetate–formic acid–water also enable much better ternary separations on stannic arsenate–cellulose layers than on cellulose alone. It was also observed that mixing stannic arsenate with cellulose significantly enhances the quality of the chromatogram in two respects – migration of the amino acids usually increases and tailing is reduced, resulting in a compact spots. This improvement is probably because of the weak cation-exchange property of stannic arsenate. On the mixed layer movement of the amino acids is governed by ion exchange and simple adsorption simultaneously, resulting in different migration of the species and thus improving the separation. It has been observed that on layers prepared by mixing stannic arsenate with cellulose the R_F values of the amino acids are usually increased. An interesting aspect of the use of the stannic arsenate–cellulose layer is that

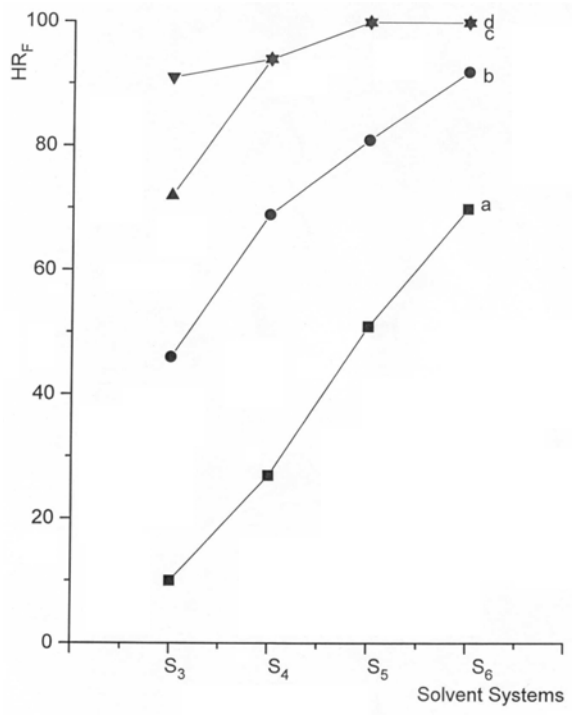

Fig. 2

Effect of formic acid concentration on the R_F values of the amino acids: a. arginine, b. alanine, c. tryptophan, d. norleucine

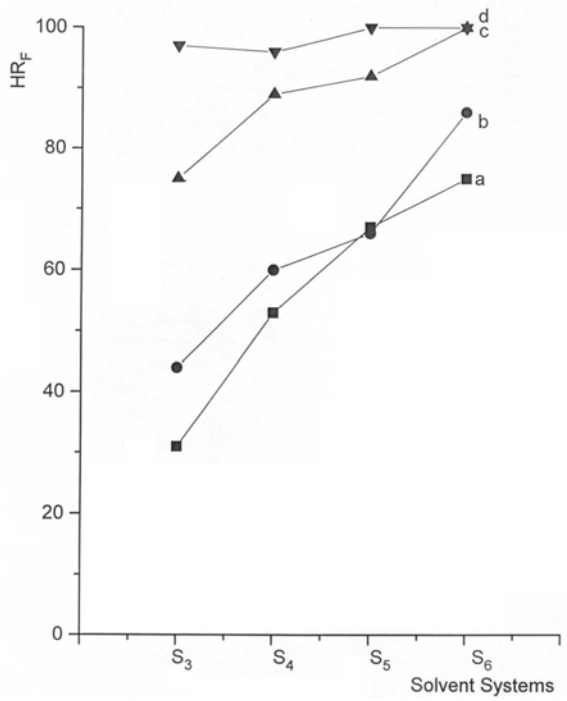

Fig. 3

Effect of formic acid concentration on the R_F values of the amino acids: a. aspartic acid, b. glutamic acid, c. methionine, d. leucine

selective separations of lysine and threonine from a mixture of other amino acids, namely valine, leucine, isoleucine, methonine, and tryptophan, have been achieved.

To demonstrate the potential of the stannic arsenate–cellulose layer for separation of amino acids, quantitative separations were performed, initially on mixtures prepared in this laboratory (Table V). The utility of the method was then demonstrated by analysis of a commercially available drug, Alamine (manufactured by Albert David), which contains several amino acids (Table VI).

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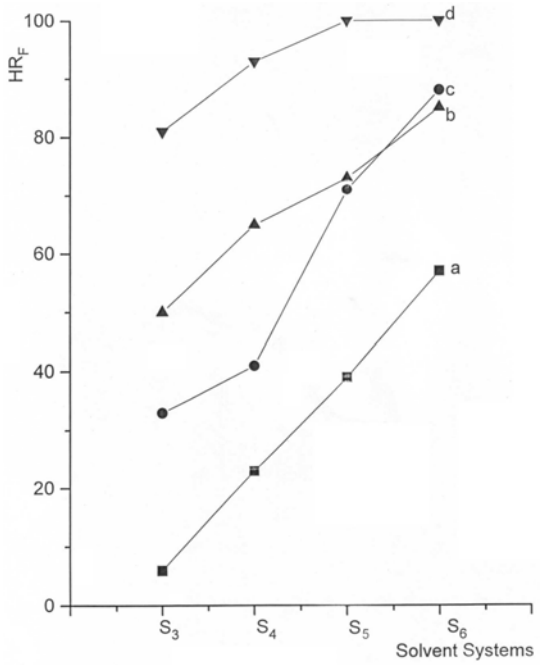

Fig. 4

Effect of formic acid concentration on the R_F values of the amino acids: a. ornithine, b. glycine, c. threonine, d. isoleucine

Table V

Quantitative separation of synthetic mixtures of amino acids on 1:4 stannic arsenate–cellulose layers developed with mobile phase S_{10}

Separation achieved	Amount taken (μg)	Amount found (μg)[a]	Error (%)
Ornithine–glutamic acid–valine	50–50–50	49.85–49.80–50.10	–0.30, –0.40, +0.20
Ornithine–glutamic acid–leucine	50–50–50	50.10–49.15–50.20	+0.20, –0.50, +0.40
Serine–glutamic acid–valine	50–50–50	49.85–49.90–49.95	–0.30, –0.20, –0.10
Serine–arginine–leucine	50–50–50	50.15–50.20–49.70	+0.30, +0.40, –0.60
Aspartic acid–glutamic acid–methionine	50–50–50	49.75–49.85–50.05	–0.50, –0.30, +0.10
Aspartic acid–glutamic acid–leucine	50–50–50	49.90–50.15–50.00	–0.20, +0.30, 0.00
Lysine–threonine–isoleucine	50–50–50	50.15–49.95–49.90	+0.30, –0.10, –0.20
Lysine–threonine from valine, leucine, isoleucine, methionine, and tryptophan	50–50	49.80–50.20	–0.40, +0.40

[a]Averages from five replicate determinations

Table VI

Quantitative separation of lysine and threonine from a mixture of amino acids in a comercially available drug (Alamine Forte capsule; Albert David) on 1:4 stannic arsenate–cellulose layers developed with mobile phase S_3

No.	Amino acid in drug	Labelled amount of compound (μg)	Amount taken (μg)	Amount found (μg)[a]	Mean recovery (% ± *SD*)
1.	Lysine	25	25	24.75	99.00 ± 0.15
	Threonine	8.3	8.30	8.20	98.80 ± 0.16
2.	Lysine	25	50	49.45	98.90 ± 0.13
	Threonine	8.3	16.6	16.35	98.50 ± 0.11

[a]Averages from five replicate determinations

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