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TLC separation of L-tryptophan using microemulsion mobile phase and its spectrophotometric determination

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The TLC system comprising silica gel as the stationary phase, and water-in-oil microemulsion (SDS+water+heptane+n-pentanol; (160 g + 8 mL + 16 mL + 25 mL) as the mobile phase, has been identified as the most favourable system for selective separation of L-tryptophan from other amino acids in the presence of metal cation impurities. The proposed method is applicable to the identification and separation of L-tryptophan from drug samples such as Astymin-m (Forte), Astymin (liquid) and Alamin (Forte). The separation up to 10 µg of L-tryptophan from milligram quantities (up to 0.77 mg) of other amino acids has also been realized. The quantitative estimation of L-tryptophan by spectrophotometry (λ_{max} 570 nm) after separation from other amino acids has been done. The recovery of tryptophan is around 98% and its limit of detection on silica gel layer is 0.13 µg.

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Thin layer chromatography (TLC) is being used for analyzing complex mixtures into individual components[1-4]. Microemulsions are thermodynamically stable, transparent and homogeneous singlet-phase solutions containing oil (non-polar solvent), water, surfactant and a co-surfactant which is generally a medium chain alcohol, amine or similar polar organic molecule[5]. Because of several favourable features such as transparent quality, small drop size, enhanced solubilization, ability to incorporate solutes within the dispersed droplets and the action as extraction media, water-in-oil microemulsion systems find exciting applications in liquid chromatography[6-8].

Analysis of amino acids has assumed importance because of their industrial and pharmaceutical applications[9-11]. TLC of amino acids using various solvent systems as mobile phase is well documented[12-19]. Most of these studies involve the use of alcohol as one of the components of mixed mobile phase. Rozylo *et al.* have demonstrated excellent separation possibilities of optical isomers of amino acids on chitin-Cu layers using methanol-water-acetonitrile as mobile phase[20]. Tryptophan enantiomers have been separated on several types of reversed phase layers[21] and on microcrystalline cellulose using aqueous solvents as mobile phase[22]. The separation of L-tryptophan from other amino acids is important because their presence offers deliterious effect on its detection by chromogenic reagents[23].

Though HPLC[24], GC[25], volumetric[26], viscometric[27], micellar electrokinetic chromatography[28] and electrophoresis[29] techniques are available for the analysis of amino acids, TLC being in-expensive is used for routine analysis.

Experimental

All experiments were performed at 30±5°C. A TLC applicator (Toshniwal, India) was used for coating silica gel on 20×3.5 cm glass plates. Chromatography was performed in 23×6 cm glass jars. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of studied compounds. A spectrophotometer (ELICO model SL 171, India) was also used.

Amino acids, heptane, cetyl trimethyl ammonium bromide (CTAB) (CDH, India); sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) BDH, England); silica gel 'G', sodium dodecyl sulfate (E. Merck, India); *n*-pentanol (Fluka AG, Switzerland); ninhydrin (Loba-chemie, India) were used. All reagents were of analaR grade. The amino acids studied were: L-tryptophan (L-Trp), L-iso-leucine (L-Ile), L-hydroxyproline (L-Hyp), L-proline (L-Pro), L-arginine (L-Arg), L-lysine (L-Lys), L-cysteine (L-Cys), L-methionine (L-Met), L-valine (L-Val), glycine (Gly) and L-serine (L-ser).

A 1% solution of L-Cys was prepared in 0.1% aqueous HCl solution and all other amino acids were prepared in demineralized double distilled water. 0.3% ninhydrin solution in acetone was used to detect all the amino acids.

The following microemulsion systems were used as mobile phase: M_1: CTAB-water-heptane-n-pentanol (8 g: 8 mL: 160 mL: 25 mL); M_2: AOT-water-heptane (8 g: 8 mL: 160 mL); M_3: SDS-water-heptane-n-pentanol (8 g: 8 mL: 160 mL: 25 mL).

Silica gel 'G' was used as the stationary phase. Silica gel plates were prepared by mixing the adsorbent with double distilled water in 1:3 ratio *w/w*. The resultant slurry was mechanically shaken for 10 min, and applied to the well-cleaned glass plates using the TLC applicator (layer thickness ~0.25 mm). The plates were air dried at room temperature and then activated by heating at 100±5°C for 1 h. After activation, the plates were stored in air-tight chamber.

The water-in-oil microemulsion used as mobile phase was prepared at 30°C by titrating a coarse emulsion of heptane (160 mL), water (8 mL) and SDS or CTAB (8 g) with *n*-pentanol (25 mL). AOT microemulsion was prepared by mixing AOT (8 g), water (8 mL) and heptane (160 mL). Various microemulsion systems were transparent, optically clear and stable at 30±5°C for several weeks.

Chromatographic studies

About 10 μL of test solution was spotted on thin layer plates with the help of micropipette. The plates were developed in the chosen solvent system by the ascending technique. The solvent ascent was fixed to 10 cm in all cases. After development was complete, the plate was withdrawn from glass jars, dried at room temperature, sprayed with freshly prepared ninhydrin solution and heated at 100±5°C for 15-20 min. All amino acids appeared as violet spots except L-Pro and L-Hyp which produce yellow spots.

For the selective separation of amino acids, an aliquot (20 μL) of mixture obtained by mixing 10 μL of L-Trp and 10 μL of other amino acids was loaded on TLC plates. The plates were developed with mobile phase M_3. The spots were detected and the R_F values of the separated amino acids were determined.

For examining the interference of heavy metal ions on separation of L-Trp with other amino acids, an aliquot (10 μL) of foreign substance (metal ions) was spotted along with the mixture (10 μL) of L-Trp and other amino acids on TLC plate and chromatography was performed as described above with M_3. The spots were detected and the R_F values of amino acids were determined.

For microgram separation of L-Trp from other amino acids, TLC plate was spotted first with 10 μL of the L-Trp (10 μg) solution followed by spotting with 10 μL of the mixture of other amino acids containing 0.1-0.77 mg (mixture of amino acids) per 10 μL on the same spot of TLC plate. The spots were dried, and the plates were developed with M_3. The separated spots were visualized and R_F values of amino acids were determined.

The identification limit of L-Trp was determined by spotting different amounts of L-Trp on the TLC plates. The plates were detected as described above. The method was repeated with successive lowering of the amount of amino acid. The lowest amount that could be detected was taken as the limit of detection.

For spectrophotometric determination of L-Trp, aliquots (10 μL) of L-Trp of different strengths (0.5-4.5%) containing 0.05-0.45 mg L-Trp were treated with 5 mL of methanol and 2 mL of 0.3% ninhydrin (in acetone) and heated in an oven at 100°C for 30 min. After cooling, the solution was diluted up to 10 mL with methanol. Absorbance of the developed colour was measured spectrohotometrically against blank reagent at 570 nm (λ_{max}) using 1 cm cells and a standard curve was plotted.

The devised TLC method was applied to the determination of L-Trp after their chromatographic separation from other amino acids. For this purpose, 10 μL of L-Trp of different strength (0.5-4.5%) containing 0.05-0.45 mg L-Trp were spotted onto TLC plates followed by spotting 0.1 mg mixture of other amino acids solution onto the same spots with micropipette and the development was made as usual with M_3. Pilot TLC plates were also run under similar conditions to ascertain the actual position of L-tryptophan spots on pilot plates, which were detected using the ninhydrin reagents. The same portion of the experimental plates was scratched out and L-Trp present in these portions was extracted with small volume of methanol, 5 mL being optimal for complete elution. The chromogenic reagent (2 mL of 0.3% ninhydrin in acetone) was added to the filtrate and the contents were heated at 100°C for 30 min. After cooling, the contents were diluted to 10 mL with methanol. The absorbance of the purple-pink colour, so developed, was measured spectrophometrically against a blank at 570 nm (λ_{max}) using 1 cm cells. The L-Trp content in spiked drug samples, after its separation from other amino acids was determined from the standard curve.

Results and discussion

The results of the present study are summarized in Tables 1-3. Surfactants provide a useful medium facilitating the selective separation of L-tryptophan. In the presence of surfactants, the side chain of amino acid plays a crucial role in its binding with

Table 1—R_F values of amino acids on silica gel layers developed with different mobile phases

Amino acids	R_F values		
	M_1	M_2	M_3
L-Trp	0.10	0.14	0.57
L-Ile	0.16	0.20	0.21
L-Hyp	0.08	0.11	0.15
L-Pro	0.04	0.08	0.10
L-Arg	0.08	0.15	0.26
L-Lys	0.07	0.12	0.19
L-Cys	0.03	0.10	0.20
L-Ser	0.06	0.10	0.16
L-Val	0.05	0.05	0.10
Gly	0.05	0.05	0.09
L-Met	0.06	0.12	0.17

Table 2—Separation of L-Trp from other amino acids, in the presence of metal cations as impurities on silica layers using mobile phase M_3

Metal cations	L-Trp	Other amino acids
Fe^{3+}	0.55	0.15
Cu^{2+}	0.56	0.15
Co^{2+}	0.55	0.13
Ni^{2+}	0.40	0.14
UO_2^{2+}	0.35 T	0.15
Al^{3+}	0.52	0.14
Cd^{2+}	0.53	0.13
Zn^{2+}	0.56	0.14
Pb^{2+}	0.50	0.10
Hg^{2+}	0.20 T	0.07
Bi^{3+}	0.22 T	0.09

T=Tailed spot R_L-R_T > 0.30. R_f of L-tryptophan in the absence of impurities is 0.57.

surfactants. L-tryptophan has the highest hydrophobicity 3400 Δft (cal/mol), whereas all others have values in the range of 2600-300 Δft (cal/mol)[30]. The highest hydrophobicity is responsible for L-tryptophan to have high affinity towards the alkane chain of the surfactant which ultimately results in higher R_f value (or mobility) of L-tryptophan. This unique micro environment of mobile phase provides the selective separation of L-tryptophan from all other amino acids. In order to select a most appropriate mobile phase, we used cationic (CTAB) and anionic (SDS and AOT) surfactants, as one of the components of water-in-oil microemulsion. The internal environment of water-in-oil microemulsion provides a small aqueous phase where amino acids can be localized to produce coloured spots with ninhydrin. Thus, amino acids are easily available to interact with ninhydrin for detection. For this reason, the resultant water-in-oil

Table 3—Spectrophotometric determination of and R_F value of L-Trp in different drug samples*

Drug samples	Amount loaded (μg)	Amount recovered (μg)	Relative recovery (%)	R_F of L-Trp	R_F of other amino acids
Astymin-m (Forte)	66	65	98.48	0.57	0.18
Astymin (Liquid)	132	129	97.73	0.55	0.18
Alamin (Forte)	150	147	98.00	0.56	0.15

*Label composition of the drugs can be obtained from the authors

microemulsion (M_1-M_3) systems were used as mobile phase to examine the mobility of amino acids on silica gel layer and the results are reported in Table 1. The table shows that the mobile phase systems (M_1 and M_2) are not useful for separation of amino acids as they fail to induce differential migration among amino acids, and L-Trp can be selectively separated from other amino acids on silica layer with M_3.

The lowest mobility of amino acids with microemulsion system (M_1) containing CTAB (cationic surfactant) may be explained on the basis of cation exchange property of silica gel.

$$[M^{n+} + m(SiOH) \rightleftharpoons m(OSi^-)_m^{n-m} + mH^+]$$

As a result of exchange of CTAB cation with H^+ of silica gel, concentration of CTAB in the mobile phase is decreased. This lowering of CTAB concentration by its sorption on silica layer results in the decrease of mobility of all amino acids with M_1. In case of M_2 and M_3, microemulsion systems containing anionic surfactants (AOT and SDS), Na^+ would be exchanged with H^+ of silica gel leaving behind the negatively charged surfactant monomer ion and H^+. Since SDS contains longer hydrophobic chain (C=12) in comparison to AOT (C=8), it shows lower selectivity towards amino acids facilitating enhanced mobility of amino acids.

Table 2 shows that the separation of L-Trp from other amino acids is possible in the presence of all metal cations in the sample except Hg^{2+}, UO_2^{2+} and Bi^{3+}. Investigating the effect of metal cations on the mobility of amino acids is an important aspect of the present study because in metalloprotein, amino acids and metal cations are the building blocks[31]. It seems that Hg^{2+}, UO_2^{2+} and Bi^{3+} have specific interaction with tryptophan leading to the formation of diffused or tailed spot.

It was observed that 10 μg of L-Trp can easily be separated from 0.77 mg of other amino acids. Thus, microgram quantities of L-Trp can be successfully separated from milligram amounts of the other amino acids using the proposed TLC system. The lowest possible detectable amount of L-Trp on silica gel layers with M_3 mobile phase was found 0.13 μg.

The method has been used to determine L-Trp after its separation from other amino acids using ninhydrin solution (0.3%) as chromogenic reagent. Absorbance of the coloured solutions was measured at 570 nm (λ_{max}). The calibration curve obtained by plotting absorbance (A) versus the concentration (C) of L-Trp shows a linear relationship up to 350 μg of L-Trp. The curve is well described by: $A = a + bC$ with coefficient of correlation (r=0.019). The developed method was used to study the recovery of L-Trp after separation from other amino acids in drug samples. The results presented in Table 3 show that relative recovery of L-Trp is always greater than 97.73%. The table exhibits the applicability of the proposed method for the selective separation of L-Trp from other amino acids in drug samples.

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