Anionic-Nonionic Surfactants Coupled Micellar Thin-Layer Chromatography: Synergistic Effect on Simultaneous Separation of Hydrophilic Vitamins

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Abstract

A micellar thin-layer chromatographic system comprising of silica layer impregnated with 0.01% sodium dodecyl sulphate (SDS) as stationary phase and 0.1% aqueous solution of Cween 80 as mobile phase was identified as the most favorable system for the mutual separation of five water-soluble vitamins (folic acid, cyanocobalamin, thiamine, pyridoxine, and riboflavin). Effects of the presence of metal cations, inorganic anions, and amines as impurities was determined. The detection and dilution limits, given in parenthesis for folic acid (1 µg and 1:0.1 × 10^4), cyanocobalamin (0.08 µg and 1:1.25 × 10^4), thiamine (0.05 µg and 1:2.0 × 10^4), pyridoxine (0.5 µg and 1:0.2 × 10^4), and riboflavin (0.5 µg and 1:0.2 × 10^4) were determined.

Introduction

Vitamins are essential dietary components required by the body in minute quantities to perform certain cellular functions, and their absence causes specific deficiency diseases. Vitamins catalyze biological reactions at very low concentrations, and lack of one or more vitamins leads to characteristic deficiency symptoms in humans. Multiple deficiencies caused by lack of more than one vitamin, such as avitaminosis, is very common in human beings. Because of their physiological importance, identification, separation, and quantification of vitamins in pharmaceutical formulations is important. Analysis of water-soluble vitamins has been performed by thin-layer chromatography (TLC) using different stationary and mobile phases (1–5). Since first use of surfactants in paper chromatography in 1963 by M. Lederer (1), surfactant-modified TLC has found wider applications in separation studies (6–9). The use of surfactants in TLC has expanded its potentiality by providing efficient separations. Three main approaches regarding the use of surfactants in TLC are evident from literature: as micellar mobile phase (10,11) where concentration of surfactant in the mobile phase exceeds the critical micelle concentration (CMC); as electrolyte in mobile phase (6,7,12) where the concentration ion of surfactants is kept below CMC value; and as impregnant of stationary phase (13–16).

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Surfactant-modified TLC provides enhanced selectivity as a result of difference in the degree of binding of separated mixture components with mobile and stationary phases. The selective solubilization of mixture components with micelles is caused by complex electrostatic, hydrophobic, donor-acceptor, and polarization interactions.

All types of surfactants (cationic, anionic, and nonionic) have been used in the mobile phase for the separation of amines (14), pesticides (17), peptides (18), dyes (19), heavy metal ions (20), vitamins (21), and steroids (22).

Alternatively, surfactants in stationary phase have also been used. Thus, following three modifications are available to utilize separation potentiality of surfactants in TLC analysis of organic and inorganic substances, including the impregnation of the stationary phase in the absence of the surfactant in the mobile phase, the impregnation of the stationary phase with the simultaneous introduction of the surfactant in the molecular form into the mobile phase, and the introduction of ionic surfactants as ion-pair reagents only in the mobile phase.

Amongst these options, the second has been least studied. The present communication is the first report on simultaneous use of micellar solutions of anionic surfactant (SDS) in stationary phase and a nonionic surfactant (Cween 80) in the mobile phase for the study of vitamins. The TLC systems, which are composed of mobile and stationary phases modified by surfactants, are valuable from points of view of cost, safety, and environmental protection because toxic organic solvents are not used.

The coupling of anionic and nonionic surfactants has provided a novel TLC system for excellent resolution of five water-soluble vitamins such as thiamine, cyanocobalamin, riboflavin, pyridoxine, and folic acid.

Experimental

Apparatus

A TLC applicator (Toshniwal, India) (20 × 3 cm glass plates and 24 × 6 cm glass jars) was used for the development of chromatographic plates. Iodine chamber was used to locate the position of the spot with a Leo microscope.

Chemicals and reagents

Silica gel G, pyridoxine (B_6), and methanol were purchased from Merck (Mumbai India); cellulose, kieselguhr G, aluminium oxide G, *N*-cetyl *N,N,N*-trimethyl ammonium bromide (CTAB), cetylpyridinium chloride (CPC), cetylpyridinium bromide (CPB), Cween 80 (Cw 80), Cween 20 (Cw 20), tween 20 (Tw 20), triton X-100 (TX-100), cobalt chloride, barium chloride, copper sulphate, zinc nitrate, mercuric chloride, ammonium chloride, aluminium nitrate, ammonium sulphate, folic acid, cyanocobalamin (B_{12}), thiamine (B_1), ascorbic acid (C), and riboflavin (B_2) came from CDH (New Dehli, India); dodecylbenzene sulfonic acid from Fluka (Milan, Italy), brij 35 from Qualigens Loba Chemie (Mumbai, India), and sodium dodecylsulphate (SDS) from BDH (Mumbai, India) were used.

Vitamins studied

Water-soluble vitamins used in the present study include folic acid, cyanocobalamin, thiamine, ascorbic acid, pyridoxine, and riboflavin.

Test solutions

1% aqueous test solutions of all vitamins (except 0.5% cyanocobalamin) were used.

Detector

Iodine vapors were used to detect vitamins.

Table I. Stationary and Mobile Phases

Symbol	Stationary phase composition	Symbol	Mobile phase composition
S1	Silica gel G slurry in 1% CTAB	M1	0.0001% Cween 80
S2	Silica gel G slurry in 1% CPC	M2	0.001% Cween 80
S3	Silica gel G slurry in 1% CPB	M3	0.01% Cween 80
S4	Silica gel G slurry in 0.00001% SDS	M4	0.1% Cween 80
S5	Silica gel G slurry in 0.001% SDS	M5	1% Cween 80
S6	Silica gel G slurry in 0.01% SDS	M6	0.1% Cween 20
S7	Silica gel G slurry in 0.1% SDS	M7	0.1% Brij 35
S8	Silica gel G slurry in 1% SDS	M8	0.1% TX-100
S9	Silica gel G slurry in 1% SDC	M9	0.1% Tween 20
S10	Silica gel G slurry in 1% DBS		
S11	Alumina slurry in 0.01% SDS		
S12	Cellulose slurry in 0.01% SDS		
S13	Kieselguhr slurry in 0.01% SDS		

Chromatographic systems

The stationary and mobile phases used are listed in Table I.

Preparation of TLC plates

Plain silica gel thin-layer plates

TLC plates were prepared by mixing silica gel G with double-distilled water in 1:3 volume ratios with constant shaking for 5 min until a homogeneous slurry was obtained. The resultant slurry was coated on the glass plates with the help of a Toshniwal applicator to give a 0.25-mm thick layer. The plates were first air-dried at room temperature and then activated by heating at 100ºC for 1 h. After activation, the plates were kept in an air-tight chamber until used.

Surfactant impregnated TLC plates

Impregnated TLC plates were prepared by mixing silica gel with aqueous surfactant solution in 1:3 ratios with constant stirring until homogeneous slurry was obtained. Thin layers of resultant slurry were prepared by following the method as described previously.

Procedure

TLC was performed on unimpregnated (or plain) and impregnated (with CTAB, CPC, CPB, SDS, SDC, or DBS) silica gel layers in glass jars. Test solutions (0.1 µL) were applied by means of micropipette about 2 cm above the lower edge of the plates. The spot was allowed to dry, and then the plates were developed in the chromatographic chamber presaturated for 30 min with the desired solvent system using ascending technique. The solvent ascent was kept up to 10 cm from the point of application. After

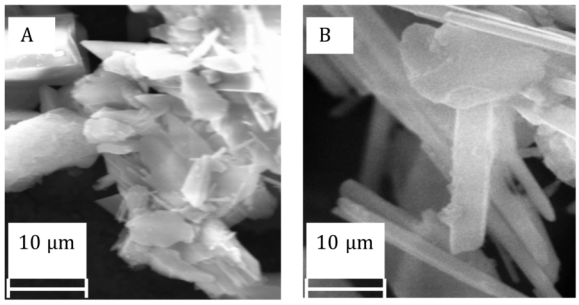

Figure 1. SEM images of pure silica gel and silica gel impregnated with SDS surfactant: (A) pure silica gel and (B) SDS impregnated silica gel.

Table II. R_f Values of Water Soluble Vitamins on Cationic Surfactant Pre-Impregnated Silica Stationary Layer Using Nonionic Surfactant as Mobile Phase

	S_1					S_2					S_3				
Vitamins	M_1	M_2	M_3	M_4	M_5	M_1	M_2	M_3	M_4	M_5	M_1	M_2	M_3	M_4	M_5
Folic acid	0.04	0.03	0.03	0.03	0.03	0.03	0.05	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.04
Cyanocobalamin	0.20	0.21	0.21	0.24	0.23	0.22	0.21	0.20	0.25	0.19	0.20	0.20	0.22	0.21	0.21
Thiamine	0.10	0.12	0.15	0.11	0.13	0.17	0.12	0.11	0.09	0.14	0.13	0.12	0.15	0.14	0.14
Ascorbic acid	0.03	0.04	0.03	0.13	0.03	0.14	0.13	0.11	0.12	0.02	1-0†	1-0†	1-0†	1-0†	1-0†
	0.99*	0.98*	0.99*	0.97*	0.99*	0.95*	0.95*	0.94*	0.95*	0.95*					
Pyridoxine	0.68	0.63	0.60	0.66	0.66	0.64	0.57	0.56	0.57	0.65	0.62	0.61	0.64	0.63.	0.64
Riboflavin	0.30	0.34	0.35	0.29	0.32	0.30	0.26	0.29	0.25	0.32	0.16	0.15	0.17	0.18	0.18

* Double spot. † Tailed spot.

development, the plates were dried at 60°C and then kept in iodine chamber to locate the position of vitamins (thiamine, ascorbic acid, pyridoxine) while folic acid, cyanocobalamin, and riboflavin are self-detected. The R_F values were calculated from the values of R_L (R_F of the leading front) and R_T (R_F of the trailing front).

$$R_F = (R_L + R_T)/2$$

Separation

For the mutual separation, equal volumes (1.0 mL each) of folic acid, cyanocobalamin, thiamine, pyridoxine, and riboflavin were mixed, and 0.2 µL of the resultant mixture was loaded on 0.01% SDS impregnated silica (S_6) TLC plates. The plates were developed with 0.1% Cw 80 (M_4) as mobile phase. The spots were detected by iodine vapors, and the R_F values of the separated vitamins were determined.

Interference

For investigating the interference of amines, inorganic anions, and metal cations on the mobility of vitamins, 0.2 µL mixture of vitamins (folic acid, cyanocobalamin, thiamine, pyridoxine, and riboflavin) was spotted on 0.015 SDS impregnated silica TLC plates (S_6) followed by the spotting of 0.2 µL of the interfering species (1% solution of cation, anion or amine) on the same spot. The chromatography was performed with S_6-M_4 (stationary phase, 0.01% SDS impregnated silica gel and mobile phase, 0.1% Cw 80) system. The spots were detected, and the R_F values of vitamins were determined.

Detection and dilution limits

The detection limits of folic acid, cyanocobalamin, thiamine, pyridoxine, and riboflavin were determined by spotting different amounts of folic acid, cyanocobalamin, thiamine, pyridoxine, or riboflavin on 0.01% SDS impregnated silica gel (S_6) stationary phase, developing TLC plates with M_4 mobile phase and detecting the spots. The method was repeated with successive lowering of the amount of vitamins. The lowest amount that could be detected was taken as the limit of detection (LOD). The limit of dilution was determined using the expression:

$$\text{Dilution limit} = 1: (\text{volume of test solution} \times 10^6)/[\text{LOD } (\mu g)]$$

Results and Discussion

The present study has following interesting features use of novel micellar TLC system comprising of anionic surfactant (SDS) in silica gel stationary phase with nonionic surfactant (Cween 80) in mobile phase in the analysis of vitamins; on-plate identification and simultaneous separation of folic acid, cyanocobalamin, thiamine, pyridoxine, and riboflavin; separation of vitamins (folic acid + cyanocobalamin + thiamine + pyridoxine + riboflavin) in the presence of impurities; and determination of chromatographic parameters like ΔR_F, α, and R_S for the separated vitamins were calculated.

Detection and dilution limits were determined for the separated vitamins. In the present study, following TLC systems were tested with the aim of identifying the most favorable system for simultaneous separation of water-soluble vitamins.

Cationic-nonionic TLC system

From the results depicted in Table II, the mobility of six water-soluble vitamins on cationic surfactant (CTAB, CPC, and CPB) impregnated silica stationary phase, using different concentrations (%) of nonionic surfactant (Cween 80) as mobile phase shows double or tailed spots for ascorbic acid. Cyanocobalamin, thiamine, and riboflavin have low R_F values, which differ marginally, and hence their mutual separation was not possible. On increasing the concentration of Cween 80 in mobile phase from 0.0001% to 1% was found non-effective to induce differential migration among vitamins as evident by marginal variation in the

Table III. R_f Values of Water Soluble Vitamins on Silica Phases Impregnated with Different Anionic Surfactants Using 0.1% Cween 80 as Mobile Phase

	R_f Values		
Vitamins	S8	S9	S10
Folic acid	0.91	0.94	0.92
Cyanocobalamin	0.27	0.27	0.20
Thiamine	0.05	0.06	0.07
Ascorbic acid	0.95	0.98	0.97
Pyridoxine	0.66	0.66	0.61
Riboflavin	0.51	0.51	0.35

Table IV. RF Values of Vitamins on Silica Layers impregnated with Different Concentrations of SDS Using M4 Mobile Phase

	R_f Values				
Vitamins	S4	S5	S6	S7	S8
Folic acid	0.99	0.99	0.99	0.99	0.91
Cyanocobalamin	0.20	0.22	0.20	0.21	0.27
Thiamine	0.08	0.08	0.05	0.07	0.05
Ascorbic acid	0.99	0.99	0.99	0.99	0.95
Pyridoxine	0.71	0.68	0.70	0.62	0.66
Riboflavin	0.45	0.39	0.42	0.33	0.51

Table V. Effect of Nature of SDS Impregnated Different Adsorbents on the RF Values of Water Soluble Vitamins With M_4 Mobile Phase

	R_f Values		
Vitamins	S11	S12	S13
Folic acid	0.02	0.97	0.99
Cyanocobalamin	0.99	0.95	0.97
Thiamine	0.99	ND*	ND
Ascorbic acid	0.03	0.99	0.99
Pyridoxine	0.24	ND	0.92
Riboflavin	0.92	0.44	0.95

*ND = Not detected.

R_F values of vitamins. Amongst various concentrations of Cween 80 examined, 0.1% Cween 80 (M_4) was selected for further studies considering the detection clarity of vitamins on TLC plates. Cationic surfactant mediated stationary phase yields poorer separations, so to obtain better surfactant mediated stationary phase, anionic surfactants were used instead of cationic surfactants.

Anionic-nonionic TLC system

In order to investigate the mobility of vitamins, anionic (1% SDS, SDC, and DBS) surfactants were taken in stationary phase with 0.1% Cween 80 (M_4) mobile phase.

Results listed in Table III show that anionic surfactant mediated stationary phases yield better results in terms of differential migration of vitamins compared to cationic-nonionic systems. Folic and ascorbic acids migrate with the solvent front as evident from high R_F value (Table III). Conversely, thiamine shows high affinity for stationary phase (i.e., anionic surfactant mediated silica layer) and remain close to the point of application (R_F = 0.06). Interestingly, mid R_F values for pyridoxine and riboflavin favors their separations from all other vitamins like folic acid, cyanocobalamin, and thiamine. With this TLC system, vitamins follow the following order of decreasing order of mobility (i.e., R_F value) ascorbic acid > folic acid > pyridoxine > riboflavin > cyanocobalamin > thiamine. Thus, the present anionic-nonionic TLC system is more effective in discriminating various vitamins on the basis of their migration pattern. As a result, several combinations of separation of vitamins are available with this system. Among anionic surfactants, for further analysis of vitamins SDS was selected and its concentration for impregnation was optimized. For this purpose, mobility pattern of vitamins was studied at different concentration levels (0.00001 to 1%) of SDS in silica layer using M_4 mobile phase. From the results shown in Table IV, S_6 (silica gel impregnated with 0.01% SDS) was selected for further study for separation of vitamins. The scanning electron microscopic (SEM) images of none modified and SDS modified silica gel shown in Figures 1A–1B clearly demonstrate the altered surface morphology of silica phase on impregnation. Figure 1A shows that silica gel particles displayed a nearly smooth surface while Figure 1B shows impregnated silica particles displayed the rod shape-like surface. Therefore, the impregnated silica gel

Table VI. Effect of Different Nonionic Mobile Phases on the Separation of Vitamins Using S6 as Stationary Phase

	R_f Values				
Vitamins	M4	M6	M7	M8	M9
Folic acid	0.20	0.20	0.20	0.15	0.19
Cyanocobalamin	0.05	0.05	0.04	0.04	0.04
Thiamine	0.99	0.99	0.99	0.99	0.99
Ascorbic acid	0.70	0.67	0.65	0.65	0.68
Pyridoxine	0.42	0.37	0.38	0.31	0.37

Table VII. Separation of Vitamins in the Presence of Impurities on SDS (0.01%) Pre-Impregnated Silica Phase (S_6) Using Cween 80 (0.1%) (M_4) as Mobile Phase

	R_f Values				
Impurities	Cyanocobalamin	Thiamine	Folic acid	Pyridoxine	Riboflavin
SO_4^{2-}	0.16	0.05	ND	0.68	0.36
SCN^-	0.16	0.05	0.99	0.70	0.37
Cl^-	0.17	0.04	0.99	ND	0.38
NO_3^-	0.20	0.04	ND	0.64	0.37
Ba^{2+}	0.20	0.05	0.99	0.74	0.46
Co^{2+}	0.17	0.04	ND	ND	0.39
Cu^{2+}	0.19	0.06	ND	0.66	0.43
Zn^{2+}	0.18	0.05	0.99	0.66	0.44
Hg^{2+}	No separation				
Cr^{2+}	0.14	0.06	0.99	0.67	0.36
Li^+	0.18	0.05	0.99	0.72	0.41
Tl^{2+}	0.18	0.06	0.99	0.66	0.37
Ni^{2+}	0.18	0.05	0.99	0.61	0.39
Al^{3+}	0.09	ND	ND	ND	0.37
Th^{4+}	0.15	0.02	0.99	ND	0.39
Pb^{2+}	0.16	0.52	0.98	0.67	0.42
MTA*	0.19	0.06	0.99	0.67	0.34
DPA*	0.16	0.06	0.96	0.66	0.36
α-NTA*	0.22	0.05	ND	0.62	0.37
T-BTA*	0.22	0.07	0.91	0.68	0.51
T-n-BA*	0.23	0.07	0.94	0.63	0.43

* MTA = Methylamine DPA = Diphenylamine a-NTAA = -Naphthylamine
T-BTA = Tert-butylamine T-n-BA = Tri-n-butylamine

Table VIII. Detection and Dilution Limits of Some Vitamins Achieved With S6-M4 System*

Vitamins	Lower limit of detection (µg)	Dilution limit
Cyanocobalamin	0.08	$1: 1.25 \times 10^4$
Thiamine	0.05	$1: 2.0 \times 10^4$
Pyridoxine	0.5	$1: 0.2 \times 10^4$
Riboflavin	0.5	$1: 0.2 \times 10^4$
Folic acid	1	$1: 0.1 \times 10^4$

*Dilution limit = 1: (volume of test solution × 10^6) / [limit of detection (µg)]

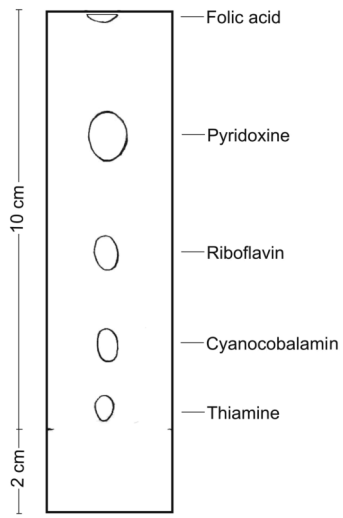

Figure 2. Separation of vitamins from their mixture (thiamine, cyanocobalamine, riboflavin, pyridoxine, and folic acid) on SDS impregnated silica layer (S6) and developed with Cween 80.

phase is effected to provide some unusual separations.

In order to broaden the scope of SDS impregnated stationary phase in TLC analysis of vitamins, certain commercially available sorbents (alumina, cellulose, and kieselguhr) impregnated with 0.01% SDS were tested as stationary phase for obtaining some new results. As expected, mobility trend of vitamins was strongly influenced by the nature of sorbent (Table V). High mobilities of cyanocobalamin, thiamine, and riboflavin and lowest mobility of folic acid or ascorbic acid were obtained on SDS impregnated alumina stationary phase. Ternary separations of vitamins can be realized on S_{11}. On SDS impregnated cellulose layer three vitamins (folic acid, cyanocobalamin, and ascorbic acid) migrate with the solvent front, riboflavin shows mid-R_F value and thiamine as well as pyridoxine could not be detected. Thus, binary separations can be obtained with this system. Poor results from the point of view of separation were obtained on kieselguhr as the entire vitamins move with the solvent front and thiamine was not detected, and hence no separation was possible. In conclusion, chromatographic system comprising of 0.01% SDS impregnated silica gel (S_6) as stationary phase and 0.1% Cween 80 (M_4) as mobile phase was identified most suitable for mutual separation of thiamine, cyanocobalamin, riboflavin, pyridoxine, and folic acid on single TLC plate (Figure 2). The formation of spots on chromatograms for folic acid were semi-circled whereas for other vitamins the spots appeared as oval-shaped. The substitution of Cween 80 by other nonionic surfactants (Cw 20, brij 35, TX-100, and Tw 20) as mobile phase yielded almost similar results in respect of mobilities of vitamins irrespective of the nature of nonionic surfactant (Table VI).

Separation in presence of impurities

To widen the applicability of developed TLC system S_6-M_4, the mutual separation of thiamine, cyanocobalamin, riboflavin, pyridoxine, and folic acid was examined in the presence of impurities (metal cations, inorganic anions and amines) as tabulated in Table VII. Folic acid could not be detected in the presence of Co^{2+}, NO_3^-, Cu^{2+}, Al^{3+}, SO_4^{2-}, and α-naphthylamine while the detection of pyridoxine was not possible in presence of Co^{2+}, Cl^-, Al^{3+}, and Th^{4+}. Al^{3+} interferes in the detection of thiamine. In the presence of Hg^{2+} five-component mixture of vitamins (folic acid, cyanocobalamin, thiamine, ascorbic acid, pyridoxine, and riboflavin) was not resolved into its components, and hence the separation is hampered. All vitamins co-migrate in the presence of Hg^{2+}.

Detection and dilution limits

The detection and dilution limits of some vitamins presented in Table VIII indicate that the proposed method is highly sensitive for detecting vitamins at trace levels.

Conclusion

The proposed novel micellar TLC system involving the use of impregnation of the stationary phase with an anionic surfactant, 0.01% sodium dodecylsulphate (SDS), and a nonionic surfactant, Cween 80, as the mobile phase has afforded good results for the separation of water-soluble vitamins. It is most suitable for the identification and mutual separation of thiamine, cyanocobalamin, riboflavin, pyridoxine, and folic acid from their five-component mixtures. The proposed method is sensitive for detecting vitamins at trace levels.

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