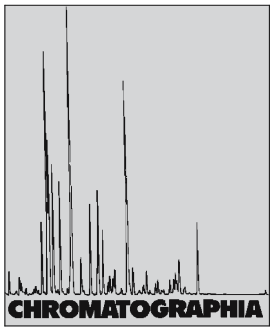
TLC Determination of Aliphatic Polyamines on Calcium Sulfate Layers



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Abstract

Biogenic polyamines are sensitive markers for various diseases including cancer. Polyamines are difficult to analyze by chromatography due to their high polarity and water-solubility so that derivatization is an essential step for their chromatographic analysis. Earlier studies have shown the efficacy of calcium sulfate ($CaSO_4$) as a TLC coating material for the separation of polar compounds. The aim of this study was to explore the potential of $CaSO_4$ for the analysis of aliphatic polyamines without derivatization. The TLC of six polyamines (ornithine, citrulline, putrescine, cadaverine, spermidine and spermine) was carried out on $CaSO_4$ and silica gel plates using 11 different mobile phases. The results showed that $CaSO_4$ is superior to silica for the separation of underivatized polyamines. The development time of the $CaSO_4$ plates was also about one-third shorter as compared to silica. Methanol was the only solvent to produce differential R_F values for the polyamines studied. Ornithine (R_F, 0–2) and citrulline (R_F, 1–3) were separated from cadaverine (R_F, 0.93), spermine (R_F, 0.85) and spermidine (R_F, 0.85). For quantitative analysis, the polyamines were eluted from the coating material scratched from the plate and the absorbance of the supernatant was measured at 550 nm. The limits of detection (LOD) and quantification (LOQ) were found to be 0.75 and 1.88 μg, respectively. The procedure was applied to the quantitative separation of polyamines in spiked human urine samples (12.5–50 μg). This is probably the first study reporting a TLC method for the separation of underivatized polyamines.

Keywords

Thin layer chromatography
Calcium sulfate layers
Separation of polar compounds
Biogenic polyamines

Introduction

Biogenic polyamines play a fundamental role in regulation of cellular growth and differentiation [1]. The induction of an early transient increase in polyamine metabolism often termed 'polyamine response' is an integral part of protective biochemical mechanisms. However, polyamine-mediated uncontrolled immune response may also exert deleterious effects on host cells [2]. The polyamine citrulline is produced by the reaction of ornithine and carbamoyl phosphate, catalyzed by ornithine transcarbamoylase whereas cadaverine is formed by the decarboxylation of lysine in the presence of lysine decarboxylase enzyme. The action of ornithine decarboxylase (ODC) converts ornithine into putrescine which is then converted into spermidine and spermine. Increased ODC activity and/or high polyamine levels have been reported in neurodegeneration [3, 4], trauma [5–7], ischemia [8] and brain tumors [9]. Polyamines accumulate in cancerous tissues and their concentration is elevated in the blood and urine of cancer patients [10]. A high concentration of spermidine in hepatocellular carcinoma tissue has been associated with recurrence of cancer after resection [11]. Stabellini et al. [12] reported higher levels of spermine, spermidine and putrescine in serum and erythrocytes of patients with breast, lung or colon cancers. Khuhawar et al. [13] observed a more than tenfold increase in the levels of cadaverine and putrescine in cancer patients as compared to healthy persons. High cytosolic citrulline levels have been reported in bladder cancer patients [14]. Polyamines are therefore regarded as sensitive biomarkers for cancer detection and assessing the success of therapy [10, 11, 15–18]. Determination of polyamines is also important for the diagnosis of urea cycle disorders [19] and assessment of food quality [20–23].

Polyamines are usually analyzed by high-performance liquid chromatography [24–26], gas chromatography [27],

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Table 1. Chemical formula and molecular weights of various polyamines studied

S. no.	Name	Chemical formula	Molecular weight
1	Ornithine	NH_2 $(CH_2)_3$ CH NH_2 COOH	132
2	Citrulline	NH_2 CO NH $(CH_2)_3$ $CHNH_2$ COOH	175
3	Putrescine	$NH_2(CH_2)_4NH_2$	88
4	Cadaverine	NH_2 $(CH_2)_5$ NH_2	102
5	Spermine	NH_2 $(CH_2)_3$ NH $(CH_2)_4$ NH $(CH_2)_3$ NH_2	202
6	Spermidine	NH_2 $(CH_2)_3$ NH $(CH_2)_4$ NH_2	145

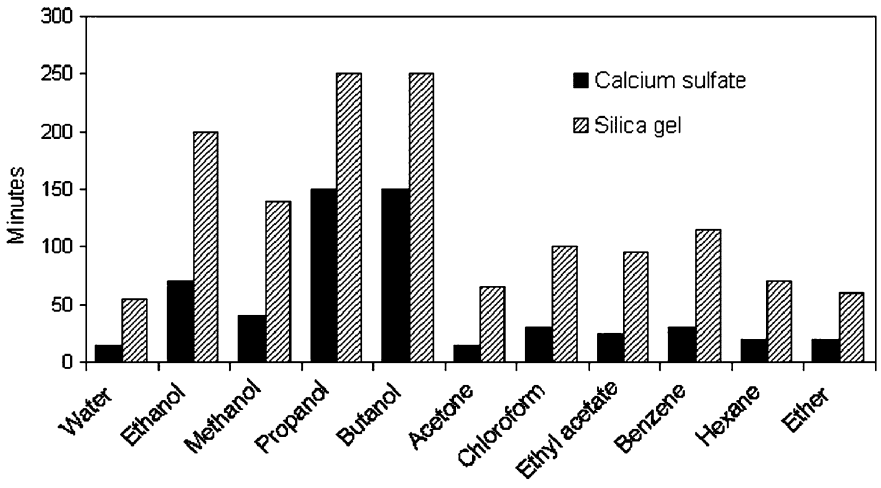

Fig. 1. Comparison of time taken by various solvents in developing TLC plates coated with calcium sulfate or silica gel

capillary electrophoresis [28, 29] and thin-layer chromatography [7, 18, 30]. However, due to their high polarity and water-solubility, they need to be derivatized prior to chromatography to avoid severe tailings and achieve a satisfactory separation. Linares et al. [31] separated dansylated putrescine, cadaverine, spermidine and spermine on silica-gel TLC plates followed by their sensitive detection using a fibre optic fluorescence instrument. Price and Gray [32] compared the suitability of dansyl chloride, dabsyl chloride and 7-chloro-4-nitrobenzoxazole for the derivatization of 14 biogenic polyamines for 2-dimensional TLC and concluded that dansyl chloride is the preferred reagent . On the other hand, Rustenbeck et al. [33] pointed out considerable errors in the quantification of dansylated polyamines due to their reactivity with the silica gel coating. Earlier we have shown that calcium sulfate ($CaSO_4$) is an excellent coating material for the separation of polar compounds by thin-layer chromatography [34, 35]. This investigation concerned the TLC potential of $CaSO_4$ for the separation and determination of aliphatic polyamines without derivatization.

Experimental

A Stahl apparatus was used to coat glass TLC plates (18 × 3 cm) with a slurry (100 g compound + 125 ml distilled water) of $CaSO_4$ or silica gel to give a uniform thickness of 0.25 mm. The plates were first allowed to dry at room temperature and then in an oven at 110°C for 1 h. Test solutions were spotted onto the plates with a fine capillary or micropipette. The solvent was removed by air drying and the plates were developed (10 cm from the origin) in glass jars (20 × 4 cm). All the polyamines were purchased from Sigma Chemical Company, USA. Anhydrous calcium sulfate and silica gel were purchased from Riedel, Germany and BDH, England, respectively. All other reagents including solvents were of analytical grade.

Serially diluted aqueous solutions of polyamines were applied onto the TLC plates and the spots were visualized by spraying with ninhydrin (0.2% alcoholic solution) followed by heating at 110°C for several minutes to ensure the plateau intensity of the colored complex. After standardizing the detection of polyamines on $CaSO_4$ coated TLC plates, six aliphatic polyamines (Table 1) were chromatographed on $CaSO_4$ and silica gel coated plates using different solvent systems (distilled water, ethanol, methanol, propanol, butanol, acetone, chloroform, ethyl acetate, benzene, hexane and ether). For tailing, the rear and front limits were measured (in centimeter) from the point of origin and the R_F values described as 'rear limit (centimeter) to front limit (centimeter)'. For compact spots, the R_F values were calculated using the formula, R_F = distance traveled by solute spot/distance traveled by solvent front.

For recovery analysis, equal volumes (15 µL) of different concentrations of polyamines (0.0125, 0.025, 0.05 and 0.1% solutions corresponding to 1.88, 3.75, 7.5 and 15 µg polyamine, respectively) were applied to the plates coated with $CaSO_4$. The plates were air dried and the spots visualized with ninhydrin as described above. The coating material was carefully scratched off the plates using a fine spatula and transferred to a 1.5 mL centrifuge tube and vortexed with 1 mL of distilled water. The tubes were centrifuged at 4,000 rpm for 2 min and the absorbance of the supernatant was measured at 550 nm against the reagent blank. The concentrations of the polyamines were determined from the respective standard curves.

To evaluate the application of this method for quantitative analysis of polyamines in biological samples, aliquots of fresh human urine (100 µL) were spiked with known amounts (12.5, 25 and 50 µg) of representative polyamines to mimic the condition of hyperpolyaminuria as seen in urea cycle disorders. The polyamines were added to the urine in the form of mixtures of citrulline and cadaverine or spermine and ornithine. The polyamine-spiked urine samples (20 µL) were spotted on the $CaSO_4$ plates, chromatographed with methanol and detected as described above. All the samples were run in triplicate and the results expressed as means and standard deviations.

Results

The results of the detection of polyamines on $CaSO_4$ coated TLC plates showed that the intensity of the colored spot was directly proportional to the concentration. The optimal durations of heating the TLC plates to achieve maximum color intensity for various polyamines were as follows: ornithine

(10 min), citrulline (7 min), putrescine (10 min), cadaverine (15 min), spermine (25 min) and spermidine (20 min). Heating for longer times did not cause deterioration of the color intensity. Polyamine solutions with a concentration of 0.006% were undetected and the lower LOD was found to be 0.75 µg. The colored spots of polyamine-ninhydrin complex were stable for more than 56 h on $CaSO_4$ coatings whereas their color gradually diminished on silica gel plates and the spots had completely disappeared after 56 h. The development time of $CaSO_4$ plates was about 2–3 times shorter than silica gel plates of the same thickness (Fig. 1).

The R_F values of polyamines on $CaSO_4$ and silica gel coatings in various solvent systems are shown in Table 2. Using distilled water as mobile phase, all the polyamines moved with the solvent front on $CaSO_4$ whereas four of the six polyamines showed massive tailing on silica gel. Among the alcoholic solvents, only methanol showed excellent potential for the TLC of these polyamines. The different movement of polyamines on $CaSO_4$ coatings using methanol as a solvent was successfully utilized for the separation of ornithine and citrulline from cadaverine, spermine and spermidine (Fig. 2). However, on silica gel coatings, methanol failed to produce differential movements of the polyamines. Other solvents including acetone, chloroform, ethyl acetate, benzene, hexane and ether were of no use for the TLC of these compounds on $CaSO_4$ or silica gel (Table 2).

The colored complexes resulting from the reaction between the polyamines and ninhydrin possess similar absorption spectra with the absorption maxima between 540–580 nm; ninhydrin on its own absorbed slightly in this region (Fig. 3). The standard curves for all the polyamines studied were linear although their slopes varied depending on the intensity of the specific colored complexes (Fig. 4). The results of recovery analysis showed that all the polyamines could be quantitatively eluted from $CaSO_4$ coatings using water as the solvent (Table 3); the limit of quantification was found to be 1.88 µg of polyamine. The results of quantitative separation of polyamine mixtures from the spiked urine samples are shown in Table 4.

Table 2. R_F values of polyamines on calcium sulfate and silica gel coatings using different solvents

Solvent (polarity, D)	Coating	R_F values[a]					
		Ornithine	Citrulline	Putrescine	Cadaverine	Spermine	Spermidine
Water (80)	$CaSO_4$	1	1	1	1	1	1
	Silica gel	1	1	7–10	3–9	0–8	1–9
Ethanol (24)	$CaSO_4$	0–1	0–2	0–10	1–7	0–4	0–9
	Silica gel	0–3	0–2	0–2	0–5	0–3	0–3
Methanol (33)	$CaSO_4$	0–2	1–3	1–4	0.93	0.85	0.85
	Silica gel	0–4	1–6	0–2	0–4	0–3	0–3
Propanol (20)	$CaSO_4$	0–2	0–2	0–10	0–8	0–2	0–5
	Silica gel	0–1	0	0	0–2	0	0–1
Butanol (18)	$CaSO_4$	0	0–2	0–10	0–4	0	0
	Silica gel	0	0	0	0	0	0
Acetone (21)	$CaSO_4$	0–1	0–1	0–8	0–1	0–1	0–3
	Silica gel	0–1	0	0–1	0–1	0–1	0–1
Chloroform (4.8)	$CaSO_4$	0	0	0–8	0	0	0
	Silica gel	0	0	0	0	0	0
Ethyl acetate (6)	$CaSO_4$	0	0	0–6	0	0	0
	Silica gel	0	0	0	0	0	0
Benzene (2.3)	$CaSO_4$	0	0	0–2	0	0	0
	Silica gel	0	0	0	0	0	0
Hexane (2)	$CaSO_4$	0	0	0	0	0	0
	Silica gel	0	0	0	0	0	0
Ether (4.3)	$CaSO_4$	0	0	0	0	0	0
	Silica gel	0	0	0	0	0	0

[a]For compact spots, R_F = distance traveled by solute spot/distance traveled by solvent front. For tailing, the R_F values described as rear limit (centimeter) to front limit (centimeter) measured from the point of origin

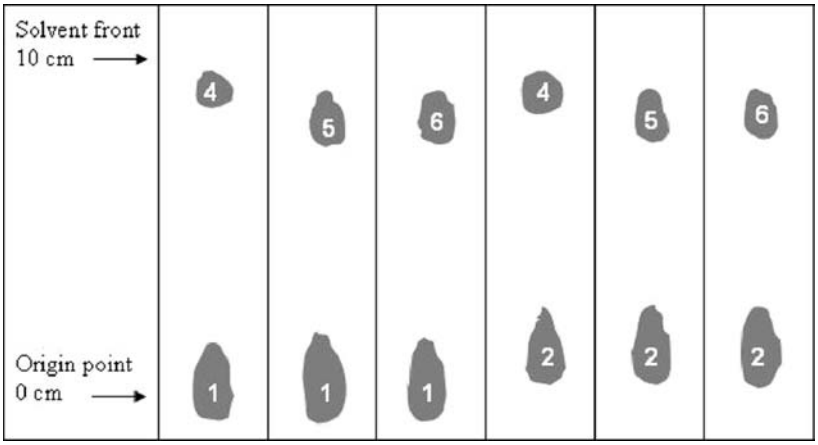

Fig. 2. Separation of ornithine (*1*) and citrulline (*2*) from cadaverine (*4*), spermine (*5*) and spermidine (*6*) on $CaSO_4$ coatings using methanol as mobile phase

Discussion

This study clearly showed that $CaSO_4$ coatings are more suitable than silica gel coatings for the detection and separation of aliphatic polyamines. We also tested the admixtures of $CaSO_4$ with silica gel (50%) or $BaSO_4$ (25 and 50%) but these coatings were not found to be as effective as $CaSO_4$ alone for the separation of polyamines. All the polyamines were successfully detected on $CaSO_4$ coated plates using ninhydrin reagent. Moreover the colored spots appeared to be permanent on $CaSO_4$ as compared to their transient stability on silica gel suggesting the superiority of the former material for quantitative work. These findings also suggest that $CaSO_4$ may also be a suitable stationary phase for the TLC of dansylated polyamines, which have been found to be unstable on silica gel plates [33]. Another advantage of $CaSO_4$ coatings was the rapid movement of solvents on these layers as compared to silica gel (Fig. 1). A longer development time using ethanol and propanol is in agreement with our earlier report [36].

Table 3. Recoveries of polyamine on $CaSO_4$ coated TLC plates

Amount applied (μg)	Amount observed (μg, Mean ± SD)						Recovery range (%)
	Ornithine	Citrulline	Putrescine	Cadaverine	Spermine	Spermidine	
1.87	2.13 ± 0.13	1.69 ± 0.19	1.32 ± 0.32	1.30 ± 0.13	1.86 ± 1.20	2.21 ± 0.50	69.3–117.8
3.75	4.12 ± 0.96	3.39 ± 0.41	3.31 ± 0.34	3.92 ± 0.97	4.23 ± 0.98	4.21 ± 0.97	88.3–112.8
7.50	6.97 ± 2.62	6.94 ± 1.23	5.95 ± 1.57	6.97 ± 1.53	7.46 ± 1.23	8.08 ± 0.63	79.3–107.7
15.00	15.95 ± 1.03	12.97 ± 0.58	13.06 ± 0.9	15.25 ± 1.00	13.16 ± 1.75	15.96 ± 0.68	86.5–106.4

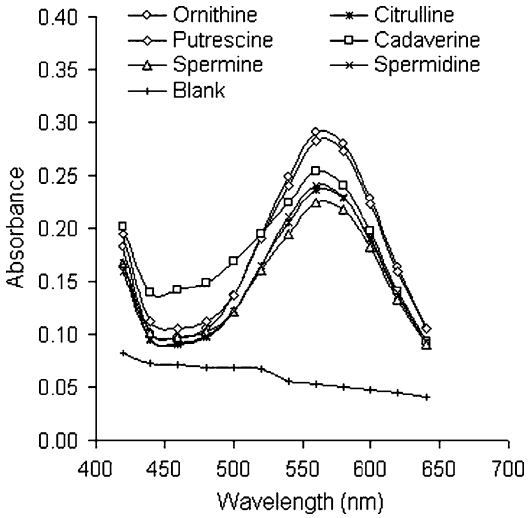

Fig. 3. Absorption spectra of various polyamines compared to blank

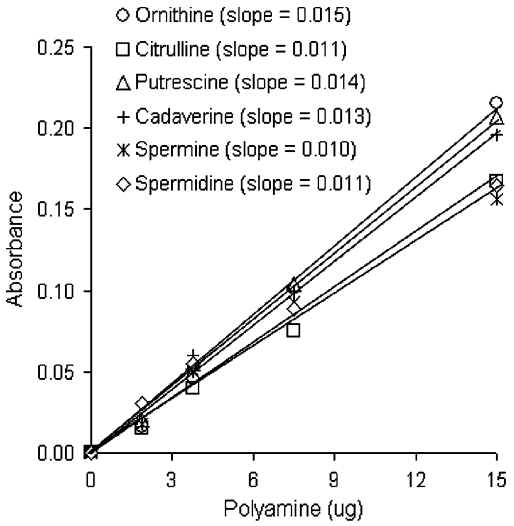

Fig. 4. Standard curves and their respective slopes for various polyamines

We used solvents of various polarities to study the movements of polyamines on $CaSO_4$ and silica gel coatings (Table 2). When water (dielectric constant = 80) was used as a solvent, all the polyamines moved with the solvent front on $CaSO_4$, apparently due to their high water solubility. On the other hand, only ornithine and citrulline showed R_F values of 1 on silica gel coatings with water as mobile phase whereas putrescine, cadaverine, spermine and spermidine had severe tailing (Table 2). This variation may be attributed to the presence of a carboxylic group in ornithine and citrulline that renders them more mobile on silica gel compared to the other polyamines. Furthermore, a high frequency of tailing of polyamines on silica gel point towards the high affinity (slow movement) of polyamines on silica. The solvents with dielectric constant ≤ 24 were found to be ineffective as all the polyamines either tailed or remained at the point of origin on both the coating materials (Table 2). Methanol (dielectric constant = 33) was the most effective solvent for the separation of polyamines on $CaSO_4$ coatings, but not on silica gel. The movement of the polyamines on TLC plates (Table 2) was not a function of molecular weight but might be associated with chemical structure (Table 1). The results of recovery analysis showed comparatively poorer recoveries of citrulline and putrescine than other polyamines (Table 3).

The chromatographic separations of polyamines on $CaSO_4$ coatings (Fig. 3) were successfully utilized for their quantitative analysis on spiked human urine samples (Table 4). The poor recoveries of citrulline and ornithine after developing the TLC plates may be attributed to their tailing as compared to compact spots of cadaverine and spermine (Table 2). The urinary excretion of total polyamines in normal individuals is low, amounting to only 5–10 mg per 24 h [37] whereas the concentration of urinary polyamines in patients with urea cycle disorders may be as high as 50 times the normal values. The excretion of citrulline has been found to be 500 mg/24 h in a patient with citrullinuria [19]. Thus, the LOQ (1.88 μg) of this procedure does not reach the sensitivity to measure the background levels of polyamines in unconcentrated urine samples from normal subjects, whereas the results of polyamines-spiked urinary samples (Table 4) suggest the application of this method for estimation of polyamines in patients with urea cycle disorders such as citrullinuria. Moreover, this procedure can also be used to determine polyamines in tumor tissue and leafy vegetables which contain high concentrations of polyamines. The levels of spermine and spermidine have

Table 4. Quantitative chromatographic separation of cadaverine from citrulline and spermine from ornithine in spiked human urine samples

Polyamine added to urine (μg)	Polyamines found in urine (μg, Mean ± SD) spiked with the mixtures of:			
	Cadaverine +	Citrulline	Spermine +	Ornithine
12.5	12.01 ± 2.82	9.40 ± 1.57	11.20 ± 1.40	12.60 ± 3.27
25.0	25.59 ± 3.77	19.02 ± 2.72	23.06 ± 4.04	21.33 ± 1.94
50.0	45.23 ± 2.08	41.27 ± 1.62	48.46 ± 1.36	43.53 ± 2.53

been reported to be 1,416 nmol g^{-1} tissue (286 μg g^{-1}) and 1,096 nmol g^{-1} (158 μg g^{-1}), respectively in experimental brain tumors [38]. The concentration of spermidine in leafy vegetables has been reported to be as high as 15 μg g^{-1} fresh weight [39].

In conclusion, $CaSO_4$ is a useful TLC coating material for the separation and determination of polyamines without derivatization. Although the procedure is not sufficiently sensitive to determine background urinary polyamine levels in normal human subjects it may be applied for screening of some inborn errors of amino acids metabolism resulting is a massive urinary excretion of polyamines. This procedure may also be used for the estimation of polyamines in tumor tissues and certain vegetables where higher polyamine levels are anticipated. Further studies are warranted to examine the usefulness of $CaSO_4$ as a stationary phase in HPLC columns to achieve better sensitivity, reproducibility, reliability and automation of polyamines assay in biological samples.

Acknowledgments

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