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ESTIMATION OF URINARY DIAMINES AND POLYAMINES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The dansylated derivatives of ammonia, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane, spermidine, histamine, and spermine were separated by one ascending development in chloroform–triethylamine (5:1) on a 250-μm silica gel 60 plate. Putrescine, cadaverine, spermidine, and spermine in human urine were quantitated by a direct scan of the fluorescent intensity of the spots corresponding to these compounds. Higher amounts of spermidine and spermine were found in the urines of cancer patients compared to the values of these substances in normal urine.

INTRODUCTION

Interest in the estimation of diamines and polyamines in physiological fluids has been stimulated by the reports of Russell *et al.*[1,2] that elevated levels of these compounds occur in the urine of patients with metastatic cancer. This finding has been confirmed by an automated procedure using an amino acid analyzer[3,4] and by gas–liquid chromatography[5].

More recently, Dreyfuss *et al.*[6] estimated urinary diamines and polyamines by thin-layer chromatography (TLC). Their method is based upon the hydrolysis of the urinary conjugates, extraction of the free bases, and conversion of the amines to fluorescent Dns (5-dimethylaminonaphthalene-1-sulfonyl) derivatives. These were separated by TLC on alumina, and were measured fluorimetrically after elution. These workers reported that the solvent system used for chromatography did not achieve good separation between the Dns derivatives of ammonia and putrescine, thus requiring that their samples be incubated with urease and aerated prior to hydrolysis in acid. The loss of amines reported to occur in their extraction procedure results in decreased sensitivity in the estimation of the amounts of these compounds.

Our protocol differs substantially from that used by Dreyfuss *et al.*[6]. Ammonia is largely removed by taking the sample to dryness in the presence of excess sodium bicarbonate. The solvent system used by us for chromatography permitted adequate resolution between the Dns derivatives of the amines and that due to residual ammonia. Extraction with isoamyl alcohol was omitted for the purpose of minimizing

losses. Quantitation was achieved by a direct scan of fluorescent intensity of the spots on the silica gel plate.

MATERIALS AND METHODS

Materials

1,3-Diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), α-(γ-aminopropylamino)-δ-aminobutane (spermidine), α,δ-bis(γ-aminopropylamino)butane (spermine), and β-aminoethylimidazole (histamine) were purchased as their crystalline hydrochloride salts from commercial sources. Dns chloride, 100 mg/ml in acetone, was purchased from Pierce (Rockford, Ill., U.S.A.).

Thin-layer plates (250 μm) precoated with silica gel 60 were purchased from E.M. Labs. (Elmsford, N.Y., U.S.A.).

Methods

Chromatography. Standard solutions of the amine hydrochlorides were made to contain 1 μmole/ml in 10^{-3} *M* HCl. Microlitre aliquots were added to 0.3 ml of saturated $NaHCO_3$ solution. Twenty-five microlitres of saturated Na_2CO_3 were added to yield a pH of 9.0 ± 0.5 followed by 0.5 ml of Dns chloride (2.7 mg/ml in acetone). Dansylation was allowed to proceed for at least 4 h or overnight in the dark at room temperature. Excess Dns chloride was converted to Dns proline by reaction with 10 μl of proline (200 mg/ml water) for 20 min. Acetone was removed by passing nitrogen over the samples (50° water-bath). The dansylated derivatives were extracted by mixing with 1 ml of benzene on a vortex mixer for 1 min. The samples were centrifuged at 484 *g* for 10 min, 0.8 ml of the benzene supernatant was transferred to 1.5-ml polypropylene tubes (Brinkmann, Westbury, N.Y., U.S.A.), dried under nitrogen (50° water-bath) and reconstituted to 80 μl with benzene. Microlitre aliquots were applied to silica gel plates activated for 1 h at 100°.

Separation of the dansylated compounds was brought about by ascending development for 14 cm in chloroform–triethylamine (100:20), a solvent mixture used by Seiler and Wiechmann[7] for the chromatography of other biogenic amines. The developed plate was sprayed with triethanolamine–isopropanol (2:8) to enhance and stabilize the fluorescence. The plates were dried *in vacuo* at room temperature for 3 h in the dark, followed by equilibration at atmospheric pressure for 30 min. Measurement of fluorescence intensity was performed with an Aminco Bowman Model 4-8202 spectrophotofluorimeter equipped with TLC scanner and XY recorder using wavelengths of 365 and 500 nm for activation and emission, respectively.

Analysis of urinary diamines and polyamines. Urine samples from hospitalized patients were collected and kept refrigerated over a 24-h period in the presence of sufficient concentrated HCl to maintain a pH of 1–2. The volume was measured, an aliquot was taken for creatinine determination, and a portion was frozen at −20° until analyzed. For analysis, samples were filtered through a 20-μm filter (Millipore HAWPO-2500; Bedford, Mass., U.S.A.) followed by hydrolysis of a small volume with an equal volume of 12 *N* HCl in a sealed glass ampul under nitrogen for 14–16 h at 110°. The contents were centrifuged at 2000 rpm for 10 min. Aliquots of hydrolyzed urine (0.4 ml) were dried *in vacuo* at 60° in centrifuge tubes adapted to the manifold of the rotary Evapo-Mix (Buchler Instruments, Fort Lee, N.Y., U.S.A.).

Saturated $NaHCO_3$ (0.3 ml) was added to the residue so as to yield a pH of 7.5–8.0 along with 10 μl of a 1/10,000 dilution of Antifoam A emulsion (Sigma, St. Louis, Mo., U.S.A.; A-5758). The samples were again dried on the Evapo-Mix. Drying was continued for an additional 5 min to remove ammonia. The residue was redissolved in 0.3 ml of water. Saturated Na_2CO_3 (25 μl) was added to yield a final pH of 9.0 $\pm$ 0.5. Dansylation and subsequent chromatography was carried out as described in the previous section.

Urine samples were always accompanied by a sample containing 5 nmoles each of putrescine, cadaverine, sperimidine and spermine which was subjected to the complete analytical procedure including chromatography on the same TLC plate. The amine concentration of experimental samples was estimated in terms of the height of the corresponding peaks in the standard sample.

RESULTS

Identification and resolution of diamines and polyamines

A series of standards in 6 *N* HCl was prepared starting with $(NH_4)_2SO_4$ and cumulatively adding 5 nmoles of 1,3-diaminopropane, putrescine, cadaverine, spermidine, histamine, and spermine. Each standard was chromatographed as described in Materials and methods, enabling each new peak to be identified with the compound added. A fluorimetric scan of all the compounds (Fig. 1) shows the peaks corresponding to the dansylated derivatives of ammonia, diaminopropane, putrescine, cadaverine, spermidine, histamine, and spermine. Peak 8 results from the hydrolytic cleavage of a side product of the dansylation reaction[8]. Diaminopropane and putrescine were only partly resolved but quantifiable. Histamine on the forward shoulder of spermidine does not interfere with estimation of the latter. Peak 8 while not characterized generally does not interfere with the estimation of spermine.

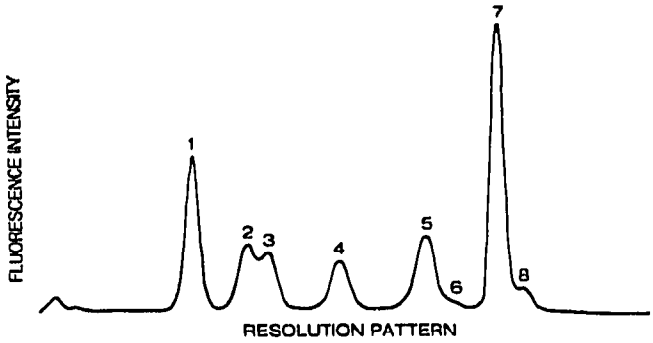

Fig. 1. Fluorimetric scan of the dansylated derivatives of: $(NH_4)_2SO_4$ (1), 1,3-diaminopropane (2), 1,4-diaminobutane (3), 1,5-diaminopentane (4), spermidine (5), histamine (6), spermine (7), and 1-dimethylaminonaphthalene-5-sulfonic acid (8) after ascending development in chloroform–triethylamine (5:1) for 14 cm on 250-μm silica gel 60.

Urinary diamines and polyamines

Chromatography. The dansylated derivatives obtained from 0.4 ml of pooled acid-hydrolyzed urine were co-chromatographed with the standard preparation used

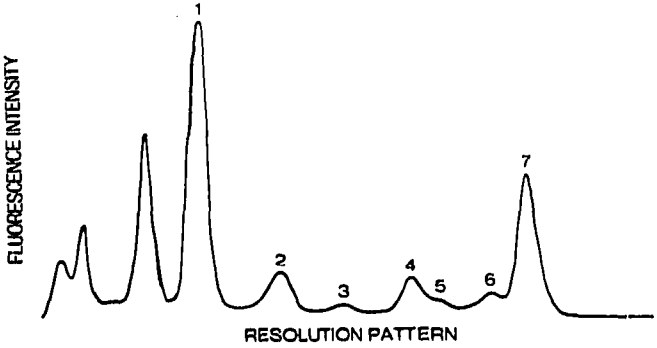

Fig. 2. Fluorimetric scan of the dansylated derivatives from human urine following TLC on 250-μm silica gel 60. One ascending development in chloroform–triethylamine (5:1) for 14 cm. NH_3 (1), putrescine (2), cadaverine (3), spermidine (4), histamine (5), spermine (6), and Dns-OH (7) are identifiable from co-chromatographed standard mixtures. Peaks preceding (1) were not characterized.

for Fig. 1. The fluorimetric scan (Fig. 2) shows that putrescine, cadaverine, spermidine and spermine may be readily identified and estimated from their peak heights. The chromatograms usually show one or more conspicuous peaks preceding Dns-amide (peak 1) in extracts prepared from urine. These have not been characterized, but their relative immobility in chloroform–triethylamine prevents interference with the more rapidly moving Dns derivatives of urinary diamines and polyamines. Standard amines added to the urine samples (spiking) and processed as described provided additional confirmation of the identity of the peaks shown in Fig. 2.

Estimation of proportionality. Varying volumes of a pooled urine sample after acid hydrolysis were analyzed as described under Materials and methods. The results

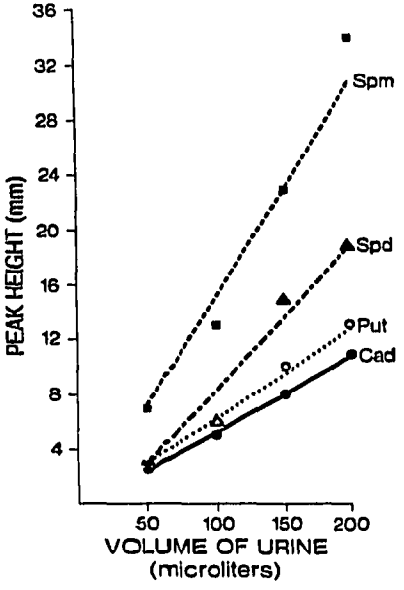

Fig. 3. Proportionality between peak heights of the dansylated derivatives of cadaverine (Cad), putrescine (Put), spermidine (Spd), and spermine (Spm) and the volumes of urine analyzed.

(Fig. 3) show approximate proportionality within the sample volumes used. However, the slopes of the curves relating peak height to the volume of sample do not go through the origin. This may reflect a small contribution to fluorescence from "streaking", *i.e.*, low background fluorescence in the direction of solvent migration as observed earlier by Dion[9].

Reproducibility of assay of urinary amines. Six 0.4-ml samples of the same pooled acid-hydrolyzed urine were subjected to the analytical procedure described under Materials and methods. The mean values and standard errors (μg/ml of urine) were: putrescine, 1.99 ± 0.13; cadaverine, 0.62 ± 0.04; spermidine, 5.08 ± 0.39; spermine, 0.75 ± 0.05 (*cf.* Table I).

TABLE I

REPRODUCIBILITY OF ASSAY

Compound	*Mean*	*Standard deviation (μg/ml)*	*Coefficient of variation (%)*	*Standard error*
Putrescine	1.99	±0.33	16.6	±0.13
Cadaverine	0.62	±0.10	16.1	±0.04
Spermidine	5.08	±0.96	18.9	±0.39
Spermine	0.75	±0.13	17.3	±0.05

Putrescine, cadaverine, spermidine and spermine in the urines of cancer patients. Urine samples from patients with neoplastic disease were analyzed as described under Materials and methods. The results are given in Table II.

TABLE II

PUTRESCINE, CADAVERINE, SPERMIDINE AND SPERMINE IN SOME CANCER URINES

Normal values[3] from the urines of ten individuals in mg per 24 h ± S.D. are: putrescine, 2.5 ± 0.6; spermidine, 2.4 ± 0.4; spermine, 0.4 ± 0.2.

Patient	*Diagnosis*	*Date*	*Putrescine (mg per 24 h)*	*Cadaverine (mg per 24 h)*	*Spermidine (mg per 24 h)*	*Spermine (mg per 24 h)*
T.W.	Malignant melanoma	8-13	1.28	0.20	1.31	0.30
		8-14	0.98	N.D.	7.63	2.22
		8-16	2.57	0.70	10.04	2.38
H.S.	Multiple myeloma	8-16	15.17	3.95	13.45	5.77
		8-17	4.73	1.96	9.10	2.90
		8-18	9.28	4.52	20.64	5.28
		8-19	9.50	2.91	14.43	3.41
M.M.	Multiple myeloma	8-13	3.85	0.90	8.20	3.69
P.M.	Multiple myeloma	8-13	2.46	1.08	10.33	5.74
B.E.	Advanced breast cancer	8-14	1.27	0.82	6.09	0.73
		8-15	2.50	0.90	6.91	1.63
		8-16	1.28	0.58	14.22	1.07
		8-17	0.68	0.62	4.79	0.86
		8-18	2.73	1.70	17.80	1.35

DISCUSSION

The higher amounts of spermidine and spermine found in the urines of cancer patients (Table II) over the values for these compounds in normal urine[3] are in agreement with earlier observations and those of Dreyfuss *et al.*[10] which are also based upon measurement of the dansylated derivatives of polyamines.

The reproducibility obtained suggests that the analysis of a sufficient number of replicate determinations of urine samples from a patient prior to treatment would allow estimation of the standard deviation for each of the diamines and polyamines under study. Saunders and Fleming[11] state that "the limits of ± 3 standard deviations can be taken as the approximate limits of error of a single result for $P = 0.95$. Deviations greater than that are not a random occurrence, but are a significant event.". It is worth noting that administration of chemotherapy (1,3-bis(2-chloroethyl)-1-nitrosourea; hydroxyurea; dimethyltriazinoimidazole carboxamide) to patient T.W. on 8-13 (Table II) was followed on subsequent days by a several-fold increase in the amounts of spermidine and spermine excreted in the urine over a 24-h period. The increases are judged significant ($P = 0.95$) in the light of the criteria noted above, and may be related to tumor cell death following the use of antitumor agents[12,13].

The greater sensitivity of the dansylation technique over that offered by the amino acid analyzer[14] is irrelevant in the case of urine samples, but may be helpful under those circumstances where the sample volume is very small, or the physiological fluid under study contains very low concentrations of polyamines.

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