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SIMPLIFIED MICRO-METHOD FOR THE QUANTITATIVE ANALYSIS OF PUTRESCINE, SPERMIDINE AND SPERMINE IN URINE

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SUMMARY

A simplified micro-method for the quantitative analysis of urinary polyamines is described. After acid hydrolysis of urine, the polyamines are converted to fluorescent 1-dimethylaminonaphthalene-5-sulfonyl (Dns; dansyl) derivatives and separated by means of thin-layer chromatography. Dns-NH_2, which has been reported to interfere with the determination of putrescine, is well separated from di-Dns-putrescine. Putrescine, spermidine and spermine are quantitated by in situ scanning of their fluorescent spots on the chromatogram.

The present method is both sensitive and reproducible. It eliminates a number of time-consuming steps and thus reduces preparative losses. Yet an adequate chromatographic resolution is obtained. Representative polyamine analyses of urine from normal volunteers and from cancer patients are reported. Elevated levels occur in the urines of pregnant women and of patients with various types of cancer.

INTRODUCTION

A relationship between polyamine levels in physiological fluids and cancer has been established. Thus, elevated levels of the polyamines putrescine, spermidine and spermine have been observed in cerebrospinal fluid of patients with central nervous system tumors [1—3], and in serum [4, 5] and urine [4, 6—10] of patients with many other forms of cancer. Present data suggest that determinations of polyamine levels in physiological fluids may provide a valuable test both as an adjunct to the diagnosis of cancer and for monitoring therapy.

Methods using thin-layer chromatography (TLC) [11, 12], gas chromatography [6] and automated high-pressure liquid chromatography [13—15] have been devised for the quantitative analysis or urinary polyamines. Several methods require tedious and time-consuming sample preparation as well as

lengthy analysis, whereas other methods require expensive instrumentation. The purpose of the present study was to develop a simple method for polyamine analysis involving a minimal number of preparative steps, thus facilitating the analysis of large numbers of clinical samples. The method described allows for minimal sample handling and rapid analysis with high sensitivity and reproducibility, and adequate separation of the polyamines from interfering compounds.

MATERIAL AND METHODS

Sample collection

Urine specimens from normal individuals (volunteers not suffering from acute or chronic disease) and from hospitalized patients with cancer (in an advanced clinical stage) were collected before the morning meal. Immediately after collection, two 10-ml aliquots were centrifuged at 1,000 *g* for 10 min at 2° to remove any cells that may have been present. Then the supernatants were analyzed for their creatinine (according to Løken [16]) and polyamine concentrations. All specimens were then stored at −25°.

Chemicals

TLC plates (20 × 20 cm) precoated with 250 μm silica gel 60 layers (Merck), and analytical grade reagents were used.

Polyamine analysis

Urine (1 ml) was hydrolyzed with an equal volume of concentrated HCl (12 *M*) in a PTFE-sealed test tube at 110° for 14—16 h. After acid hydrolysis, the sample was neutralized with solid Na_2CO_3 and centrifuged at 2,000 *g* for 5 min. A 200-μl aliquot of the supernatant was supplemented with 400 μl of Dns-Cl (30 mg/ml acetone). To the reaction mixture was added 100 μl of a saturated Na_2CO_3 solution and the mixture was sonicated for 2—3 h in an ultrasonic cleaner. The excess of Dns-Cl was converted to Dns-proline by reaction with 100 μl of the amino acid (250 mg of L-proline per ml). This reaction was quantitative after 3 min of sonication. The excess of Dns-Cl must be removed inasmuch as it is easily hydrolyzed by silica gel to 1-dimethylamino-naphthalene-5-sulfonic acid (Dns-OH), which causes blue-green fluorescent streaks on the chromatogram. The sulfonamides (Dns-amides) were extracted into 500 μl of toluene and the layers were separated by centrifugation. The principle for the dansylation technique has been described in detail by Seiler [17, 18] and by Seiler and Wiechmann [19].

TLC separation of Dns-amides

Aliquots (5—20 μl) of the toluene extracts were applied to pre-activated (110° for 1 h) silica gel 60 plates divided into 12 bands by scoring. A 25-μl Hamilton micro-syringe pipet with disposable PTFE tips was used for the application. Toluene was used as solvent since it does not move the Dns-amides chromatographically and thus results in small application spots [18]. Dansylated polyamine standards at 3 concentrations were included on each plate. These concentrations were selected so that they encompassed the range found

in the urine samples. The dansyl derivatives were separated in 1 h by one-dimensional ascending chromatography in chloroform—triethylamine (5:1) according to Seiler and Wiechmann [20]. After chromatographic separation, the plate was carefully sprayed with 20 ml of a solution of triethanolamine—propan-2-ol (1:4) according to Seiler and Wiechmann [21], and dried in vacuo for 16 h at room temperature in a desiccator containing silica gel as desiccant. This procedure considerably increased the fluorescence intensity and the stability of the Dns derivatives. Apparently triethanolamine reduces the adsorption of the dansyl derivatives to the polar and acidic silica gel and thus increases the quantum yield of fluorescence [17]. The removal of water and possibly some volatile quenching substances by desiccation may contribute to the increased fluorescence intensity [17]. To avoid quenching of the fluorescence by water adsorption during scanning, the plates were equilibrated for 30 min at room atmosphere.

Instrumentation

After equilibration the TLC plate was analyzed by fluorometry in situ in an Aminco-Bowman spectrophotofluorometer (model J4-8950) equipped with a TLC scanner and an XY recorder. This instrument uses two monochromators and therefore both the activation and emission spectra can be determined directly on the TLC plates. Thus valuable qualitative information about the separated substances is provided in addition to the quantitative information. The scanning accessory used, operates by measuring the reflected fluorescence light, at variance with the device used for example by Seiler [22] in which fluorescence measurements are achieved by transmittance. The excitation maximum was at 340 nm and the emission maximum at 505 nm for the dansylated polyamine derivatives. Scoring of the TLC plates allowed for perfectly linear development of the chromatograms, a pre-requisite for obtaining accurate results with the scanning device used.

RESULTS AND DISCUSSION

The present method shows that it is possible to analyze urinary polyamines quantitatively without previous concentration of the urine and without removal of ammonium contained in the urine. Furthermore, the method allows for minimal sample preparation, and rapid analysis with high sensitivity and reproducibility.

Reduced sample preparation was achieved by omitting several concentration steps as well as the extraction of polyamines (as free bases) into 1-butanol or isoamyl alcohol (3-methyl-1-butanol). These steps are not obligatory for the analysis of urinary polyamines. Furthermore, the removal of ammonia by urease treatment or evaporation in alkaline solution was found to be unnecessary and was omitted for the purpose of minimizing losses. Other workers, however, have not achieved adequate separation between the Dns-derivatives of ammonia and putrescine, thus requiring that their urine samples be either incubated with urease and aerated before hydrolysis in acid [11] or dried in the alkaline state [12]. However, with the present method, excellent separation between these two derivatives is obtained (Fig. 1). Recoveries of polyamines

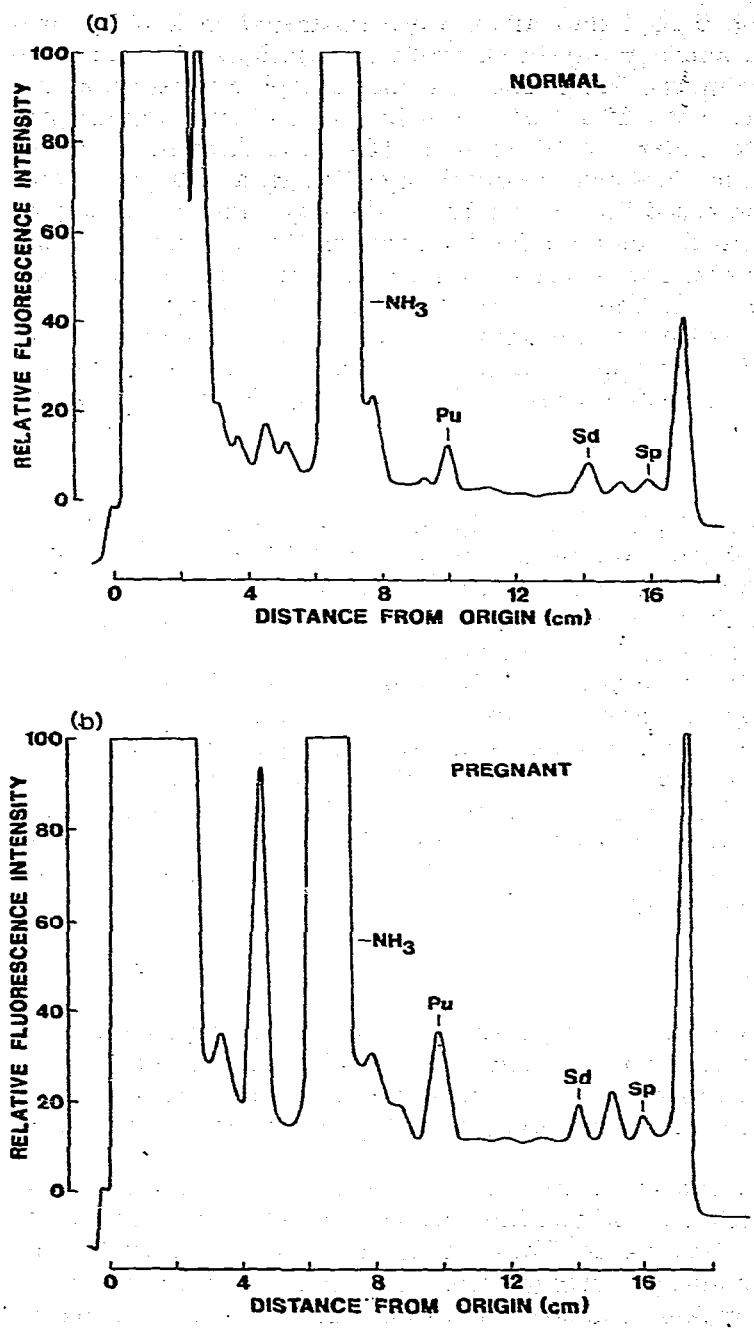
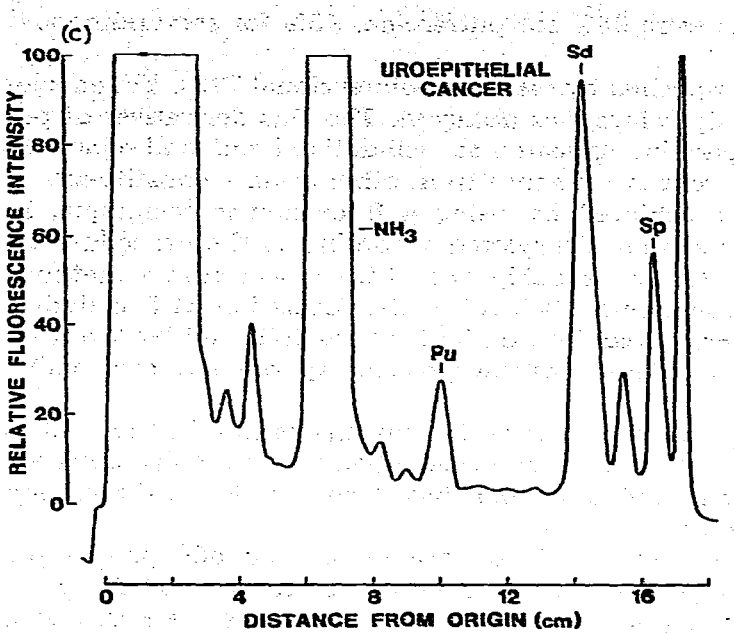
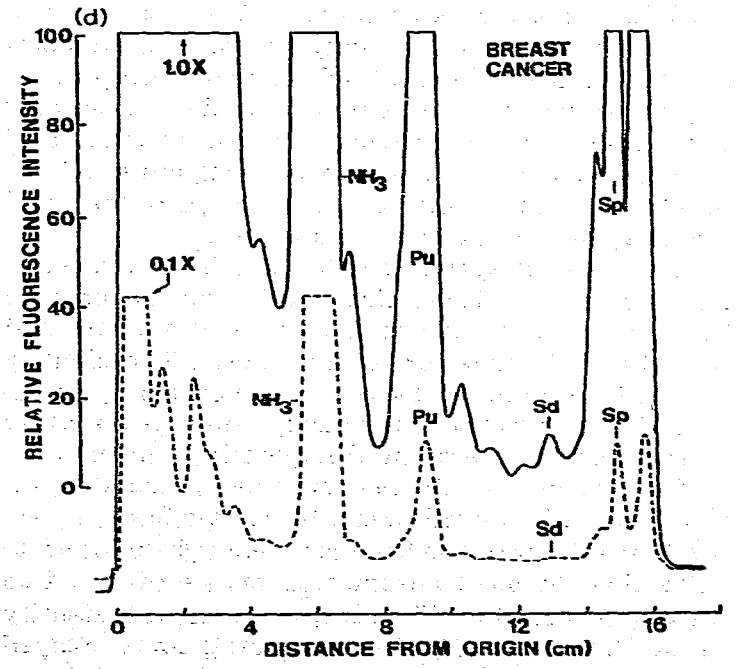


Fig. 1. Chromatographic separation of the dansylated derivatives of putrescine (Pu), spermidine (Sd) and spermine (Sp) contained in (a) urine from a normal female with a 5.8 m*M* creatinine concentration; (b) urine from a woman in the 8th month of pregnancy with a 6.1 m*M* creatinine concentration; (c) urine from a patient with uroepithelial cancer with a 5.4 m*M* creatinine concentration; and (d) urine from a patient with disseminated mammary carcinoma with a 6.8 m*M* creatinine concentration. The dotted curve (0.1x) represents a 10-fold damping of the normal deflection (1.0x).


(c)
UROEPITHELIAL CANCER
RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
0
$-NH_3$
Pu
Sd
Sp
0
4
8
12
16
DISTANCE FROM ORIGIN (cm)


(d)
BREAST CANCER
1.0X
0.1X
RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
20
0
NH_3
NH_3
Pu
Pu
Sd
Sd
Sp
Sp
0
4
8
12
16
DISTANCE FROM ORIGIN (cm)

added to the urine samples were 98% for putrescine, 95% for spermidine and 89% for spermine.

Rapid analysis was accomplished by using one-dimensional TLC. Yet an adequate separation of the polyamines was obtained. The Dns derivatives of putrescine, spermidine and spermine appeared as well-defined and well-separated spots and there was no apparent interference from other urinary constituents.

The high sensitivity was achieved by using a fluorometric technique, in which the urinary polyamines were dansylated according to the procedure described by Seiler [17—22]. This is probably one of the most sensitive methods for the determination of polyamines. It involves the formation of Dns derivatives of polyamines and their separation on TLC plates followed by the measurement of the fluorescence intensity of the spot corresponding to each amine by scanning in situ.

For obvious reasons, this method allows for the analysis of free as well as total (free plus conjugated) polyamines in urine. When the free polyamines are to be determined, the only modification that has to be done is the elimination of the hydrolysis and neutralization steps.

Linear calibration curves were obtained between 25 and 500 pmoles per spot. The coefficients of variation for the analysis of mixtures of the polyamines in this range (25—500 pmoles) were better than 10% (CV = S.D./mean, n=5). To obtain this reproducibility special precautions have to be observed at two steps of the procedure: first, the dansylated derivatives have to be applied without the TLC plate being touched with the pipette tip; and second, the TLC plate must be carefully and evenly sprayed with the reagent which increases the fluorescence intensity and stability of the Dns derivatives. When the relative merits of methods developed for the analysis of urinary polyamines are compared, it is apparent that the present method provides definite advantages over gas chromatographic techniques, which require tedious and time-consuming clean-up steps. Compared with automated ion-exchange chromatography, TLC spectrophotofluorometry appears to have the advantage of being more reliable in running. Furthermore, automated amino acid analyzers adapted for polyamine analysis are very expensive both to obtain and to maintain.

Figs. 1a—d show comparative chromatograms (similar creatinine values) of the separation of putrescine, spermidine and spermine in urine specimens from two normal individuals (a non-pregnant woman and a pregnant woman) and from two cancer patients (uroepithelial cancer or breast cancer). The identity of the putrescine, spermidine and spermine peaks was corroborated by inclusion of commercial products. The chromatograms usually show peaks other than those of putrescine, spermidine and spermine. Frequently, a peak is observed just before the peak of putrescine, which appears to be 1,3-diaminopropane. When added to urine it coincided with this peak. In no case has the peak which appears to be due to 1,3-diaminopropane been large enough to interfere with the quantitation of putrescine. In most chromatograms we observed an extra peak between spermidine and spermine. Thus far, however, the identity of this peak has not been revealed. Occasionally, the chromatograms displayed an additional peak near the peak for cadaverine. We have made no effort to identify positively the compound responsible for this peak. Only on rare occasions have these additional peaks interfered with the determination of putrescine, spermidine and spermine.

TABLE I

POLYAMINE CONCENTRATIONS IN THE URINE OF NORMAL INDIVIDUALS

Group	Putrescine	Spermidine (μg/mg creatinine)	Spermine*
Normal	2.9	2.0	0.5
	1.9	1.5	±
	2.4	1.9	±
	2.3	1.7	–
	3.9	2.7	1.1
	2.7	2.4	0.5
	3.3	1.9	0.6
	2.0	2.2	–
	4.1	2.6	0.9
	4.3	2.9	1.7
	2.5	1.8	±
	3.7	2.5	0.8
Mean ± S.D.	3.0 ± 0.84	2.2 ± 0.44	
Pregnancy**	5.1	1.8	0.6
	4.3	2.1	–
	3.5	2.5	0.7
	7.0	3.0	0.9
	3.7	1.9	+
Mean ± S.D.	4.7 ± 1.4	2.3 ± 0.49	
Postpartum, 3 days	3.6	2.4	–
6 days	2.9	2.6	0.8
9 days	2.3	2.1	–

* +, definitely detectable, but not quantifiable; ± just detectable; – not detectable

** Specimens were collected during the last month of pregnancy.

The concentrations of the polyamines were estimated with the present method in urine of normal individuals (Table I) and of patients with a variety of disease entities (Table II). Putrescine and spermidine were found in all urine samples analyzed whereas spermine was not detectable in some of the samples. Furthermore, we observed that pregnant women, in addition to patients with cancer in an advanced clinical stage, showed elevated urinary polyamine levels. However, it appears that only putrescine increases significantly during pregnancy. In cancer patients the spermidine and/or spermine concentrations were markedly increased, in addition to the putrescine concentration, when compared with the levels of these compounds in normal urine.

Elevated levels of the urinary polyamines have now been observed in a number of independent studies involving large numbers of patients [4, 6—10]. The results obtained have led to an increasing interest in extracellular polyamines and suggest a possible use of urinary polyamine levels as a clinical test in the diagnosis, management and follow-up of patients with cancer. To meet the need for a simpler, yet reliable method we have developed the present technique, which obviates some of the drawbacks noticed for similar analytical procedures.

TABLE II

POLYAMINE CONCENTRATIONS IN THE URINE OF PATIENTS WITH VARIOUS TYPES OF CANCER IN ADVANCED CLINICAL STAGES

All specimens were collected from patients with active disease in an advanced clinical stage, and always before treatment.

Diagnosis	Putrescine	Spermidine (μg/mg creatinine)	Spermine
Hematological tumors			
Acute lymphoblastic leukemia	31.5	24.0	4.2
Acute myelogenous leukemia	8.3	7.8	3.6
Hodgkin's disease	12.1	15.3	2.1
Lymphosarcoma	6.4	8.7	1.5
Reticulum cell sarcoma	11.9	10.2	6.6
Multiple myeloma	7.7	3.8	0.9
Solid tumors			
Undifferentiated small cell carcinoma (oat cell) of the lung	5.5	5.1	0.5
Mammary carcinoma	48.9	2.9	22.0
Transitional cell carcinoma of the bladder	4.6	20.7	8.9
Osteogenic sarcoma	7.1	4.8	1.1
Malignant melanoma of the skin	9.3	5.1	1.4

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