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DETERMINATION OF 5-DIMETHYLAMINONAPHTHALENE-1-SULFONYL DERIVATIVES OF URINARY POLYAMINES BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

NESBITT D. BROWN, R. BRICKLEY SWEET, JOHN A. KINTZIOS, H. DAVID COX and BHUPENDRA P. DOCTOR

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012 (U.S.A.)

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SUMMARY

A sensitive and specific method for the determination of diamines and polyamines by ion-pair high-performance liquid chromatography is described. The 5-dimethylamino-naphthalene-1-sulfonyl derivatives of putrescine, 1,6-diaminohexane, spermidine and spermine are separated on a μBondapak C_{18} reversed-phase column with 1-heptanesulfonic acid and acetonitrile as the mobile phase. All compounds are eluted within 30 min using a programmed solvent gradient system. The method has a lower detection limit of 1 pmole on column.

Because of the simplicity of the method, its application provides a better means for closely monitoring patients undergoing treatment for various types of genito-urinary neoplastic diseases.

INTRODUCTION

A recent review of the literature has indicated that many new analytical procedures have been developed during the last five years for measuring the concentration of diamines and polyamines in various types of physiological fluids [1—4]. However, much of the work reported in the literature uses techniques of previously developed methods. The combined use of high-performance liquid chromatography (HPLC) and fluorometry [5—7] has contributed much to these recent developments. At the same time, the original work of Seiler and Wiechmann [8], who used 5-dimethylaminonaphthalene-1-sulfonyl chloride to derivatize di- and polyamines, has improved significantly upon the sensitivity of this method.

Because of the refinements in the reported procedures, an increased interest

has developed among researchers to apply these new methods to problems pertaining to various diseases states involving polyamines.

In this report, we describe a relatively simple and specific ion-pair HPLC method for quantifying 5-dimethylaminonaphthalene-1-sulfonyl derivatives of diamines and polyamines in derivatized urine specimens. The purpose of this study is to offer to the clinician, an extremely sensitive method which may be used as a tumor marker technique in detecting and closely monitoring patients with various types of genito-urinary neoplastic diseases.

MATERIALS* AND METHODS

A Waters Model APC/GPC-204 liquid chromatograph was used throughout this study. The system consisted of two Model 6000A high-pressure pumps, a Model 660 solvent programmer, and U6K loop injector. An Aminco Fluoro-Colorimeter equipped with a 365-nm excitation and a 510-nm emission filter was employed for fluorometric detection. A Houston Instrument Omni-Scribe A5000 dual pen recorder and a Columbia Scientific Industries Supergrator-3 integrator recorded and integrated all peak areas. Spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) mixed with PIC B-7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, Mass., U.S.A.) was used in a gradient mode for separating the various polyamines. Standard solutions of each compound were prepared by using 99% putrescine, 99% spermidine, 97% spermine and 98% 1,6-diaminohexane (Aldrich, Milwaukee, Wisc., U.S.A.). Dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, 100 mg/ml, Pierce, Rockford, Ill., U.S.A.) was used to derivatize the urinary polyamines.

PROCEDURES

Dansylated polyamines were prepared as follows: Two hundred microliters of hydrolyzed urine, prepared as described below is pipetted into 13 mm × 100 mm silanized glass tubes. Twenty microliters of a 100 n*M*/ml solution of 1,6-diaminohexane (internal standard), 280 μl of 0.5 *M* carbonate buffer (pH 9.2), 100 mg of anhydrous potassium carbonate, and 500 μl of 10 mg/ml of dansyl chloride in acetone were added and thoroughly mixed. The tubes were sealed with parafilm and the samples were incubated in the dark at 54° for 60 min. At the end of the incubation time, the reacted samples were allowed to cool to room temperature. The dansylated polyamines were extracted into 1 ml of ethyl acetate. The samples were thoroughly mixed. Five μl of the ethyl acetate extract containing the dansylated polyamines were injected onto the column for analysis.

A prepacked 300 × 3.9 mm I.D. μBondapak C_{18} column was employed to chromatograph the dansylated polyamines. μBondapak C_{18} is a 10-μm particle size packing material, which is designed for both analytical and semi-pre-

*The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

parative separations. The mobile phase consisted of 0.02 *M* solution of 1-heptanesulfonic acid combined with acetonitrile. The PIC B-7 reagent was prepared by mixing 40 ml of the pre-package reagent with 460 ml of glass distilled water. The pH of the solution was 3.40. A concave gradient (curve No. 8, solvent programmer) was used to elute the various dansylated polyamines from the column. Curve No. 8 may be produced in any dual pumping chromatographic system by using the below formulae.

$$\% \text{ flow from pump B} = (FC - IC)\left(\frac{t}{T}\right)^m + IC \quad (1)$$

$$\% \text{ flow from pump A} = 100\% - \% \text{ flow from pump B} \quad (2)$$

where: FC = final concentration; IC = initial concentration; t = time into the run; T = time for the total gradient run; m = 3.00.

Gradient parameters were 50% acetonitrile and 50% 1-heptanesulfonic acid at zero time. Upon injection, the acetonitrile was increased from 50% to 80% within a 20-min period. Total analysis time was 30 min. Flow-rate for the dual pumping system was 2 ml/min. Column pressures ranged between 1200 and 1500 p.s.i. All separations were performed at ambient temperatures. 1,6-Diaminohexane was used as an internal standard. Each specimen was run in duplicate to ensure reproducibility. Peak areas were measured by an on-line computing integrator. The detection limit of the method was 1 pmole on column with a signal-to-noise ratio of 3 to 1.

SAMPLES

Urine specimens collected from 20 normal subjects and 85 patients undergoing therapy for a variety of urologic malignancies were used for this study. One-milliliter volumes of urine were mixed with an equal volume of concentrated hydrochloric acid and incubated at 100° for 14 h. Analyses were performed immediately or several days later.

RESULTS AND DISCUSSION

The importance of early detection in various types of neoplasm is difficult to overemphasize. The constant search by investigators to develop a simple and specific test to reveal the presence of neoplastic diseases, long before the clinical symptoms become apparent has always been a major goal of the modern clinician. It is the purpose of this report to investigate the usefulness of the urinary polyamines for developing such a test.

An ion-pair reversed-phase HPLC procedure was developed to separate polyamines in urine specimens from normal subjects and patients with known malignancies. From a series of standard solutions and experimental samples, the application of the new method is demonstrated by the chromatograms depicted below. Figs. 1 and 2 represent the separation of a standard solution containing the dansylated derivatives of putrescine, spermidine, and spermine. 1,6-Diaminohexane was incorporated into both the standards and experimental samples for normalizing the values obtained for each separation. The chroma-

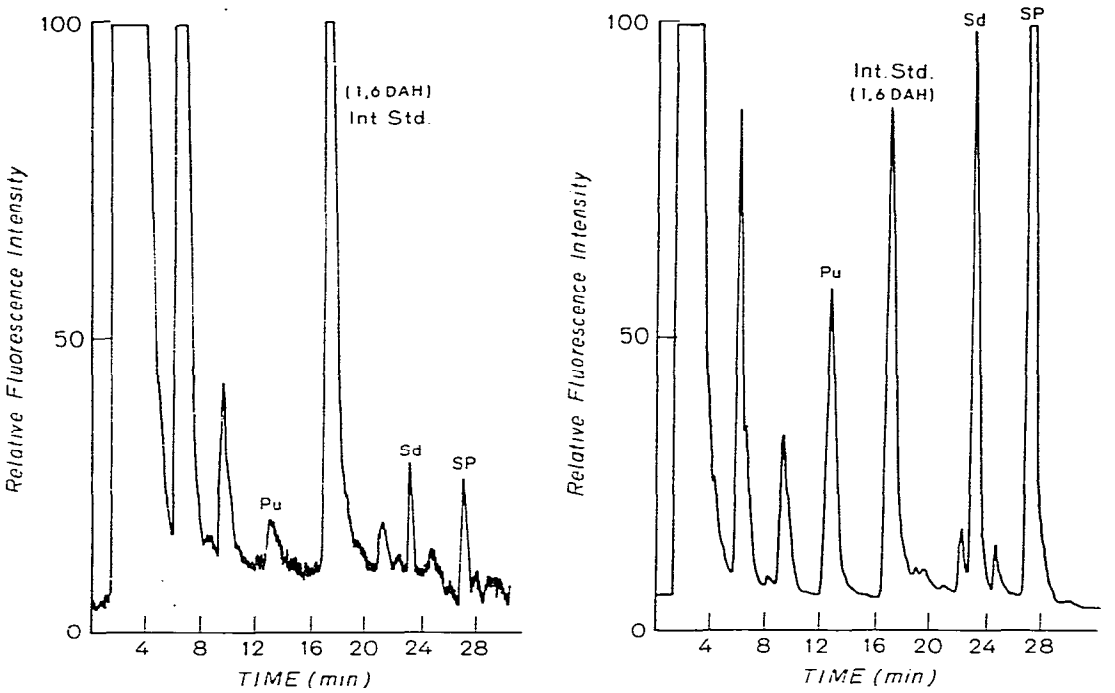

Fig. 1. Separation of a standard solution containing 1.10 pmole of putrescine (Pu), spermidine (Sd), and spermine (Sp). Column: 30 cm x 3.9 mm μBondapak C_{18}. Mobile phase: gradient mode 50% acetonitrile—50% 1-heptanesulfonic acid (zero time) 50%—80% acetonitrile (20 min), 2 ml/min. Internal standard: 1,6-diaminohexane (1,6DAH). Meter multiplier: 0.1.

Fig. 2. Chromatogram of a 100-pmole sample of polyamine standard detected at 365-nm excitation and 510-nm emission. Meter multiplier: 0.1. Abbreviations as in Fig. 1.

togram shown in Fig. 1 represents the lower detection limit of the method (1.10 pmole). A higher concentration (100 pmole) of the standard solution was also applied to the column to determine the optimum operational range of the method (Fig. 2). Linearity was observed for all concentrations of polyamines used in this study (25 pmole—1 nmole). The correlation coefficients for putrescine, spermidine, and spermine were 0.923, 0.961 and 0.942, respectively.

Based on these initial separations, urine specimens with polyamines values between 25–100 pmole were also analyzed. Figs. 3 and 4 are chromatograms showing the separation of dansylated polyamines prepared from the urine of normal and abnormal subjects. The normal urine samples were analyzed to establish baseline values for putrescine, spermidine, and spermine. From the results obtained, a 2—3-fold increase was noted between the control group and the experimental group. The results are shown in Table I.

In the patients with known carcinomas of the kidney, prostate, bladder and testis, a two-fold increase in the mean values of putrescine and spermidine were noted. At the same time, no appreciable differences were seen between the spermine values of the two groups. Sanford et al. [9] have suggested that a significant increase in the concentration of putrescine and spermidine in

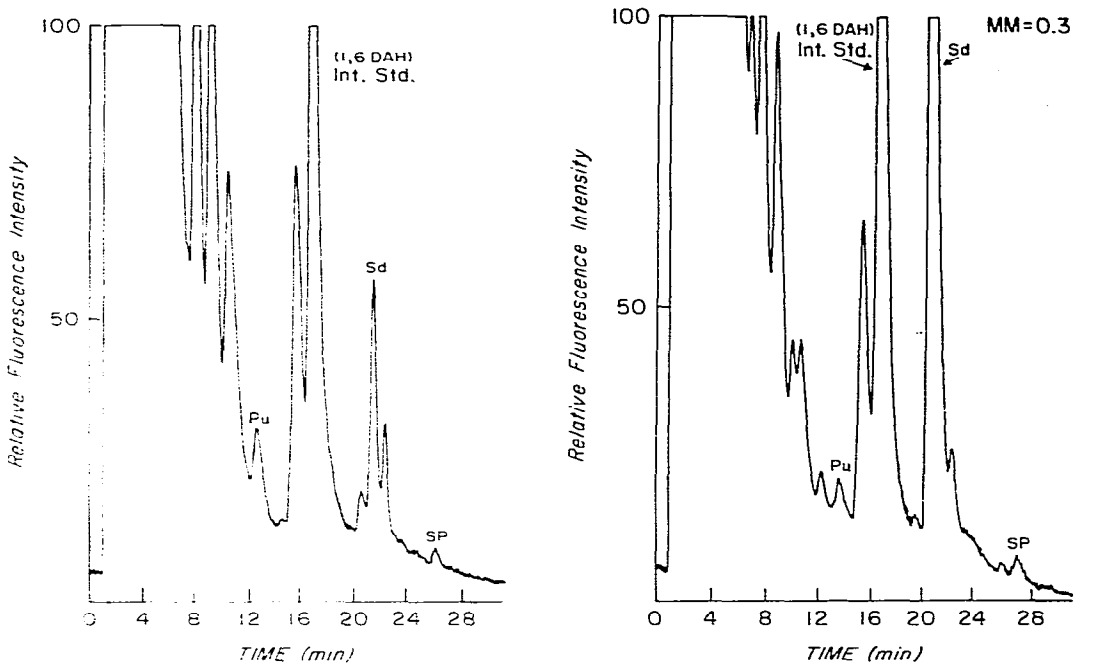

Fig. 3. Chromatogram showing a dansylated urinary polyamine specimen from a normal subject containing 1,6DAH (internal standard). Sample volume: 5 μl. Meter multiplier: 0.3. Column temperature: ambient conditions. Abbreviations as in Fig. 1.

Fig. 4. Chromatogram of a dansylated urine specimen from a carcinoma patient. Meter multiplier: 0.3. Abbreviations as in Fig. 1.

patients with genito-urinary diseases from that of the normal subject is a reliable indicator for use as a tumor marker.

Our HPLC technique is capable of accomplishing the parameters prescribed for making a distinction between normal subjects and patient with known genito-urinary carcinomas. Therefore, the use of this new analytical procedure holds great promise as a tumor marker technique. It achieves the degree of sensitivity and specificity desired in polyamine profiling which has been unattainable in earlier methodologies.

TABLE I

URINARY POLYAMINES VALUES (mg/24 h) OF 20 NORMAL SUBJECTS (NS) VERSUS 85 GENITO-URINARY CARCINOMA PATIENTS (CP)

	Putrescine		Spermidine		Spermine	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
NS	0.22—3.18	0.98 ± 0.49	0.26—18.66	4.57 ± 1.02	U.D.*—0.75	0.31 ± 0.09
CP	0.25—5.38	2.09 ± 0.70	0.45—47.96	9.24 ± 2.98	U.D.*—1.47	0.23 ± 0.13

*U.D. = Undetectable.

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