Title
Liquid chromatographic analysis of diamine and polyamines in biological samples

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Publication Date
1983

Peer reviewed|Thesis/dissertation

LIQUID CHROMATOGRPHIC ANALYSIS OF DIAMINE AND POLYAMINES IN BIOLOGICAL SAMPLES

by

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B.S., TAIPEI MEDICAL COLLEGE, 1979

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF CLINICAL LABORATORY SCIENCE

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

[redacted]

Date **University Librarian**

Degree Conferred: MARCH 20, 1983

ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. Pokar M. Kabra for his guidance and assistance in the development of this project. I am also grateful to Dr. Laurence J. Marton and Mr. Warren P. Lubich for providing various biological samples, and assistance in method comparison. I also wish to thank Dr. S. F. Nussenbaum for his encouragement and guidance during my graduate study.

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ABSTRACT

A sensitive and simple fluorometric method for the determination of polyamines in biological fluids by liquid chromatography is described. The polyamines, after derivatization with dansyl chloride, are first chromatographed on a Bond Elut C_{18} column, and then separated on a reversed-phase column with a gradient elution. The recoveries for putrescine, cadaverine, spermidine and spermine are 94%, 95%, 100% and 98%, respectively, when 5 µg/ml of each is added to hydrolyzed urine. The coefficient of variation of intraassay precision is as follows: 2.4 %, 2.5%, 3.2% and 4.1% for 1.97 µg/ml of putrescine, 0.85 µg/ml of cadaverine, 1.37 µg/ml of spermidine and 1.25 µg/ml of spermine, respectively; 1.91%, 1.62%, 2.98% and 4.00% for 18.31 ug/ml of putrescine, 17.31 µg/ml of cadaverine, 18.46 µg/ml of spermidine and 18.03 µg/ml of spermine, respectively. While the interassay precision is 2.6%, 4.5%, 2.3% and 6.0% for 1.81 µg/ml of putrescine, 0.34 µg/ml of cadaverine, 1.35 µg/ml of spermidine and 0.42 µg/ml of spermine, respectively; 2.0%, 1.9%, 1.6% and 3.0% for 18.09 µg/ml of putrescine, 16.50 µg/ml of cadaverine, 17.72 µg/ml of spermidine and 17.27 µg/ml of spermine, respectively. This method was compared with ion-exchange chromatography using a modified amino acid analyzer method for urine, tissue and CSF samples. The correlation coefficients between these two methods are as follows: 0.986 for urine putrescine, 0.971 for urine spermidine, 0.996 for tissue putrescine, 0.987 for tissue spermidine, 0.896 for CSF putrescine and 0.946 for CSF spermidine. Detection limits of 0.12, 0.11, 0.08 and 0.06

pmole are achieved for putrescine, cadaverine, spermidine and spermine, respectively. In interference studies, normetanephrine was found to have the same retention time as the internal standard. Although dextroamphetamine partially interfered with putrescine, it could be separated by slowing the gradient elution.

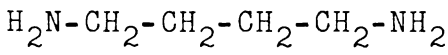
INTRODUCTION

Spermine was first isolated by Lewenhoeck in 1677 (1). Since this discovery, the naturally occuring polyamines, spermidine and spermine, and the related diamines, putrescine and cadaverine, have been the subject of diverse investigation. After the initial reports by Russell and coworkers (2,3) indicating that cancer patients excrete increasing amounts of the polyamines, much attention was focused on the determination of these amines in physiological fluids. Recently, it has been also reported that the polyamines may be useful indicators of the efficacy of chemotherapy and that their potential usefulness may extend to the early prediction of relapse (4-11).

Polyamines are ubiquitous in biological materials, although the relative amounts of putrescine, spermidine and spermine differ markedly in different cells (12). In general, prokaryotic cells have a higher concentration of putrescine than spermidine and lack spermine. Eukaryotic cells usually have little putrescine, and have spermine as well as spermidine (1). The structures of these compounds are shown in Figure 1.

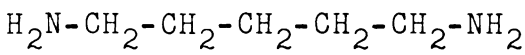
1. Biosynthesis

The first pathways for the polyamine biosynthesis were reported by Tabor and Tabor for E. coli (13). The primary precursors of the polyamines in both microorganisms and mammalian tissues are ornithine and L-methionine. The former is converted to putrescine by decarboxylation, while L-methionine is activated by ATP to S-adenosyl-L-methionine, which in turn donates the propylamino moiety for the synthesis of the higher



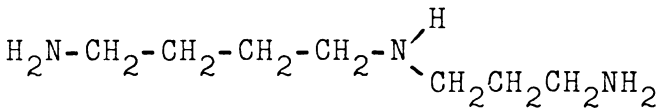
1,4-Diaminobutane

(Putrescine)

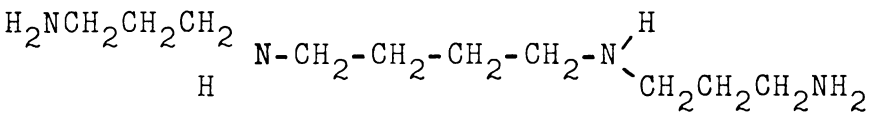


1,5-Diaminopentane

(Cadaverine)



Spermidine



Spermine

Figure 1: Structures of naturally occuring diamine and polyamine

polyamines. The pathway of biosynthesis (14-20) is shown in Figure 2.

In mammalian tissues, four enzymes are responsible for the synthesis of putrescine, spermidine and spermine: L-ornithine decarboxylase (EC 4.1.1.17), S-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) and two propylamine transferases (spermidine and spermine synthase). The first two enzymes catalyze the decarboxylation of L-ornithine and S-adenosyl-L-methionine, respectively. Spermidine synthase and spermine synthase catalyze the transfer of the aminopropyl group to putrescine and spermidine to form spermidine and spermine, respectively.

Numerous investigators have indicated that a dramatic increase in orthine decarboxylase activity take place during rapid growth and tissue proliferation (18). An increase of putrescine and spermidine usually parallels the enhancement of ornithine decarboxylase activity. When growth declines, the activity of ornithine decarboxylase decreases, suggesting a modulation of enzyme synthesis and a rapid turnover (18). Russell and Synder (21) used the rate of decline of ornithine decarboxylase after drug treatment to determine enzyme turnover and half-life, and showed that ornithine decarboxylase had an exceptionally short half-life; the shortest reported for a mammalian enzyme (18,21,22). This unique property of ornithine decarboxylase supports the view that it fulfills an important regulatory role (18).

Cadaverine, a diamine derived from lysine, is not involved in the same biosynthetic pathway as are the other polyamines but may be present in small quantities in normal urine (23). There

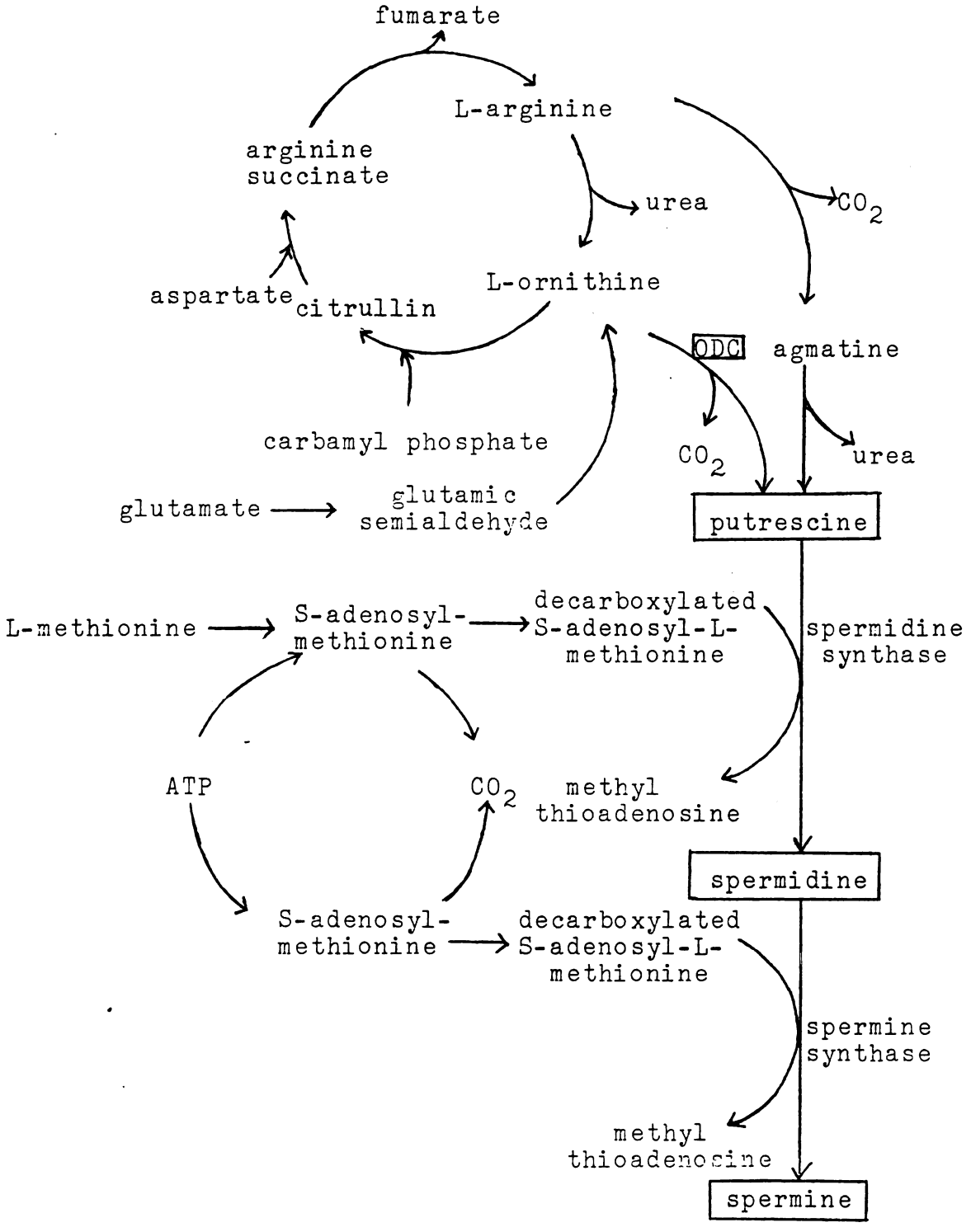


Figure 2: Biosynthetic pathway of the polyamines

is little experimental evidence to indicate that mammals have the capacity to synthesize cadaverine from lysine. It is speculated that the synthesis of this diamine is accomplished by bacterial flora and that it is then absorbed into blood and excreted in the urine (19).

Certain bacteria and higher plants have an alternative pathway for polyamine biosynthesis; the conversion of agmatine into putrescine by L-arginine decarboxylases which are absent in mammalian tissue (15,24).

2. Metabolism

The metabolism of polyamines involves oxidative and nonoxidative reactions. They are shown in Figure 3.

(A) Oxidative Pathway:

(a) Oxidation of Polyamines:

There are two polyamine oxidases involved in the oxidation of polyamines: plasma amine oxidase (EC 1.5.3.3) (or serum spermine oxidase) and tissue polyamine oxidase.

The mechanism of polyamine oxidation by plasma amine oxidase was studied by Tabor et al. (26). Plasma amine oxidase catalyzes the oxidative deamination of the aminopropyl groups of spermidine and spermine to the corresponding mono- and di-aldehyde metabolites. Under this catalysis, spermine yields both a mono- and a di-aldehyde which produces spermidine and acrolein by a non-enzymatic β-elimination reaction, or further oxidation of this di-aldehyde to form spermic acid, N,N'-bis(2-carboxyethyl)-1,4-diaminobutane. Spermidine yields a monoaldehyde metabolite which produces putrescine and acrolein by a non-enzymatic β-elimination reaction, or further oxidation of this metabolite to generate

putrescine and isoputreanine (25,27).

A second polyamine oxidase was recently discovered by Höltta" (41) in rat liver peroxisomes. This enzyme catalyzes the release of 3-aminopropionaldehyde from spermine and spermidine to produce spermidine and putrescine, respectively (14,15,25). A comparative study of tissue polyamine oxidase is lacking. From the limited data available, one obtains the impression that the enzyme is probably ubiquitous in mammalian tissues. Its activity is usually comparable to those of spermidine and spermine synthase so that it is unlikely to be rate limiting in polyamine catabolism (27).

(b) Oxidation of Diamines:

Putrescine is thought to be metabolized by two different pathways involving oxidative deamination by amine oxidase. In a nonmitochondrial pathway, i.e. in metabolic compartments not containing an appropriate aldehyde dehydrogenase such as the circulatory system, diamine oxidase (EC 1.4.3.6) oxidizes putrescine to 4-aminobutyraldehyde, which then undergoes cyclization to form Δ^1-pyrroline or is further oxidized to 4-aminobutyrate (GABA) (25,28). The other metabolic pathway (mitochondrial) involves the acetylation of putrescine to form acetylputrescine, followed by its oxidation by mitochondrial monoamine oxidase to N-acetyl-4-aminobutyrate and subsequent deacetylation to 4-aminobutyrate (15,25).

In addition to oxidative deamination, putrescine also can be hydroxylated to form 2-hydroxyputrescine, which is then oxidized to pyrrole by diamine oxidase (25).

(B) Non-oxidative pathway:

Putrescine, spermidine and spermine can be monoacetylated by a nuclear acetyltransferase (29). Recently, a cytoplasmic enzyme was also found to have the capability of acetylating polyamines (29,30). The monoacetyl derivatives of putrescine, spermidine (both 1-N-acetyl and 8-N-acetyl), and spermine have been found in human urine (31-34). In unhydrolyzed human urine, approximately 95% of the polyamines are present in the form of monoacetyl conjugates (14,35). 1-N-acetylspermidine, but not 8-N-acetylspermidine, has been also isolated from human serum (36,25). It has been suggested that polyamine acetylation is important in effecting or regulating elimination of polyamines from cells and in excretion of polyamines from the body (37-39).

Monoacetylputrescine has been shown to be an intermediate in the catabolism of putrescine in those organs which do not contain diamine oxidase (39). More recently, evidence had accumulated that 1-N-acetylspermine; N^1,N^{12}-diacetylspermine and 1-N-acetylspermidine were better substrates for polyamine oxidase than the nonconjugated polyamines (40,41). It was therefore proposed that the acetylation of spermidine may serve not only to produce an excretable derivative, but may be the first step in the degradation of polyamines and thus be essential in the regulation of polyamine turnover (40,19).

There are some other reports indicating that polyamine conjugates other than acetyl derivatives exist. Haddox and Russell (42) studied the polyamine content of nucleolar proteins (released by acid hydrolysis) and concluded that putrescine, spermidine and spermine are differentially conjugated to selective nucleolar proteins (42). Chan, Seale, Rosenblum and others (43-

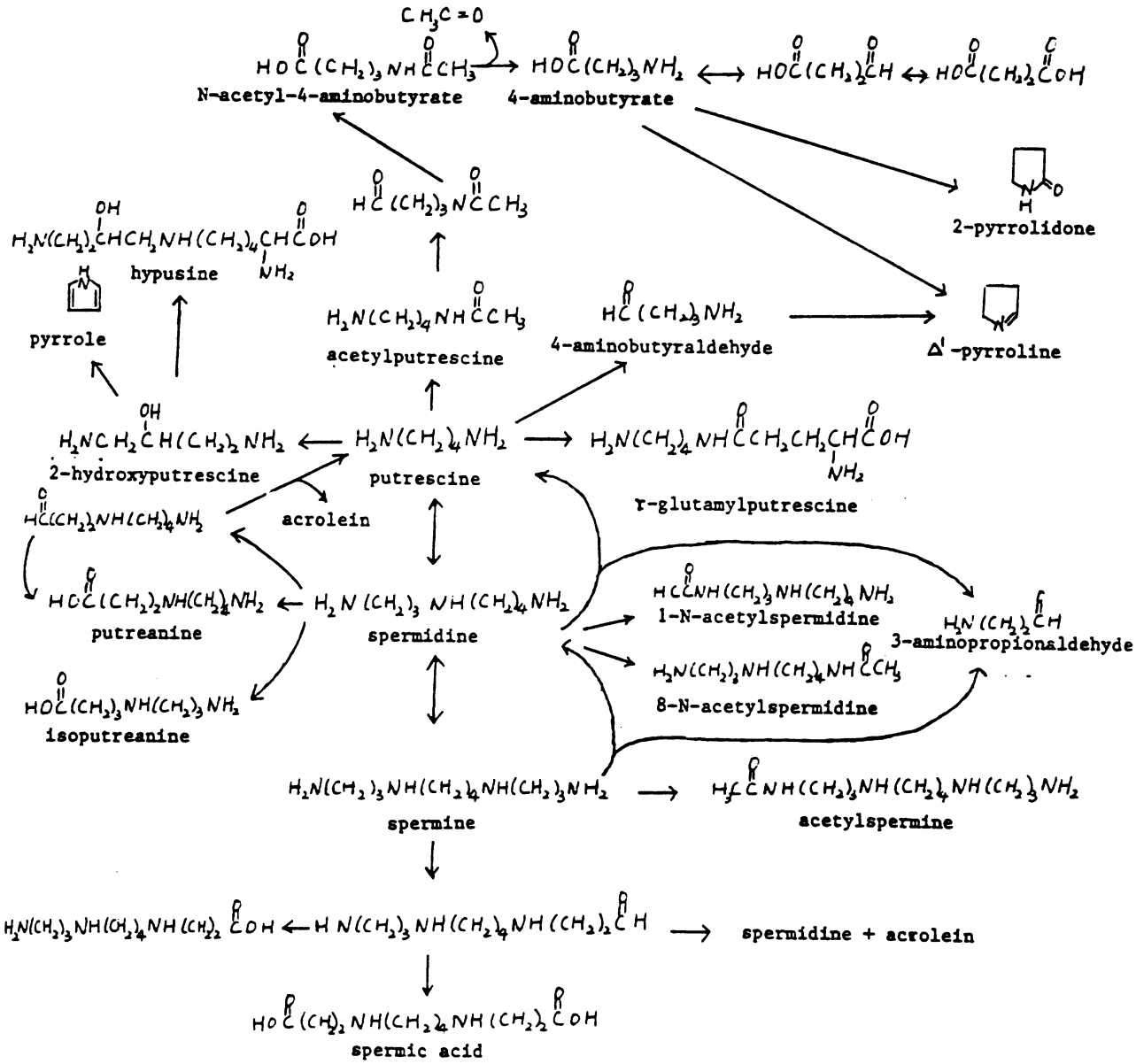


Figure 3. Polyamine Metabolism in Man and Higher Animals

46) reported the presences of putrescine-peptide and spermidine-peptide conjugates in plasma and amniotic fluid. Aigner-Held et al. (47) also showed the presence of Schiff base conjugates of pyridoxal phosphate with diaminopropane, putrescine, cadaverine, spermidine and spermine in healthy human urine.

3. Clinical Significance of Polyamines

In 1971, Russell et al. (2,3) reported that the urinary excretion of polyamines was higher in cancer patients than in normal controls or nontumor patients and suggested that polyamine excretion level could serve as a tool to monitor cancer and as a screening test for the early detection of cancer. These reports stimulated a flurry of investigational efforts to determine the relationship of polyamines in body fluids to etiology, diagnosis and therapy of malignant disease.

As mentioned earlier, polyamines are widely distributed in all living cells. The exact biological role of these compounds has not been fully elucidated, but these amines and their biosynthetic enzymes are elevated in neoplastic tissue, and in response to stimuli which produce growth in normal tissue (48). Therefore, it has been postulated that these amines play an important role in cellular metabolism related to cell growth (18,48,49), RNA and protein synthesis (17,18,20,48,49), etc. Increased intracellular polyamine concentrations are associated with increased rates of cell proliferation. Thus, not surprisingly, elevated polyamine levels are frequently found in the urine, serum and CSF of cancer patients (2,3,4,9,17,50-57). A good correlation between cancer and polyamine elevations has been found in patients with Burkitt's lymphoma, colorectal car-

cinoma, medulloblastoma, acute leukemia and multiple myeloma (16,19,23,50,58). Unfortunately, the increased polyamines are not entirely specific for cancer. Abnormal amount of polyamines are also present in a wide variety of disease states other than cancer (4,9,17,59-66,93). For example, pernicious anemia, hemolytic anemia (Coomb's positive), sickle cell anemia, rheumatoid arthritis, polymyositis, chronic obstructive lung disease, alcoholic cirrhosis, acute hepatitis, acute and chronic infections, psoriasis, cyctic fibrosis, systemic lupus erythematosus, polycythemia rubra vera, atherosclerosis, renal failure and viral carditis, etc. may all cause elevated urinary polyamine levels. The sensitivity of the polyamine assay as a tumor markers is also limited. Low sensitivity is found for a variety of malignancies, including cancers of breast, lung, stomach and pancreas, certain tumors of the central nervous system, and perhaps malignant melanoma (17).

In general, it seems that polyamine determinations have a minor role in the early diagnosis of cancer, although they appear to be more useful in monitoring the progression of known tumors (11,57). Polyamine determinations may be also useful for monitoring the cancer patient undergoing therapy or surgery. Variation in the levels of polyamines might suggest response to treatment, while unchanged levels might indicate resistance to therapy (9,14,17,62,95). Durie et al. (62) studied 124 patients with hematological and solid neoplasma and reported that an increase in urinary spermidine with sucessful therapy can be used as a marker of cell kill, and that the pretreatment (baseline) polyamine value can be used to evaluate a number of

tumor characteristics including tumor size, growth rate, and prognosis (62).

However, to date, absolute clinical utility for this assay has been demonstrated for only one use, monitoring CSF concentrations to predict the progression or response to therapy of medulloblastoma (10,11,76). Marton et al. (10) showed a good correlation between CSF polyamine levels and clinical status determined by neurological examination, radionuclide and computerized tonographic scans, myelography, and CSF cytology in 15 of 16 patients with medulloblastoma. In patients harboring medulloblastoma, elevated CSF polyamine levels are an accurate indicator of tumor progression and the earliest indication of recurrence (57).

ANALYTICAL REVIEW

1. Ion-Exchange Chromatography

This is a frequently used technique for the separation of polyamines. Automated ion-exchange chromatography has been widely explored and several methods based on the amino acid analyzer have been reported (50,67-76). The separation of the polyamines can be achieved by cation exchange chromatography due to the different molecular weights of the polyamines and the fact that their ionic charge can be influenced by pH and ionic strength of the eluting buffers.

Marton et al. (67) measured polyamines in serum and urine on an amino acid analyzer by forming ninhydrin derivatives. Amino acids were bound less strongly to the cation-exchange resin than were the polyamines. Thus they could be removed by first eluting with a buffer of lower ionic strength. polyamines were then eluted, derivatized, and detected by colorimetry at 570 nm. With ninhydrin as the detection reagent, the sensitivity was in the nanomole range. In 1975, Marton et al. (70) replaced ninhydrin with o-phthaldehyde. The sensitivity was incresed by 6- to 10-fold. With primary amines, o-phthaldehyde produces intensely blue fluorescence condensation products with an excitation maximum at 340 nm and an emission maximum at 455 nm. This method has a lower limit of detection of 3 to 6 picomoles of putrescine or spermidine and 12 to 15 picomoles of spermine. The assay was linear for the polyamines over the range of 8-200 pmol (70). In the updated version of this method, a modified amino acid analyzer is used to determine polyamine

levels in cerebrospinal fluid in addition to other biological fluids and tissue homogenates (77). These fluids are hydrolyzed with 6N HCl, lyophilized using a CVC concentrator (Savant Instrument, Inc.), reconstituted with 4% 5-sulfosalicylic acid, and injected onto a cation exchange column. The polyamines are eluted with a step-gradient of potassium chloride and citrate buffers. Post-column reaction with o-phthaldehyde yields a fluorescent derivative that is detected and quantitated (77). This assay is linear over the range of 50 to 1000 pmoles. The C.V. of within-day precision is 6.9% and 4.1% for putrescine and spermidine in CSF samples, respectively. Day-to-day precision is 10.7% and 8.2% for putrescine and spermidine, respectively.

Fluorescamine also forms a highly fluorescent product when reacted with primary amines (78). Greater sensitivity is obtained with fluorescamine than with ninhydrin (78,79). Veening et al. (71) used fluorescamine to assay for urinary polyamines. The sensitivity of fluorescamine for 1,3-diaminopropane and cadaverine was acceptable, however, the sensitivity for spermidine and spermine was less than that of ninhydrin. Possible reasons include interference of the secondary amino group with the fluorescamine reaction, or a quenching effect on the activation of fluorescence in the fluorescamine/polyamine complexes.

Adler et al. (72) described a method for the rapid determination of di- and polyamine in urine and whole blood. Samples were deproteinized with sulfosalicylic acid, hydrolyzed with barium hydroxide, neutralized with sulfuric acid, and left at room temperature overnight before analysis of the supernatant.

The polyamines were concentrated and separated from amino acids on a small bed of ion-exchange resin that then served to load the samples on a two channel, automated ion-exchange chromatography apparatus. As many as 100 samples could be analyzed in a 24-h period. The method was thought to be applicable to the analysis of urine and whole blood sample, but further development was needed for application to serum samples.

Gehrke et al. (73) reported a chromatographic method for the analysis of di- and polyamines using a Beckman Model 121M amino acid analyzer with ninhydrin detection. Linearity of of the ninhydrin color response was observed from 17 pmoles to 25 nmoles. Recoveries of 94-97% were achieved in day to day routine operation with a relative S.D. of 4 to 7%.

Recently, methods for the analysis of acetylated polyamines have been reported. Abdel-Monen and Ohno (31,32,94) suggested that the measurement of acetylated polyamines might be a more specific means of detecting cancer than total polyamine determinations. Mach et al. (76) separated free polyamines and their acetylated derivatives on Durrum DC-6A cation-exchange resin with an automated amino acid analyzer based on a stepwise elution with a sodium chloride-sodium citrate buffer system. Fluorescence detection used o-phthaldehyde. Reproducibility and sensitivity were excellent. However, the measurement of the acetylated and free polyamines using this method required two separate runs. The first run separated and measured acetylputrescine and the second, the combined acetylspermidine derivatives and the free polyamines. This method did not separate the two isomers of acetylspermidine; N^1- and N^8-acetyl-

spermidine cochromatographing as a single peak.

Prussak and Russell (96) determined urinary acetylated polyamines using a single-step high-performance liquid chromatographic method. This procedure utilized an ion-exchange column for the separation of the acetyl derivatives and the compounds were quantitated by fluorescence after reaction with o-phthaldehyde. No interference with the acetylpolyamine derivatives was noted. The two isomers of acetylspermidine were completely separated with 8-N-acetylspermidine eluting at 46.5 min and 1-N-acetylspermidine at 60 min. Total analysis time was 63 min and total run time was 74.5 min.

2. Liquid Chromatography

Liquid chromatography is a potentially useful alternative to the previously described techniques. It has the advantage of being faster, more cost-efficient and in some cases more sensitive.

Brown et al. (80) separated urinary polyamines with ion-pair high-performance liquid chromatography. The dansylated derivatives of putrescine, 1,6-diaminohexane (I.S.), spermidine and spermine were separated on a μ Bondapak C_{18} reversed-phase column with 1-heptanesulfonic acid and acetonitrile as the mobile phase. All compounds were eluted within 30 minutes using a programmed solvent gradient system. The detection limit of this method was 1 pmole with a signal-to-noise ratio of 3 to 1, and linearity was observed from 25 pmoles to 1 nmole. Recently, these investigators used ion-pair reversed-phase liquid chromatography to determine polyamines in red blood cell extracts (81). All compounds were eluted within 28 min and the

detection limit was 25 fmole.

Vandemark et al. (82) separated dansylated polyamines in urine by reversed-phase chromatography with a methanol and water mobile phase. The derivatized polyamines were eluted within 12 min and monitored at an excitation wavelength of 340 nm and an emission wavelength of 515 nm. 0.5 ng of putrescine, spermidine and spermine could be detected.

Seiler and Knödgen (83) determined blood putrescine, spermidine and spermine by reversed-phase chromatography. These compounds were converted to dansyl derivatives and separated on a Micropak CH-10 column with water and acetonitrile as the mobile phase. The sensitivity of the method is 30 pmoles.

Wagner et al. (84) used reversed-phase ion-pair liquid chromatography with dual detection for the simultaneous determination of the S-adenosyl methionine (SAM) analogues and the polyamines in tissue extracts. The separation was obtained with a gradient elution and by optimizing the concentration of octanesulfonic acid used as the ion-pair agent, and the ionic strength, the pH, and the acetonitrile content of the eluent. The SAM analogues were analyzed by UV detection at 254 nm and the polyamines by fluorescence detection after post-column derivatization with o-phthaldehyde.

Kneip et al. (85) utilized a cation-exchange chromatographic method using o-phthaldehyde and ultraviolet detection at 280 nm for the determination of free polyamines. Specimens such as reticulocytes, and heart muscle have been analyzed sucessfully using this chromatographic procedure.

Shipe and Savory (86) measured polyamines in plasma and

erythrocytes by HPLC with post-column derivatization with o-phthaldehyde. The polyamines were separated on an Aminco PA cation-exchange resin. Sensitivity of the method was less than 10 pmoles of each polyamine, and recovery averaged 93.9%. The precision of the analysis was 2% (R.S.D.). A good correlation was obtained with a reference chemical ionization gas chromatographic-mass spectrometric procedure.

Hayashi and Sugiura and their co-workers (87,88) measured urinary polyamines as tosylated derivatives on a column of Zipax Permaphase ETH by HPLC. An Amberlite IRA-410 (OH^-) column and a Dowex 50W-X8 (H^+) column were used for the pre-purification of polyamines from hydrolyzed urine samples. A gradient elution with acetonitrile and water was utilized to separate the tosylated polyamines. Detection was at 254 nm.

Samejima et al. (89) assayed the polyamines as fluorescamine derivatives in rat liver, human serum and urine. The samples were deproteinized and hydrolyzed after adding the internal standard. The resultant filtrates were subjected to CM-cellulose column chromatography, reacted with fluorescamine and then analyzed by liquid chromatography. It was possible to assay less than 100 pmoles of each amine with a R.S.D. of about 5%. In this method, the derivatives of putrescine and cadaverine and those of spermidine and spermine were analyzed separately because of insufficient resolution of the derivative of spermidine from that of cadaverine. Therefore, use of this method require a column chromatographic separation of putrescine and cadaverine from spermidine and spermine prior to reaction with fluorescamine.

Kai et al. (90) tried a rather unique approach. The polyamines, after clean-up by cellex P column chromatography, were converted to their fluorescamine derivatives in the presence of nickle ion which inhibited the reaction of interfering amines with fluorescamine. The derivatives were separated simultaneously by reversed-phase chromatography with linear gradient elution. The lower limits of detection were 10 and 5 pmoles for spermidine and the others in 0.5 ml of serum, respectively.

Recently, HPLC methods for the separation and quantitation of acetylpolyamines have been developed (91,92). Abdel-Monem and Merdink (91) measured monoacetylputrescine, N^1-AcSpd and N^8-AcSpd in urine separating the dansyl derivatives of the amines on a three column system. The first two columns used for isolation and the third for quantitation. The HPLC method of Seiler and Knödgen (92) utilized a single reversed-phase column for separation and detection of polyamines after forming ion-pairs with octanesulfonate. This method has the advantage of measuring both the free compound and the acetylpolyamine derivatives. However, the accurate measurement of urinary acetylputrescine was not possible due to the presence of an unknown interfering compound.

3. Gas-Liquid Chromatography

GLC was applied to the analysis of aliphatic diamines for the first time by Smith and Radford (97), using carbowax 20 M as a liquid phase and chromosorb W coated by KOH as a solid support (97,98). Gehrke et al. (99) performed GLC analysis of the polyamines involving an initial acid hydrolysis of the urinary sample followed by clean-up on cation-exchange resin

and conversion of free bases to their N-trifluoroacetyl derivatives.

Makita et al. (100) assayed polyamine as pentafluorobenzoyl derivative, using two GLC columns and election capture detectors. This method appeared to be very sensitive, but it failed to detect spermine. In 1978, Makita et al. (101) used Amberlite CG-120 cation-exchange resin to isolate the polyamines from interfering substances in hydrolyzed urine. The polyamines were then derivatized with isobutyl chloroformate and analyzed by temperature-programmed gas chromatography.

Beninati et al. (98) developed a method for the determination of putrescine, spermidine and spermine in animal tissue by GLC. The polyamines were extracted either from tissue homogenates or from standard solutions with butanol. Chromatography was performed without converting polyamines to their volatile derivatives on a GLC column prepared with Corning glass beads coated with 1% KOH and 4% Carbowax 20 M, with D-amphetamine and benzoamphetamine as internal standards. Beninati et al. (102) applied the same method, with some modification, to the analysis of polyamines in hydrolyzed urine. Distilled water was the final solvent injected into the GLC. The detector response was linear between 5 and 100 ng.

Rattenburg et al. (103) determined urinary polyamines using electron capture detection. Urine samples were hydrolyzed and purified on Porapak Q and Dowax 50X2 columns. Following evaporation of eluate, pentafluoropropionyl derivatives were made and analyzed gas chromatographically using temperature programming. This procedure gave a detection limit of 10 pg.

Polyamines extracted from urine could be measured accurately and reproducibly at the picomole level.

Recently, Bakowski et al. (8) described a method for the rapid estimation of the isobutyloxycarbonyl derivatives of plasma polyamines. Isobutyloxycarbonyl derivatives of putrescine, spermidine and spermine were analyzed on a mixed phase, 1.5% SE-30, 0.15% FFAP, column by temperature-programmed gas chromatography using a nitrogen-sensitive glass bead detector. The minimum concentrations detected in 1 ml of plasma were 10 pmol of spermidine and 20 pmol of spermine. Interfering peaks limited the level of detection of putrescine to 100 pmol.

4. Gas Chromatography-Mass Spectrometry

The GC-MS analysis of polyamines was first reported by Walle (104). Urinary polyamines were derivatized with trifluoroacetic anhydride and subjected to GC-MS. Each polyamine was identified by scanning the total ion current of the individual peak and comparing it with the mass spectra of reference compounds. Complete mass spectra could be obtained from as little as 50 to 100 ng of material eluted from the gas chromatographic column.

Smith and Daves (105) developed a GC-MS method, based on the technique of selected ion monitoring which utilized deuterated analogs as internal standards. This method allowed for the quantitative analysis of spermine, spermidine, putrescine and cadaverine levels as low as 1 pmole. The enhancement in sensitivity was accomplished by using a large excess ($\gtrsim$100 pmoles) of the deuterated analogs to improve chromatographic band profiles. Selected ion monitoring analysis using the high mass

$(M-CF_3)^+$ ions has the advantage of increasing specificity while decreasing interference from background ions. Subsequently, Smith et al. (35) applied this method to serum. After silica gel column clean-up serum polyamines were derivatized and subjected to chromatography. The trifluoroacetylated polyamines were chromatographed on a column packed with 3% OV-17 on 80/100 chromosorb W-HP. The trimethylsilylated polyamines were chromatographed on a column packed with 1.5% OV-101 on 100/120 Gas Chrom Q. Polyamines were quantitated from the peak height ratios of labeled to unlabeled polyamines from a standard curve.

Recently, Shipe et al. (106) developed an accurate and highly sensitive gas-chromatographic/mass-spectrometric procedure for determining polyamines in plasma and erythrocytes. Deuterium-labeled analogs of putrescine, spermidine and spermine were synthesized for use as internal standards. Trifluoroacetyl derivatives were detected with negative-ion chemical ionization/mass spectrometry combination with multiple ion monitoring. The limits of sensitivity ranged from 0.25 to 1.0 pmol of analyte injected into the instrument.

5. Thin-Layer Chromatography

Seiler et al. (35) reported the first separation of dansyl derivatives of biogenic amines by thin layer chromatography. Polyamines were separated on a silica gel G plate by two dimensional TLC and the chromatogram was viewed under UV light.

Dreyfuss et al. (107) separated dansylated polyamines by TLC on an aluminum G plate, then quantitated these derivatives fluorimetrically after elution. The two methods above were

interfered with by urea and ammonia, and neither method allowed for the separation of putrescine, spermidine and spermine with a single chromatographic system.

Gittins and Cooke (108) developed an assay, based on the same underlying principle, but in which problems previously mentioned had been overcome. Derivatized samples were applied to silica-gel 60 HPTLC plates and developed in a cyclohexane/ethylacetate (3:2, v/v) solvent system. Quantitation was with a Perkin-Elmer MPF-2A fluorescence spectrophotometer equipped with a thin-layer scanning attachment.

Fleisher and Russell (109) and Heby and Andersson (110) separated dansyl derivatives of di- and polyamines on a 250-µm silica gel 60 plate using chloroform-triethylamine (100:20) as a development solvent. The developed plate was sprayed with triethanolamine-isopropanol (2:8) to enhance and stabilize the fluorescence. The measurement of fluorescence intensity was performed with an Aminco Bowman Model 4-8202 spectrophotofluorimeter equipped with a TLC scanner and an XY recorder.

Seiler et al. (33) measured dansylated polyamines from urine by TLC. Urine was first applied to a Dowax 50W-X8 column for pre-purification, and then derivatized with dansyl chloride. The dansyl derivatives of acetylated polyamines were developed in a chloroform-tetrachloromethane-methanol (70:30:5) solvent system for 2 runs. Dansyl derivatives of free or total polyamines were developed in cyclohexane-ethylacetate (1:1) (2 runs) followed by cyclohexane-ethylacetate (3:2) (1 run). After development, the TLC plate was sprayed with a triethanolamine-propanol solution to enhance and stabilize fluorescence intensi-

ty, and quantitated with a Camag TLC scanner.

Bardoez and Karsai (111) combined thin-layer ion-exchange chromatography with video densitometry to determine polyamines in tissue extracts without any previous purification. Tissue extracts and standard solution were applied to Fixion 50X8 ion-exchange thin layer chromato-sheets (Na^+) for chromatography. After chromatography the sheetes were dried and developed with ninhydrin reagent containing cadmium acetate. The concentration of polyamine was then determined by measuring the density of the ninhydrin spots in a Telechrom-S OE 976 video densitometer.

6. Radioimmunoassay

Antibodies against polyamines were first prepared by Quash et al. (124), and used in radioimmunoassay techniques in 1975 (112). At present, fairly specific RIAs have been developed for spermidine and spermine by the Bartos and their coworkers (112,113). In the method of Bartos et al. (112), antispermine antibodies were produced by immunizing New Zealand white rabbits with thyroglobulin spermine conjugate, using multiple-site injection and macrophage-harvesting techniques. (3H) spermine, antiserum and serum are incubated at $4^{\circ}C$ for 2 hours. After incubation, labeled bound and free spermine are separated by charcoal adsorption and centrifugation. The supernatant containing sciltillation fluid was then counted for radioactivity. The values for spermine in known samples were read from standard curve which were constructed from the percentage bound (3H) spermine plotted in semilog fashion against the corresponding concentration of unlabeled spermine. A sensitivity of 200 pg was

achieved for human serum. This method is simple, rapid and very sensitive, but unfortunately the antispermine antibody cross-reacted with spermidine (22%), putrescine (1%), diaminopropane (6%) and cadaverine (0.16%).

Recently, Bartos et al. (35, 114) described a procedure for the purification of antipolyamine antibodies using affinity chromatography. Spermine-sepharose gel complex was used as an immunoadsorbent to isolate spermine antibodies. The cross reactivity was significantly reduced and the sensitivity of the assay increased from 1 to 0.05 pmol (35).

In 1978, Bartos et al. (113) reported a method of radioimmunoassay for spermidine in human serum. The specific antispermidine antibodies were produced following immunization of New Zealand white rabbits with a spermidine-thyroglobulin conjugate of high (>200:1) hepten-carrier molar ratio. The specificity of this antibody was tested against cadaverine, putrescine, spermidine, monoacetylputrescine, N^8-acetylspermidine and other compounds of similar molecular structure. Negligible cross-reactivity was observed with putrescine (2.1%), cadaverine (0.6%), spermine (0.3%) and diaminopropane (0.1%), and no cross-reaction was found with monoacetylputrescine, N^8-acetylspermidine, histamine, L-lysine and L-ornithine. The assay requires 20 µl of serum and has pmol sensitivity. The coefficient of variation for intraassay precision was 12.8% with a mean value of 0.29 nmole/ml. Interassay precision was found to be 16.2% with a mean value of 0.37 nmole/ml.

7. Enzymatic Methods

Bachrach and Oser (115) developed an enzymatic method for

the assay of 0.05 to 0.40 μmole of spermidine. They used dried Serratia marcescens cells to oxidize spermidine to 1,3-diaminopropane and Δ^1-pyrroline. Δ^1-Pyrroline was then reacted with o-aminobenzaldehyde to produce 2,3-trimethlene-1,2-dihydroquinolium which is yellow and has an absorption maximum at 435 nm. Subsequently, Bachrach and Reches (116) described another method for the assay of combined spermidine and spermine based on the oxidation of polyamine by serum amine oxidase followed by addition of N-methyl-2-benzothiazolone hydrazone hydrochloride to form a colored product.

Unemoto et al. (117) described a fluorometric method for the estimation of spermidine and spermine based on the finding that one of the oxidation products of spermidine and spermine when reacted with beef plasma amine oxidase further reacted with resorcinol to produce a fluorescent compound with an absorption maximum at 520 nm.

Harik et al. (118) developed an enzymatic isotopic microassay procedure for the measurement of putrescine. This method depended on the decarboxylation of S-adenosyl-L-{carboxy-^{14}C}methionine by Baker's yeast (Saccharomyces cerevisiae) S-adenosyl-L-methionine decarboxylase in the presence of varying amounts of putrescine. The quantity of $^{14}CO_2$ evolved was a linear function of the amount of putrescine present. Recently, Harik and Marton (119) used this method with minor modification to determine CSF putrescine, and compared it with a chromatographic method using an amino acid analyzer. It was found that putrescine levels obtained by the enzymatic-isotopic method correlated relatively well with free putrescine level, but

grossly underestimated the results obtained for total putrescine values.

Braganca et al. (120) described a micromethod suitable for the assay of polyamines in concentration of 2 to 30 nmole. This method is based on the oxidation of polyamines by purified fractions of crude L-amino acid oxidase from Russell's viper venom. Using a combination of two enzyme fractions, one which oxidized polyamines and amino acids and another which oxidized only amino acid, the technique could accurately determine polyamine concentrations in extracts of sera which may not be free of amino acids.

Matsumoto et al. (121) developed a new photometric assay for total diamines and polyamines in human urine using soybean seedling amine oxidase (SSAO) as an enzyme reagent. The amines were purified from urine by cation-exchange chromatography and incubated with SSAO. Hydrogen peroxide formed in the oxidase reaction was measured photometrically by coupling 4-aminoantipyrine with phenol in the presence of peroxidase.

8. Electrophoresis

Russell et al. (3) measured polyamines with electrophoresis. Urine sample was hydrolyzed and extracted by butanol. The butanol layer was evaporated to dryness and the residue was redissolved in 0.2 ml of 0.1 N HCl. A 10- to 50-ul aliquot of this solution was subjected to high-voltage paper electrophoresis at 80 V/cm for 2 hours, and the amines were quantified after staining with ninhydrin reagent.

Lipton et al. (4,52,58) described a similar method for polyamine determination. After hydrolysis, the sample was extracted

and redisolved in 0.1 N HCl, spotted on Whatman 3 MM chromatography paper and subjected to high voltage electrophoresis for 1.5 hours at 3500 V using a Savant Flat Plate electrophoresis system. The paper was stained with ninhydrin and eluted for 30 min in 5 ml of a 1 H_2O : 5 glacial acetic acid : 4 ethanol-Cd acetate (2 mg/ml) mixture and quantitated using a Beckman spectrophotometer at 505 nm.

High-voltage paper electrophoresis is time-consuming, rather insensitive and nonspecific. Spermine values measured after staining of the electrophoretograms with ninhydrin were considerably higher than those obtained with other methods (9,122). An as-yet-unidentified urinary constituent co-migrated with spermine under the conditions of the electrophoretic separation. This method had little importance for the routine assay of urinary polyamines and had been abandoned for years. Recently, Fujita et al. (123) reported an improved method for high-voltage electrophoresis of urinary polyamines. They eliminated interfering substances from spermine by cation-exchange column chromatography instead of with butanol extraction. The polyamines were then separated by high-voltage paper electrophoresis, reacted with ninhydrin, and detected with a dual-wavelength thin-layer chromatography scanner by the zigzag scanning method. They were able to completely separate putrescine, cadaverine, histamine, spermidine and spermine. However, the sensitivity of assay (0.6 to 0.8 nmole of each polyamine) was still inadequate for use in blood or CSF analysis.

9. Comment

Most of the methods here are complicated and required tedi-

ous sample preparation or highly specialized equipment. At present, ion-exchange chromatography with an amino acid analyzer is frequently used for the analysis of polyamines in physiological fluids, but the long analysis time required and the cost of instrumentation limit the use of this technique for routine assay. HPLC has been used sucessfully for the assay of urinary polyamines, but have thus far been inadequate for CSF levels, the one fluid for which a clear-cut clinical application exists.

This thesis describes a simple, rapid, precise, and highly sensitive liquid chromatography method which correlates excellently with a tested amino acid analyzer technique for urine and tissue extracts, and appears quite promising for its application to CSF.

MATERIALS AND METHOD

1. Apparatus

A model Series 3 liquid chromatograph equipped with a Model 650-10S fluorescence spectrophotometer and a Model LC 100 column oven (all from Perkin-Elmer Corp., Norwalk, CT 06856) was used. A reversed phase column "Ultrasphere ODS 5 um" 15 cm × 4.6 mm (Altex Scientific Inc., Berkeley, CA 94710) was mounted in the oven. The chromatograms were recorded on a "B.D.41" recorder (E&K Scientific Products Inc., Saratoga, CA 95070). Samples were injected into a Rheodyne 7105 value (Rheodyne, Berkeley, CA 94710) mounted on the chromatograph. A temperature controlled multi-temp-block heater (Lab-Line Instruments Inc., Melrose Park, ILL.) was used for incubation during derivatization. Vac-Elut and Bond Elut C_{18} columns were purchased from Analytichem International Co., Harbor City, CA 90710. Eppendorf micro test tubes (1.5 ml polypropylene) and an Eppendorf centrifuge were obtained from Brinkman Instruments Inc., Westbury, N. Y. 11590.

2. Reagents and Standards

All inorganic reagents were analytical (AR) grade.

Methanol-distilled in glass, Burdick & Jackson Laboratory, Inc., Muskegan, MI 49442.

Acetonitrile-HPLC grade, J.T. Baker Chemical Co., Phillipsburg, NJ 08865.

Hydrochloric acid-concentrated.

Sodium carbonate-saturated aqueous solution.

Dansyl chloride-100 mg/ml in acetone, Pierce Chemical Co., Rockford, IL 61105. Prepare a working solution by diluting

0.2 ml to 2 ml with acetone to yield a concentration of 10 mg/ml. Wrap the glass tube with foil and store at 4°C.

Polyamine standards-Cadaverine.2HCl, spermidine.3HCl and spermine.4HCl were from Sigma Chemical Co., St. Louis, MO 63178; putrescine.2HCl from Calbiochem-Behring Co., La Jolla, CA 92037. Dissolve 10 mg of each in 10 ml of 0.1 N HCl and dilute 100 fold to a concentration of 0.001 mg/ml. Store at 4°C.

1,6-Diaminohexane-Internal standard Sigma was prepared by dissolving 10 mg of 1,6-diaminohexane in 10 ml of 0.1 N HCl and diluting 100 fold to a concentration of 0.001 mg/ml. Store at 4°C.

Mobile phase-Pump A contained degassed acetonitrile, and pump B degassed deionized water. Various mixtures of these mobile phase solvents were obtained using gradient modes on the liquid chromatograph.

3. Procedure

A. Preparation of Samples

a. Urine:

To 200 ul of urine in a 1.5 ml Mini-Aktor tube add 200 ul of 12 N HCl and cap using teflon tape on the threads. Hydrolyze at 110°C for 14-16 hr and freeze dry the hydrolysate. Dissolve residue in 200 ul of 4% 5-sulfosalicylic acid and centrifuge for 5 min. Transfer 50 ul of supernatant to a polypropylene tube and derivatize the sample using the reaction conditions described below.

b. CSF:

To 1.0 ml of CSF in a 1.5 ml Mini-Aktor tube add 1 drop 3.0 N HCl and freeze dry. Add 0.5 ml of 6.0 N HCl to residue, cap

tightly and hydrolyze at 110°C for 14-16 hr. Freeze dry hydrolysate, dissolve residue in 150 µl of 4% 5-sulfosalicylic acid and centrifuge for 5 min. Transfer 50 µl of supernatant to another polypropylene tube for derivatization.

c. Tissue:

Cells are washed free of tissue culture medium with phosphate buffer saline. Spin cells into a pellet, pour off saline, and absorb excess saline with a Kim-wipe being careful not to touch the pellet. Add 200-250 µl of 8% 5-sulfosalicylic acid for every 10^6 cells in the pellet. Sonicate for 20-30 seconds and allow to stand in ice for 60 min, mixing once at 30 min. Transfer material to 400 µl microfuge tubes and spin for 5 min at 12,000 g. Transfer 50 µl of supernatant into another polypropylene tube for derivatization.

B. Derivatization and Extraction

Fifty microliters of 1,6-diaminohexane (I.S.), 200 µl of saturated Na_2CO_3 and 200 µl of dansyl chloride (10 mg/ml) are added to 50 µl of any of the supernatants in a polypropylene tube. Cap the tube and vortex for 15 seconds. Incubate at 70°C for 10 min. Cool the tube to room temperature and transfer contents to a Bond Elut C_{18} column. After the column has drained, wash with 2 column volumes of water. The dansylated polyamines are then eluted with 500 µl of methanol. Ten µl of methanol eluate is injected onto the HPLC for analysis.

C. Chromatography

The following gradient parameters were selected for the separation of urine and tissue polyamines: 0 time, 45% acetonitrile and 55% H_2O; after injecting the sample the concen-

tration of acetonitrile is increased from 45% to 80% in 14 min, then from 80% to 90% in 1 min and finally kept at 90% for the last 5 min. At the end of gradient run, the column is equilibrated with 45% acetonitrile for 6 min. The flow rate is set at 2 ml/min and the column is at 50°C. The column effluent is monitored at an excitation wavelength of 340 nm and an emission wavelength of 515 nm at a sensitivity 0.3. Figure 4 shows a chromatogram of putrescine, cadaverine, 1,6-diaminohexane, spermidine and spermine standards. Each peak represents about 1 ng of polyamine injected. For CSF polyamines analysis, the following parameters were selected: 0 time, 50% acetonitrile and 50% H_2O; after injecting the sample acetonitrile concentration is increased from 50% to 80% in 15 min, then from 80% to 90% in 1 min and 90% for the last 4 min. Figure 5 and 6 show the chromatograms of polyamines in tissue and CSF samples.

D. Calculations

Since the polyamine peaks are sharp and symmetrical, peak height measurement can be employed for quantitation. The amount of polyamine in the unknown sample is calculated according to the following formula:

$$\text{Conc. of polyamine} = \frac{\text{P.H. of polyamine in sample}}{\text{P.H. of internal standard}} \times \text{R.F.}$$

$$\text{R.F.} = \frac{\text{P.H. of internal standard}}{\text{P.H. of polyamine standard}} \times \text{conc. of internal standard}$$

(P.H., peak height; R.F., response factor)

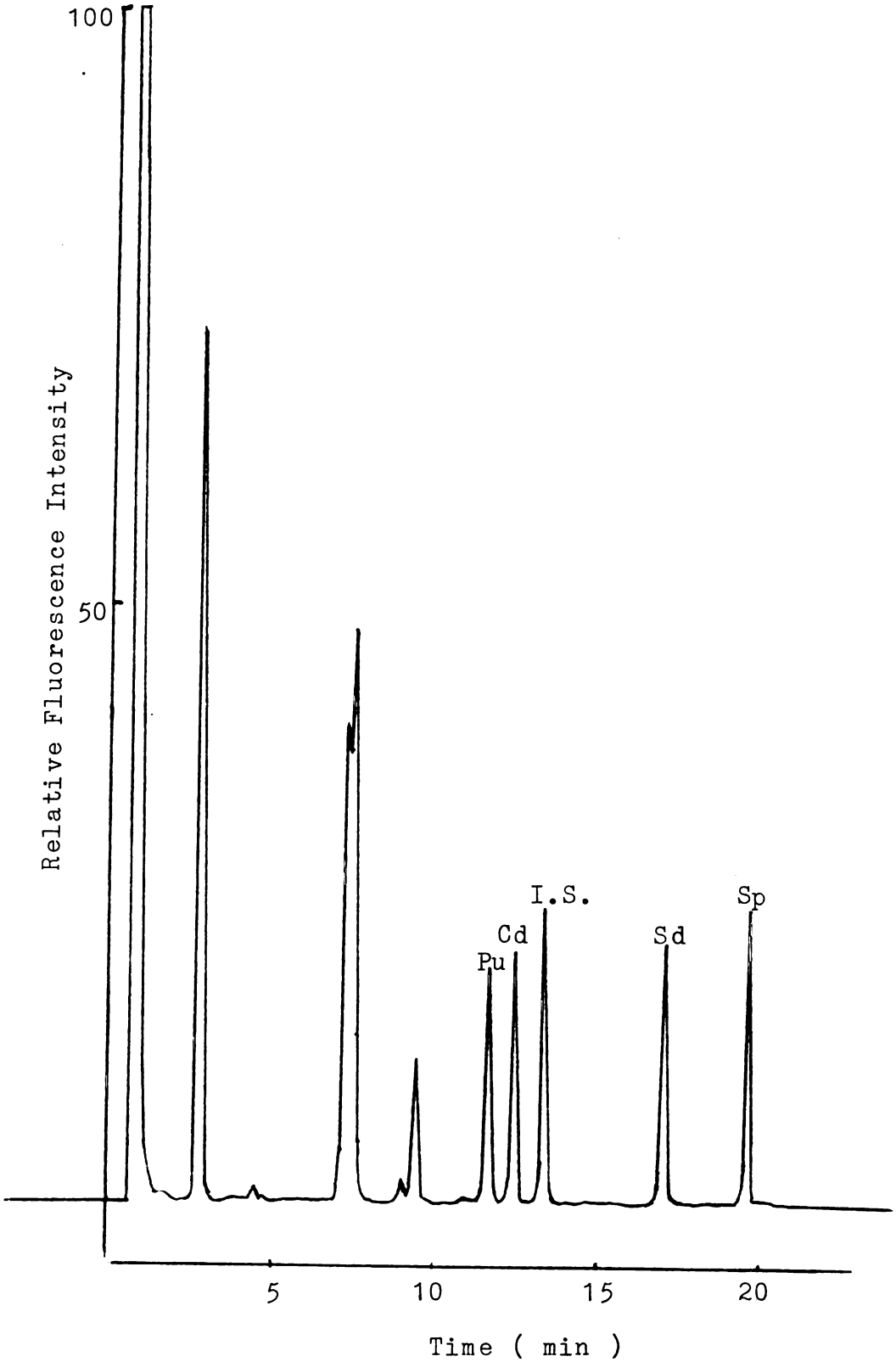


Figure 4. Chromatogram of the dansyl derivatives of putrescine (Pu), cadaverine (Cd), Spermidine (Sd) and spermine (Sp) standards separated on an "Ultrasphere ODS 5 µm" column at 50°C. Each peak including I.S. represents about 1 ng of polyamine injected.

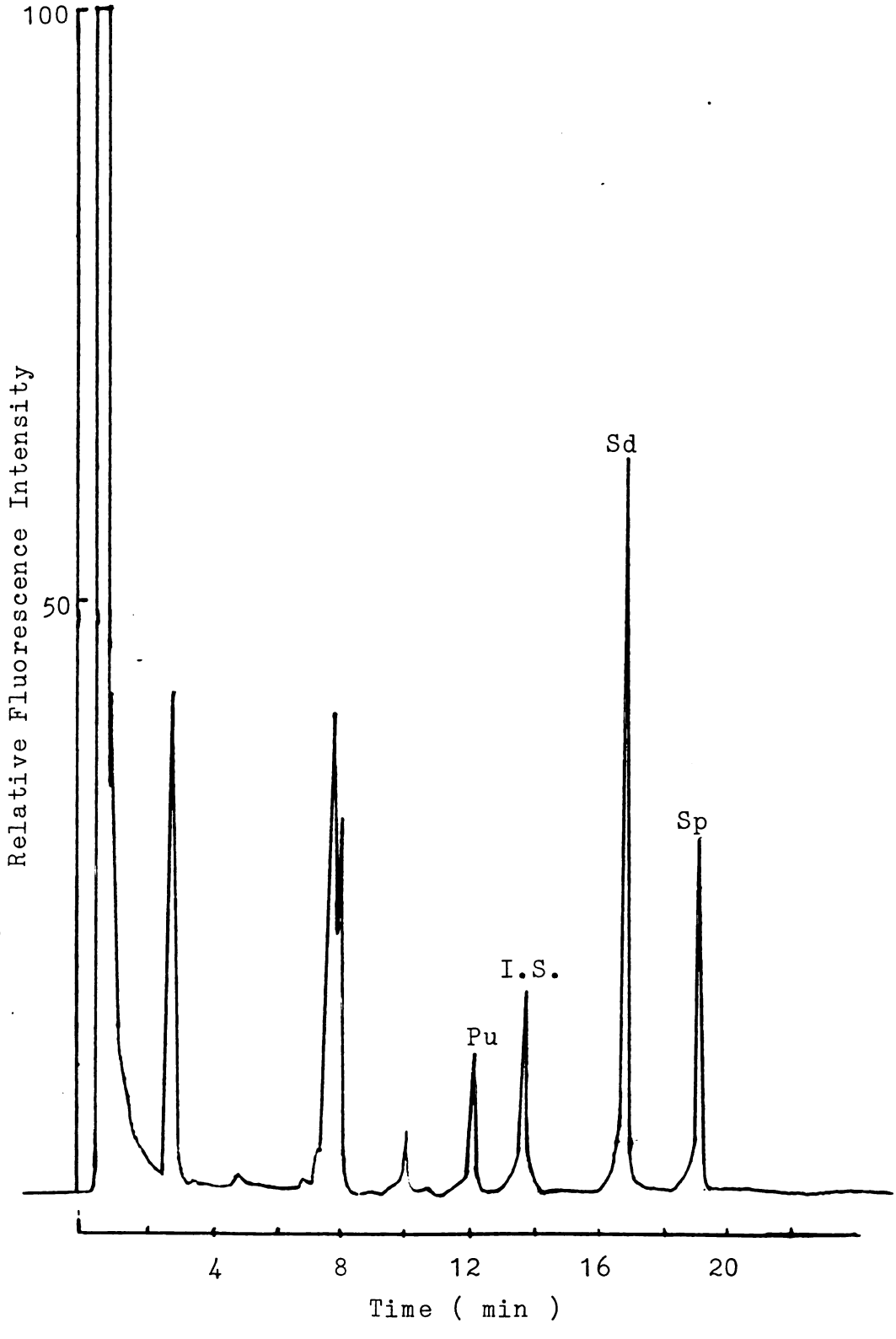


Figure 5. Chromatogram of dansylated polyamines in tissue.

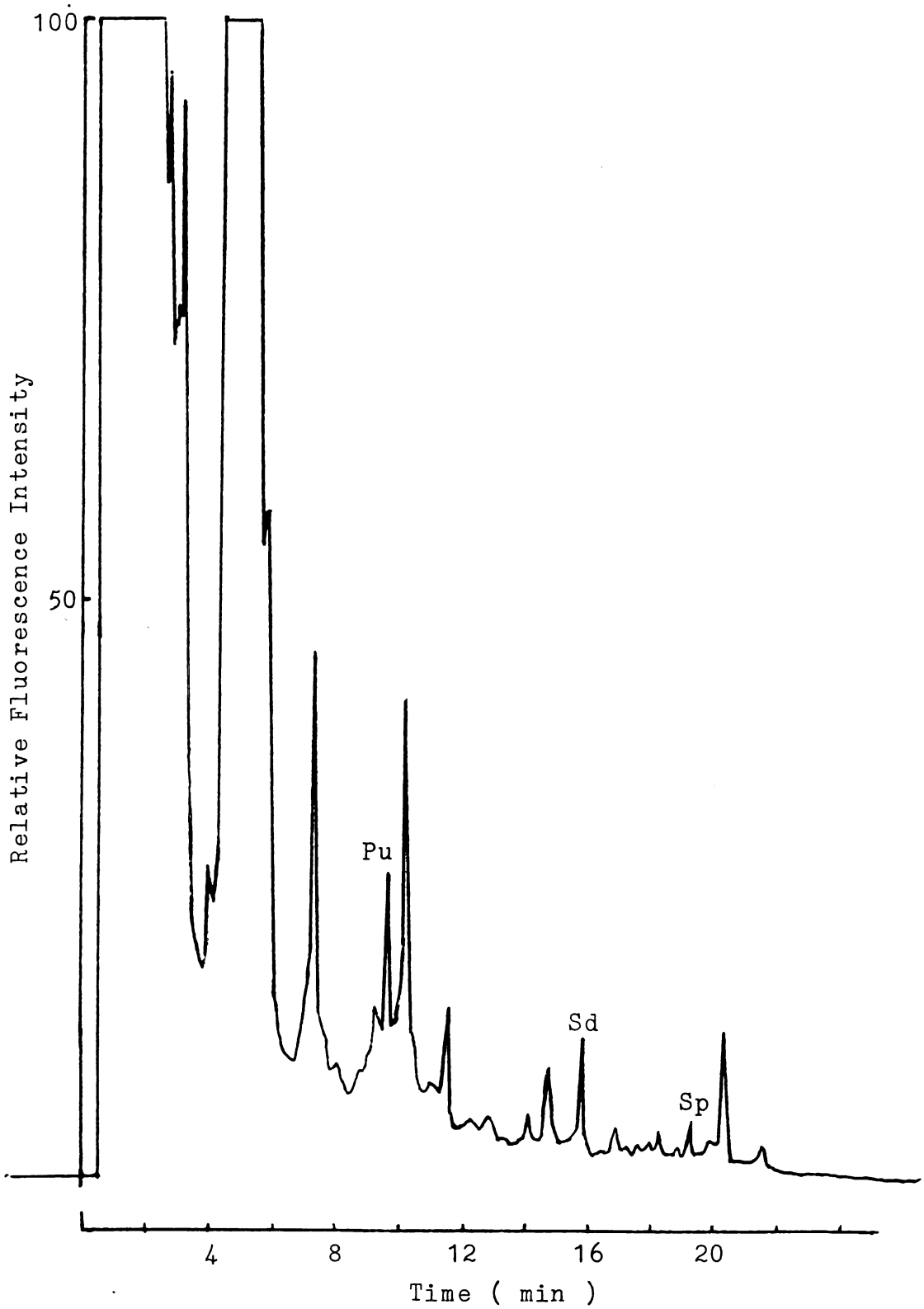


Figure 6. Chromatogram of dansylated polyamines in CSF.

RESULTS AND DISCUSSION

1. Recovery

Five aliquots containing known amounts of polyamines were added to hydrolyzed urine and processed as described above. Their recoveries were calculated and given in Table 1.

Table 1. Recoveries of Polyamines (n=5)

Compound	Amount Added (µg/ml)	Recovery (µg/ml)	(%)	Range (%)	C.V. (%)
Putrescine	1	0.93	93	90-97	2.79
	2	1.93	96	94-100	2.60
	5	4.72	94	91-97	2.55
	10	9.80	98	94-103	3.61
	20	19.08	95	92-108	2.73
Cadaverine	1	0.92	92	91-94	1.41
	2	1.92	96	94-102	3.53
	5	4.76	95	93-98	2.02
	10	9.56	96	92-101	3.96
	20	18.88	94	91-97	2.44
Spermidine	1	0.97	97	92-108	7.07
	2	1.94	97	95-100	2.24
	5	5.00	100	95-108	4.80
	10	9.58	96	93-97	1.87
	20	19.68	98	95-105	4.23
Spermine	1	0.94	94	91-98	3.19
	2	1.92	96	94-98	1.71
	5	4.88	98	91-105	5.50
	10	9.54	95	91-101	3.82
	20	19.44	97	90-103	6.02

2. Linearity

Standard curves were constructed by plotting the peak height ratios vs concentration of polyamines. There was a linear relationship between peak height ratio and concentration up to 30 µg/ml (186.2 nmol/ml), 30 µg/ml (171.3 nmol/ml), 30 µg/ml (117.8 nmol/ml) and 20 µg/ml (57.4 nmol/ml) for putrescine, cadaverine, spermidine and spermine, respectively. The curves are shown in Figures 7, 8, 9 and 10.

3. Precision

Within-run precision was assessed by processing aliquots of urine samples containing polyamines in the concentrations shown in Table 2. Ten samples were processed as a batch and the coefficient of variation (C.V.) for the polyamines were < 3%, < 3%, < 4% and < 5% for putrescine, cadaverine, spermidine and spermine. Day-to-day precision was similarly evaluated and C.V.s were < 3%, < 5%, < 5% and < 6% for putrescine, cadaverine, spermidine and spermine, respectively. The results are shown in Table 3.

Table 2. Within-run Precision (n=10)

Polyamine	Concentration (µg/ml) Mean ± 1 S.D.	C.V. (%)
Putrescine	1.97 ± 0.47	2.39
	9.81 ± 0.14	1.47
	18.31 ± 0.35	1.91
Cadaverine	0.85 ± 0.22	2.54
	8.45 ± 0.95	1.13
	17.13 ± 0.28	1.62

Polyamine	Concentration (μg/ml) Mean ± 1 S.D.	C.V. (%)
Spermidine	1.37 ± 0.43	3.15
	9.39 ± 0.15	1.62
	18.46 ± 0.55	2.98
Spermine	1.25 ± 0.51	4.10
	8.07 ± 0.18	2.21
	18.03 ± 0.72	4.00

Table 3. Day-to-Day Precision (n=10)

Polyamine	Concentration (μg/ml) Mean ± 1 S.D.	C.V. (%)
Putrescine	1.81 ± 0.47	2.63
	10.03 ± 0.26	2.54
	18.09 ± 0.36	2.01
Cadaverine	0.34 ± 0.15	4.50
	8.55 ± 0.25	2.88
	16.50 ± 0.31	1.89
Spermidine	1.32 ± 0.31	2.26
	9.71 ± 0.40	4.11
	17.72 ± 0.28	1.60
Spermine	0.42 ± 0.25	6.00
	8.68 ± 0.40	4.64
	17.27 ± 0.52	3.03

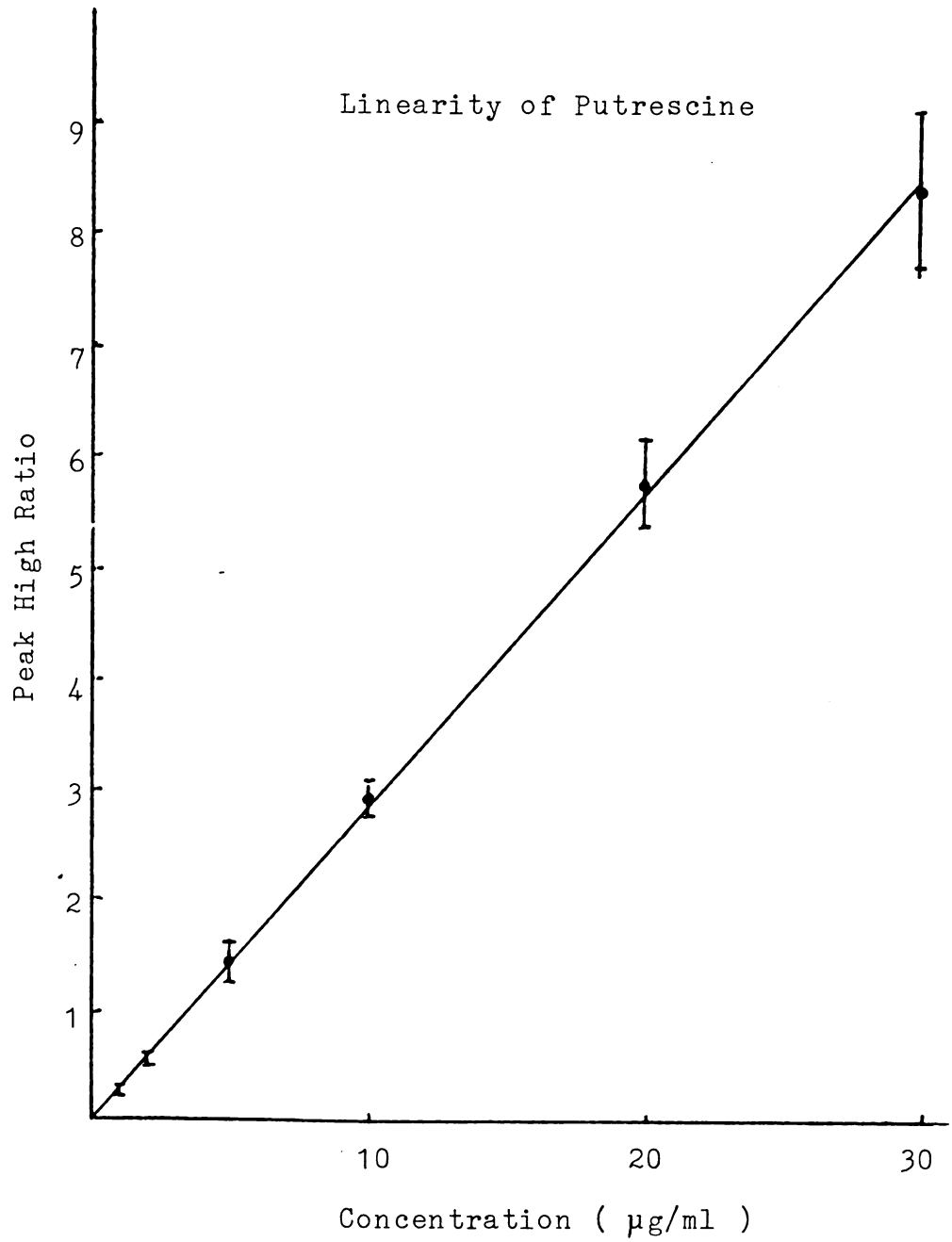


Figure 7. Peak high ratio of putrescine vs. concentration.

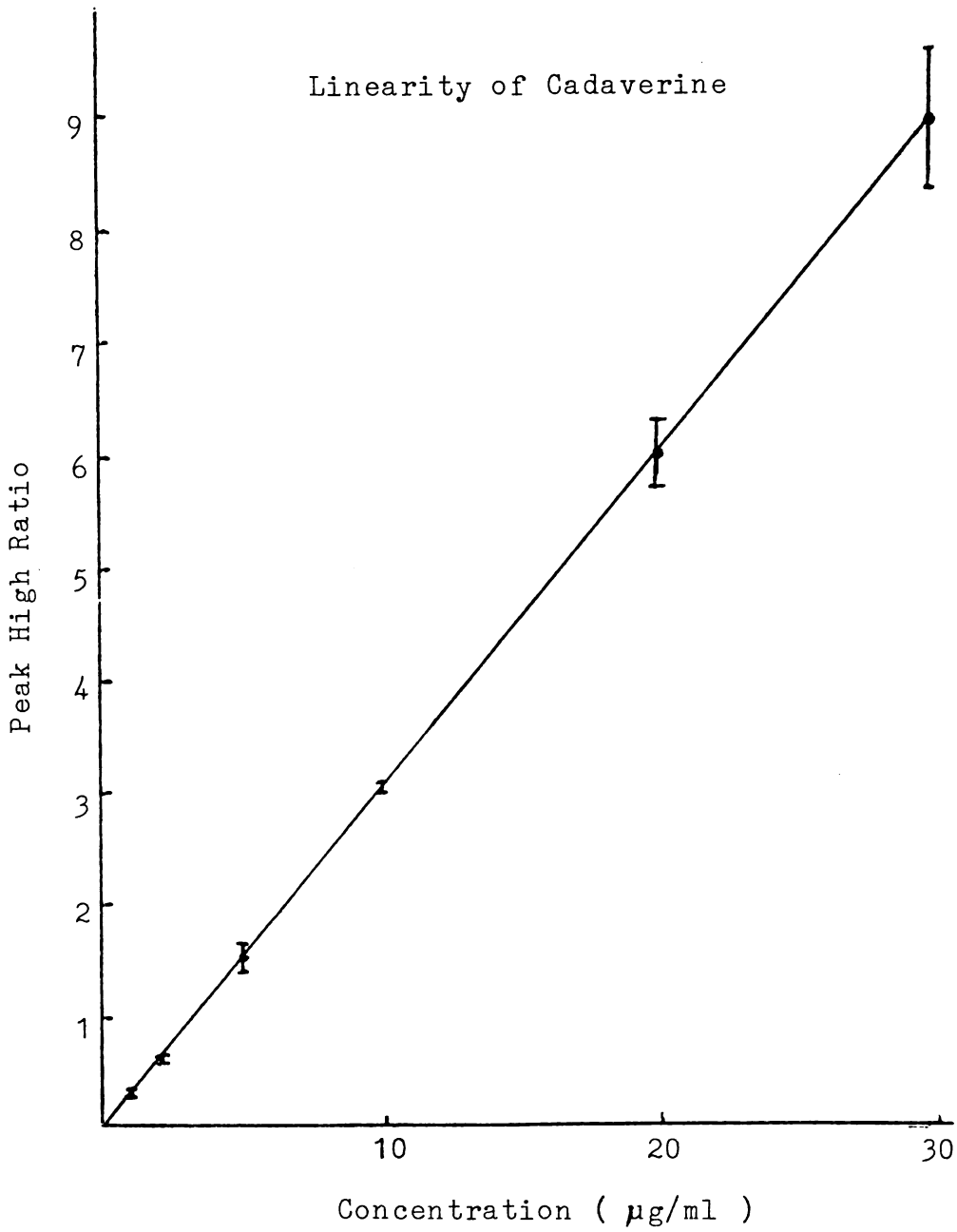


Figure 8. Peak high ratio of cadaverine vs. concentration.

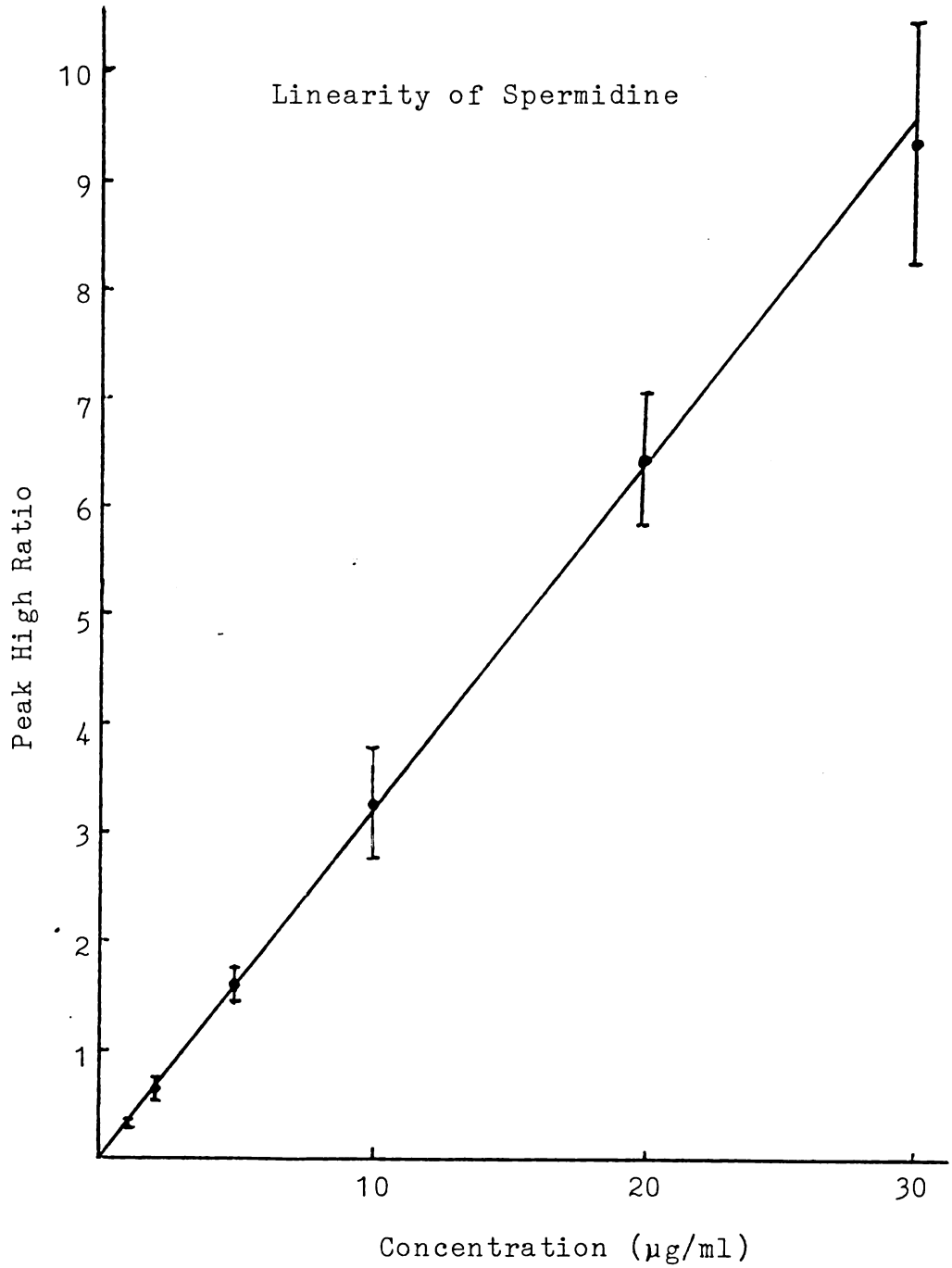


Figure 9. Peak high ratio of spermidine vs. concentration.

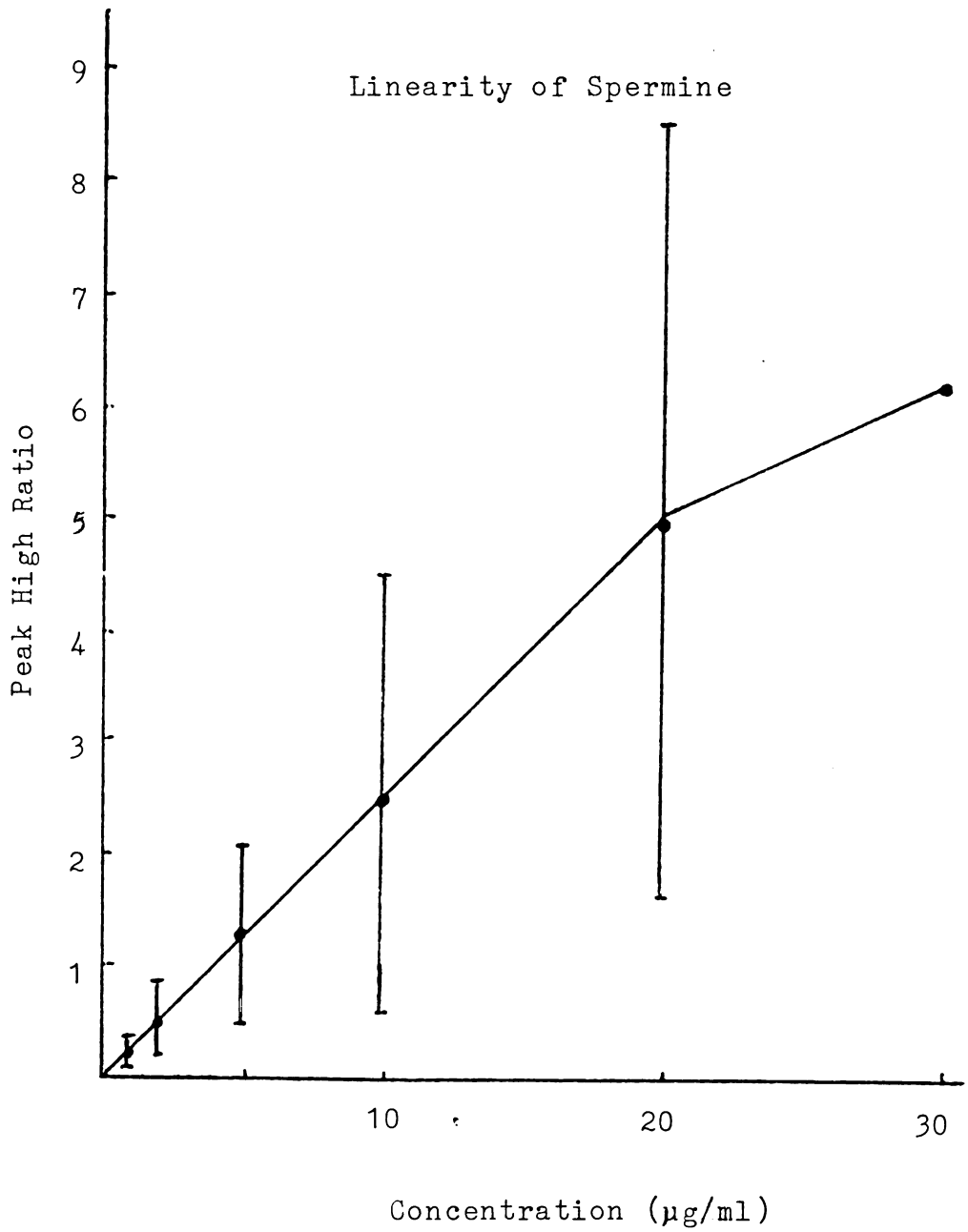


Figure 10. Peak high ratio of spermine vs. concentration.

4. Detection Limits

Minimum detection limits for dansylated polyamines were determined by injecting 20 pg of each polyamine and determining the signal-to-noise ratio. Less than 20 pg (equal to 0.12, 0.11, 0.08, 0.06 pmol for putrescine, cadaverine, spermidine and spermine, respectively) could be detected at a signal-to-noise ratio of 4 (Figure 11).

5. Method Comparison

The accuracy of this assay was assessed by comparing the results obtained with this method to those obtained with an ion-exchange chromatographic method (77). Fifty-one urine samples were analyzed using both methods. Regression analysis for the two methods for putrescine and spermidine are shown in Figure 12 and 13. Tissue and CSF samples were also compared and are illustrated in Figure 14 to 18.

6. Background

Five hydrolyzed urine samples without added internal standard were assayed to see if there were any interfering peaks at the elution point of the internal standard. A small peak eluted at the same time as the internal standard, but it was inconsequential for accurate quantitation. Figure 19 illustrates this peak in hydrolyzed urine. Tissue samples analyzed by this method were found to have cleaner backgrounds than urine and CSF. No interfering peak was present in the chromatograms of tissue extracts. For tissue extracts the gradient could be shortened such that the analysis time is reduced to 15 min.

7. Interference

The compounds listed in Table 4 were tested for possible interference. Normetanephrine was found to have the same elution time as the internal standard. Attempts to separate normetanephrine from the internal standard by replacing water with phosphate buffer and changing the pH of the phosphate buffer were unsucessful. Dextroamphetamine partially interfered with putrescine, but it could be separated by slowing the gradient elution.

Table 4. Retention Times of Polyamines and Some Drugs

Compound	Retention Time (min)
Putrescine	11.7
Cadaverine	12.5
1,6-Diaminohexane (I.S.)	13.5
Spermidine	17.2
Spermine	19.8
Tobramycin	18.4
Gentamicin	19.5, 20.5, 20.9
Amikacin	*N.D.
Deoxyepinephrine HCl	*N.D.
5-Hydroxytryptamine HCl	14.6
Agmatine sulfate	8.8
1,3-Diaminopropane	11.2
Histamine	13.0
Dopamine	*N.D.
Normetanephrine	13.5
Norepinephrine	18.2
Epinephrine	19.1
Metaamphetamine	14.0
Dextroamphetamine	11.4

*N.D., Not Detectable

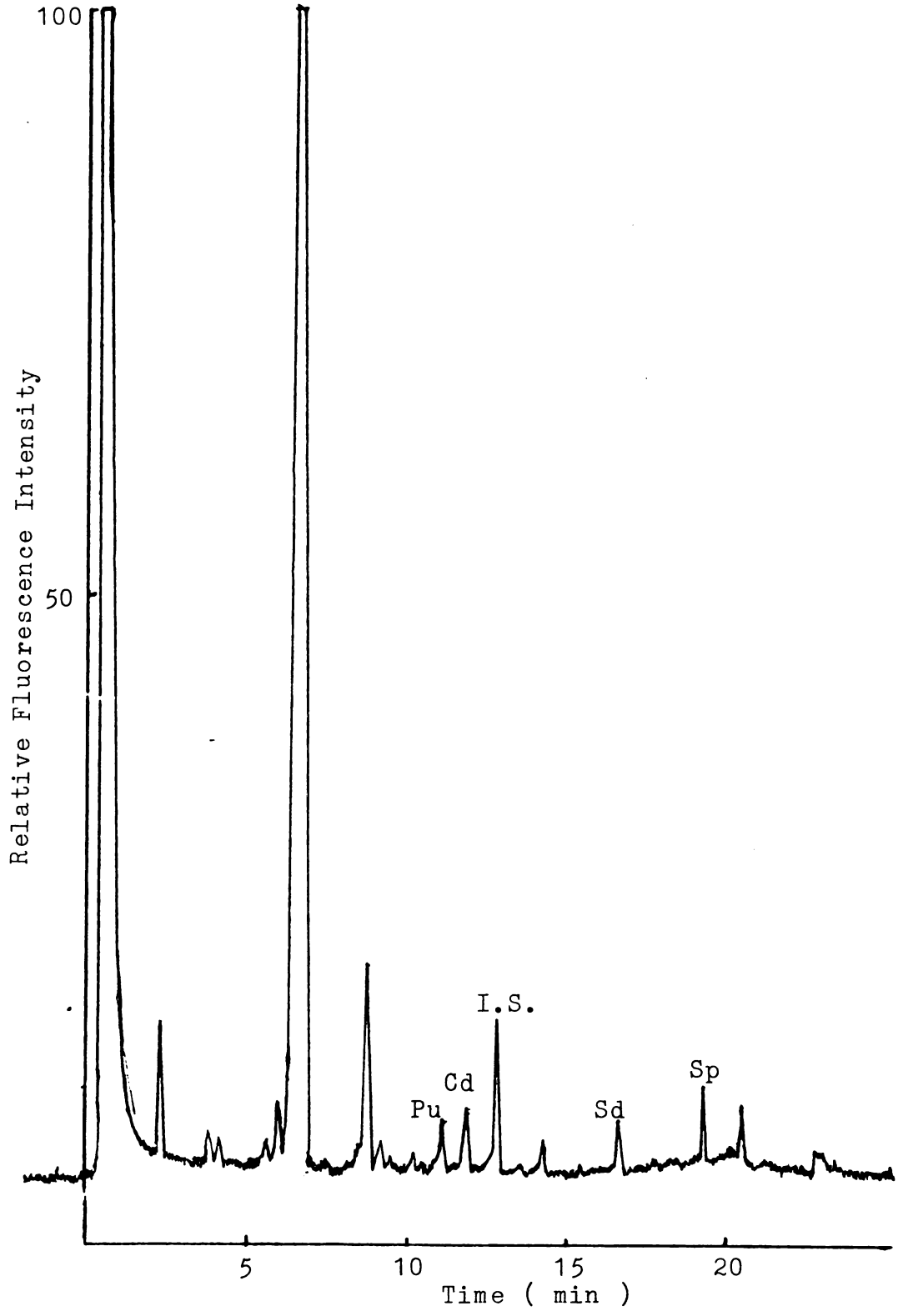


Figure 11. Chromatogram defining detection limits of polyamines. The peaks for putrescine, cadaverine, spermidine and spermine represent 0.12, 0.11, 0.08 and 0.06 pmol injected, respectively. The sensitivity range of the detector was set at 3.

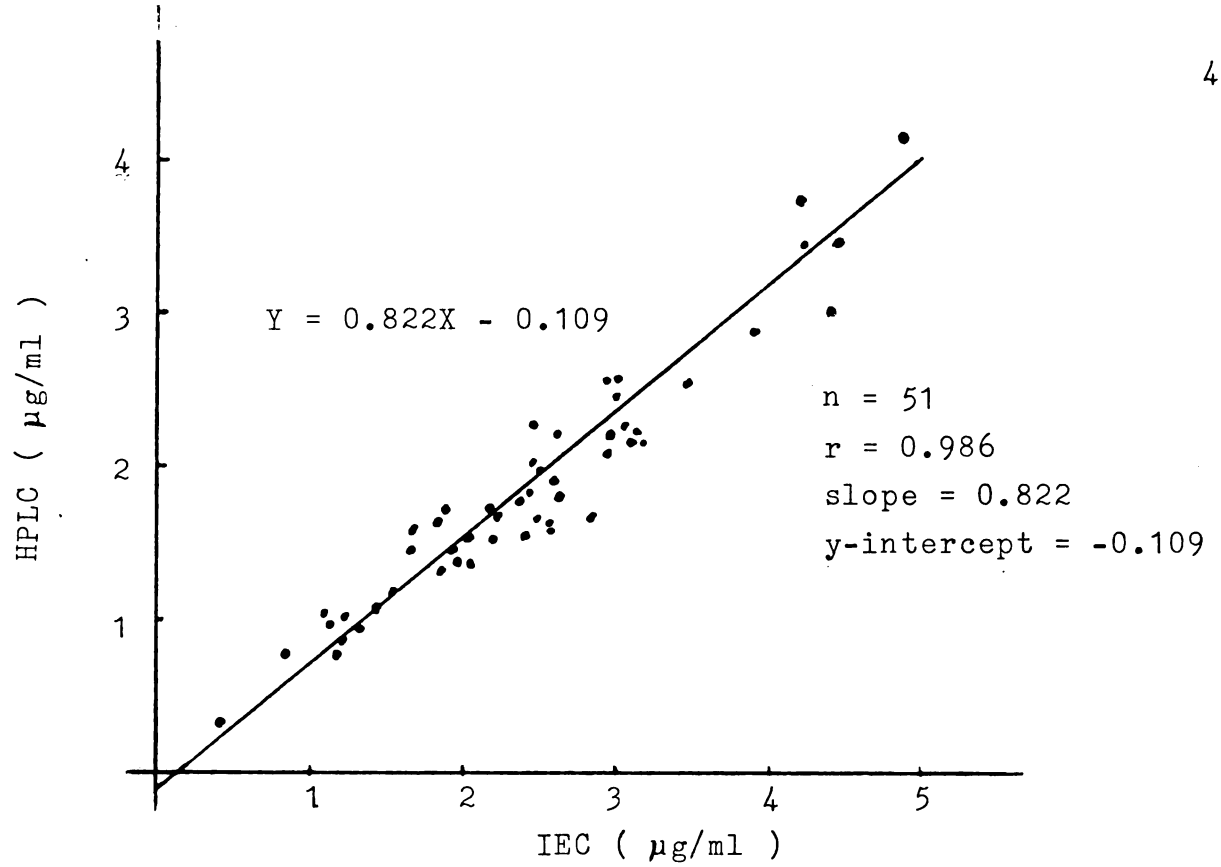


Figure 12. Urine putrescine comparison between HPLC and IEC methods.

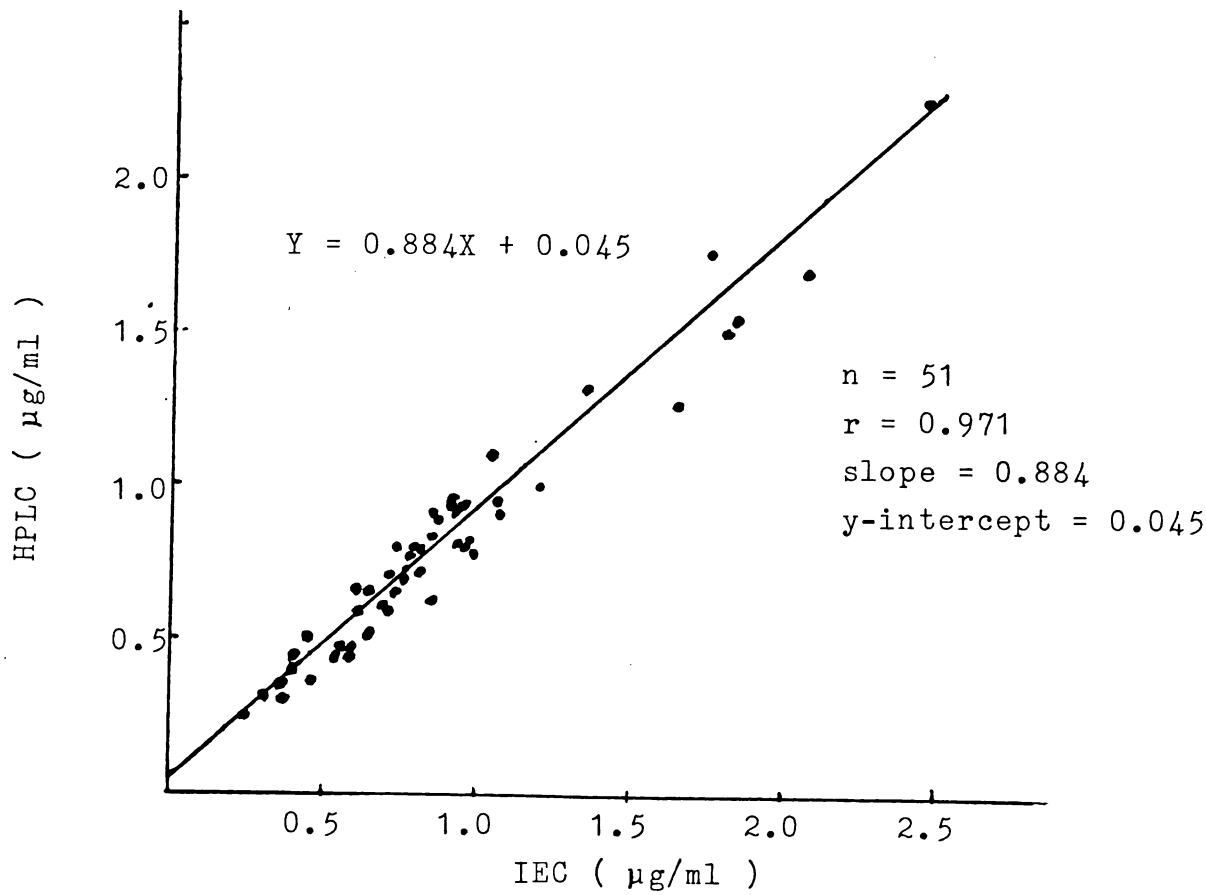


Figure 13. Urine spermidine comparison between HPLC and IEC methods.

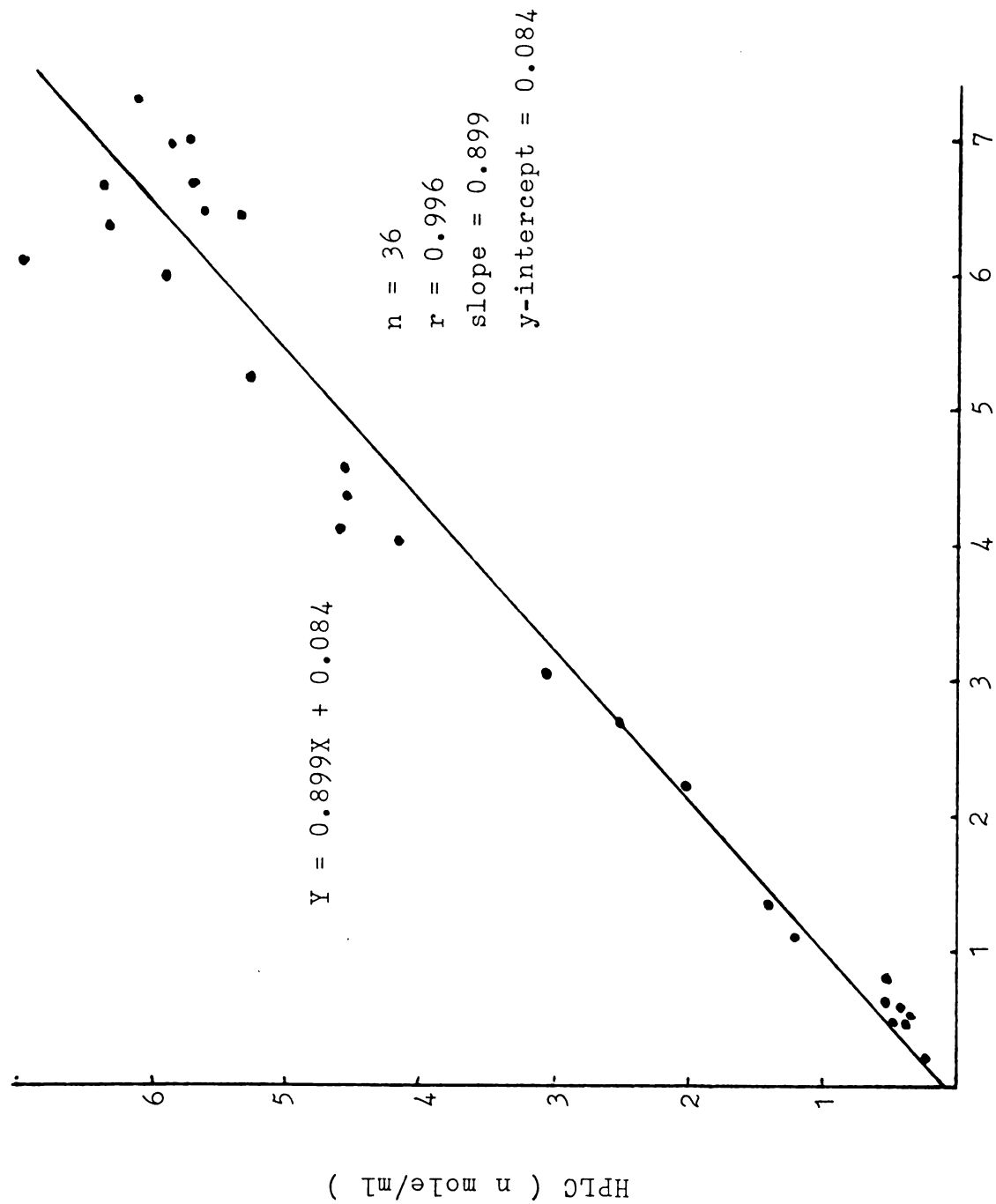


Figure 14. Tissue putrescine comparison between HPLC and IEC methods.

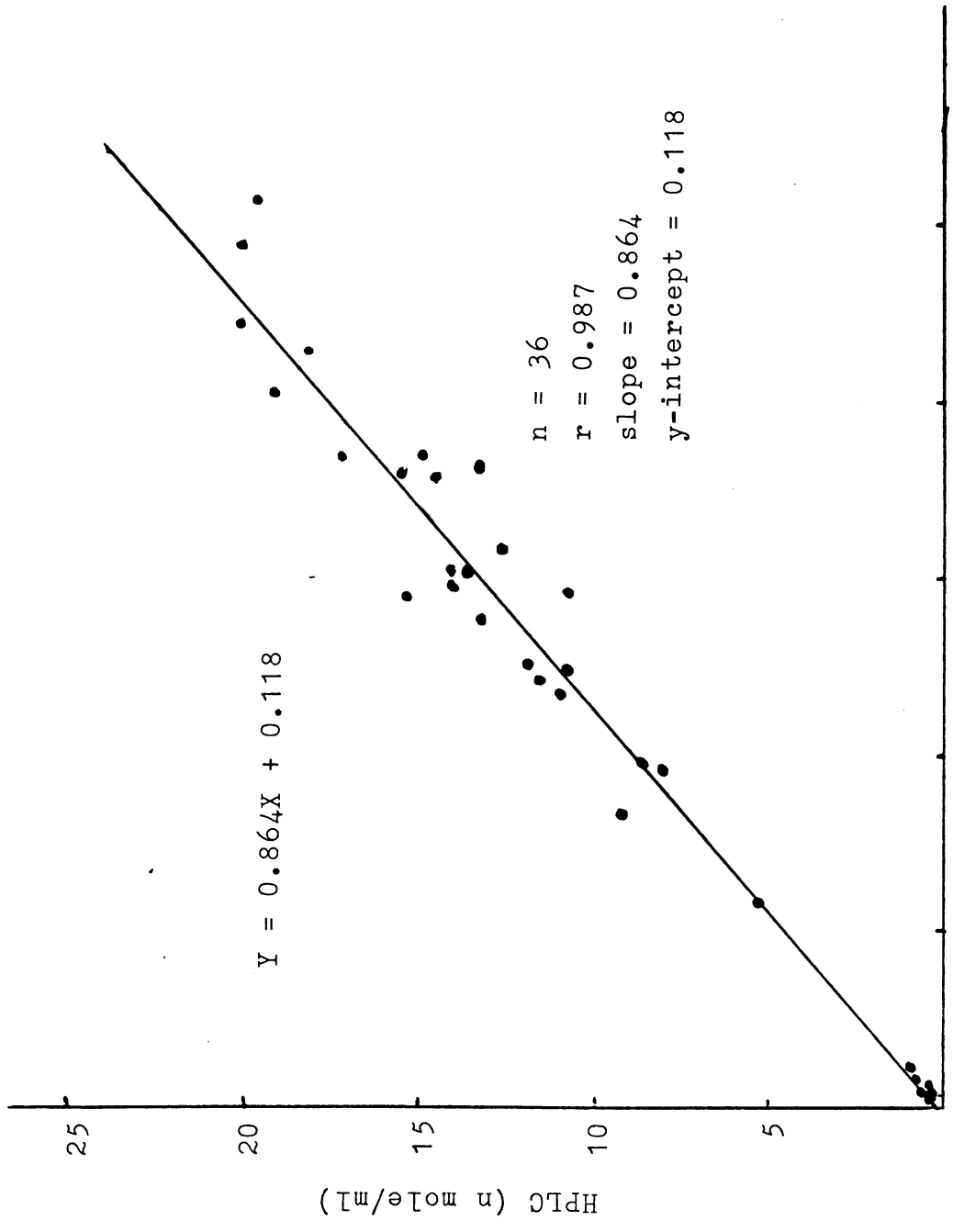


Figure 15. Tissue spermidine comparison between HPLC and IEC methods.

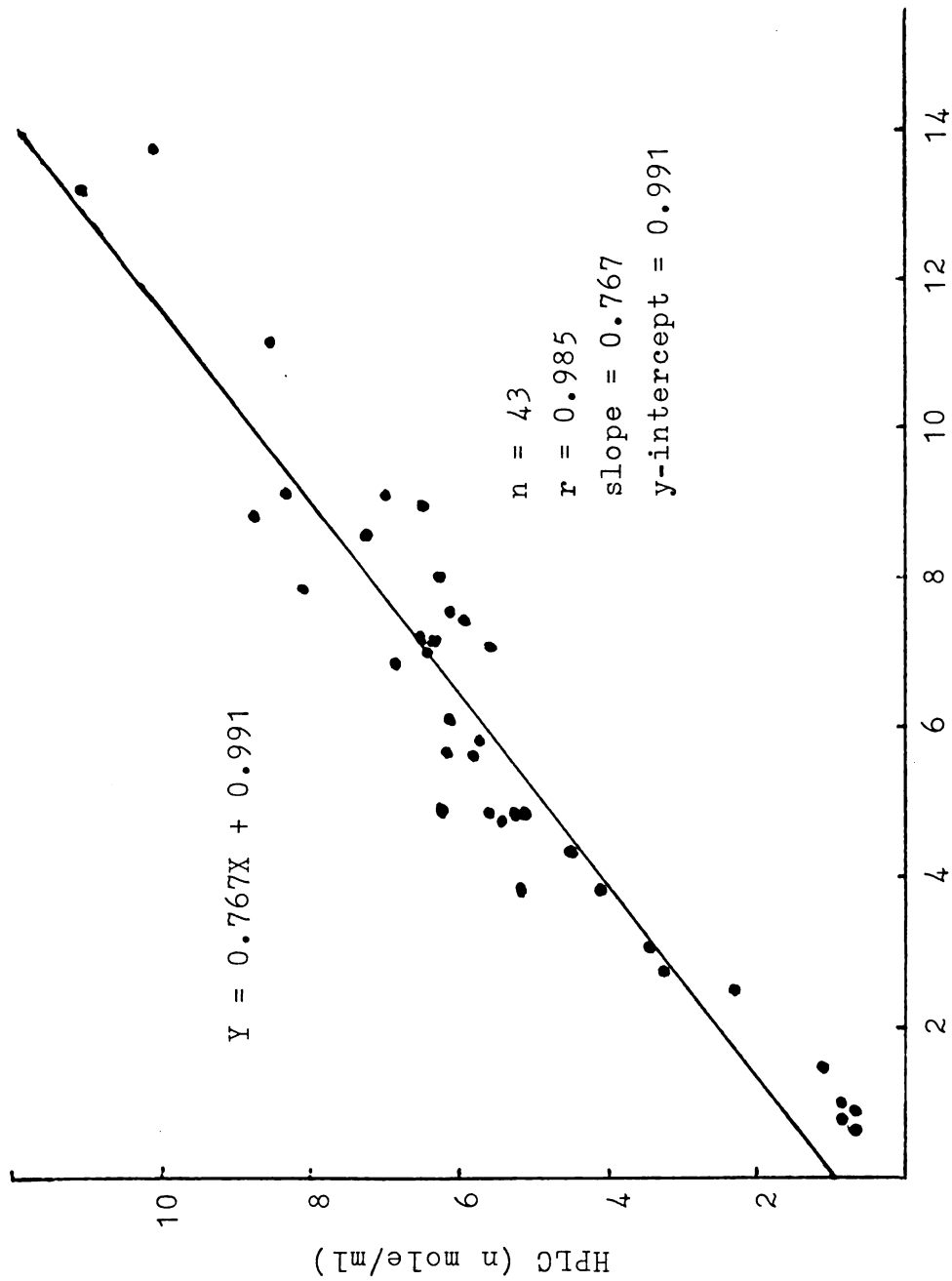


Figure 16. Tissue spermine comparison between HPLC and IEC methods.

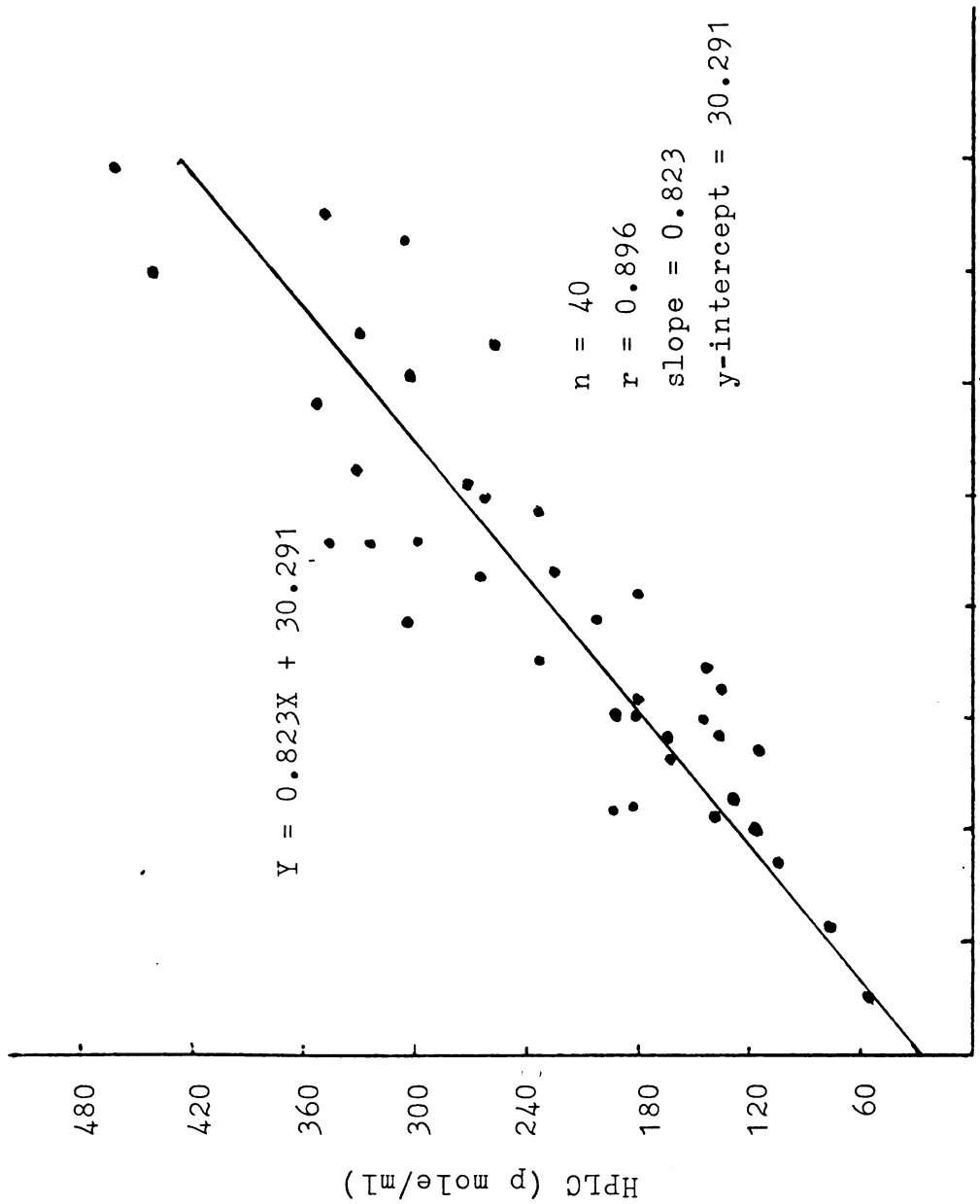


Figure 17. CSF putrescine comparison between HPLC and IEC methods.

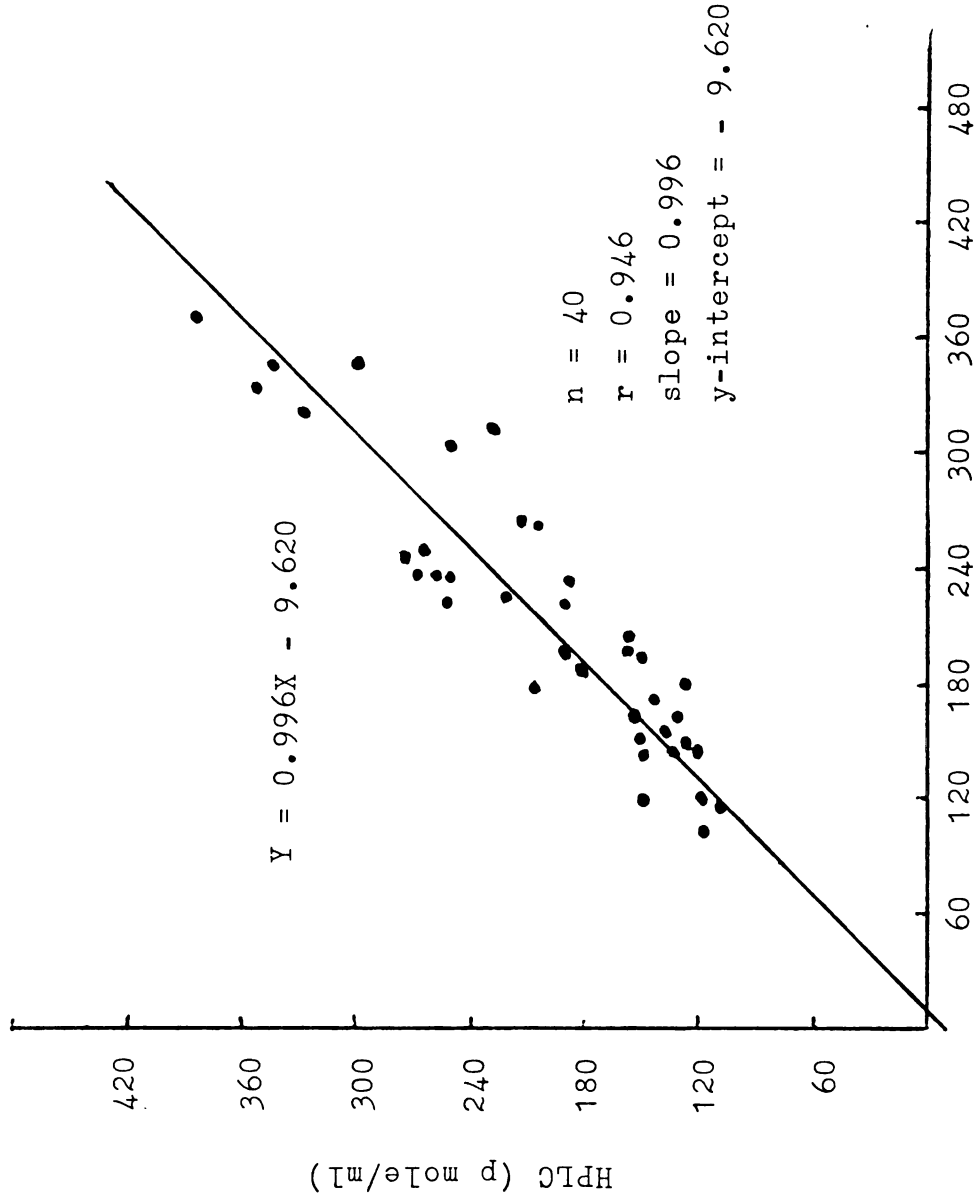


Figure 18. CSF spermidine comparison between HPLC and IEC methods.

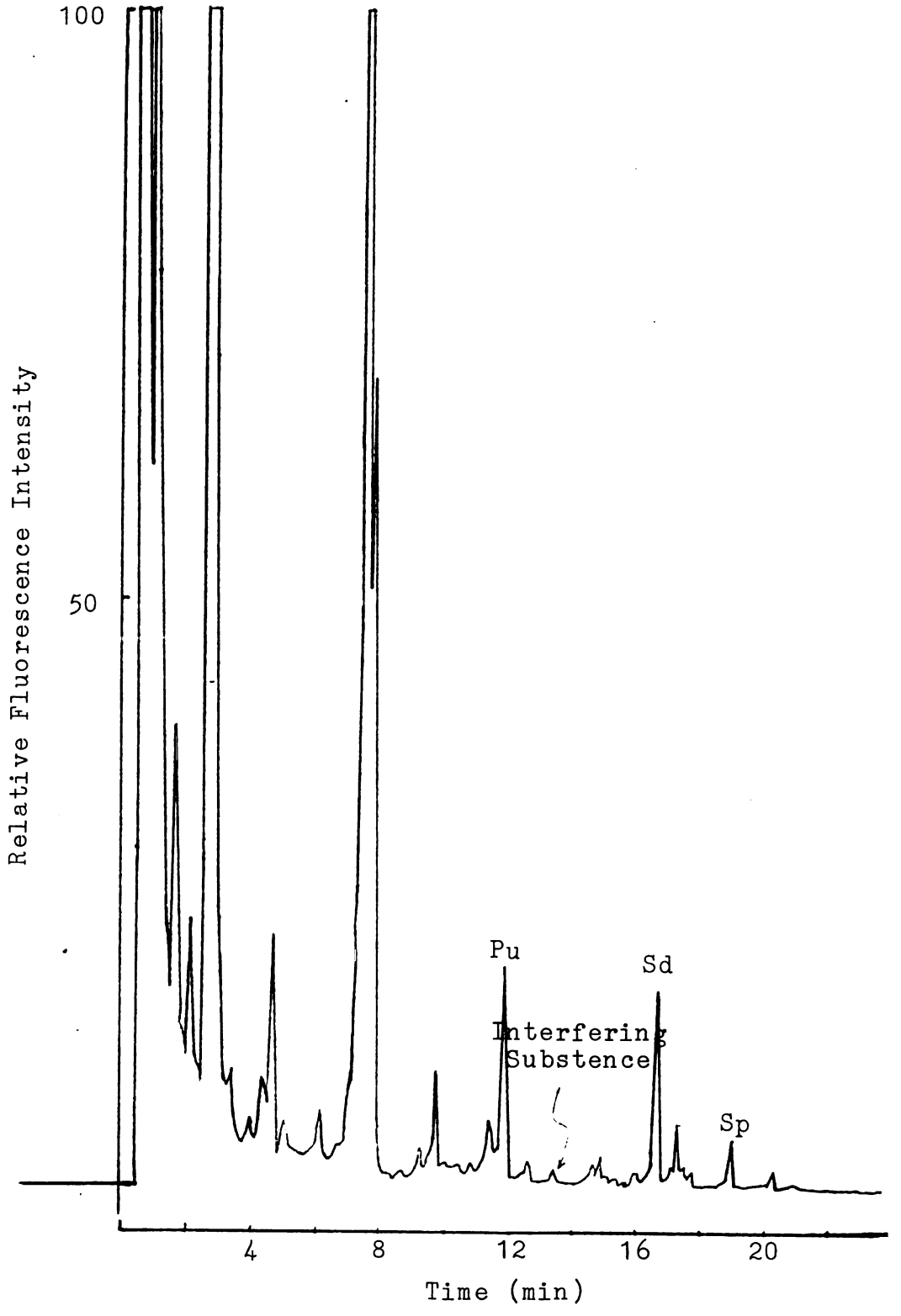


Figure 19. Chromatogram of hydrolyzed urine withot added internal standard. Injection volume, 10 µl. Detector sensitivity, 0.3.

8. Stability

Dansyl chloride is light-unstable. Thus, the stability of the dansylated polyamines were studied. Samples were wrapped with foil and stored at 4°C after the first analysis. One month later, the samples were assayed again and the differences in the two results calculated (Table 5). Putrescine, cadaverine and spermidine derivatives were found to be stable during this storage period; the difference being less than 3%. Spermine was less stable.

Table 5. Stability of Dansylated Polyamines

Polyamine	*C_1	**C_2	% Difference
Putrescine	10.09	10.01	-0.8%
	17.97	18.14	+1.0%
Cadaverine	8.84	8.69	-1.7%
	16.78	16.68	-0.6%
Spermidine	9.75	9.53	-2.3%
	17.62	17.92	+1.7%
Spermine	9.06	8.17	-9.8%
	17.68	15.79	-10.7%

*C_1, concentration obtained from initial analysis.
**C_2, concentration obtained one month later.

9. Acetylated Polyamines

The acetylated spermidines, N^1-acetylspermidine and N^8-acetylspermidine, were chromatographed using this procedure. Figure 20 shows the chromatogram of N^1- and N^8-acetylspermidine standards. Although several different gradient programs were

tried, these compounds still could not be separated completely. Figure 21 shows a chromatogram obtained with one of the modified gradient programs. Unhydrolyzed urine and hydrolyzed urine were compared using this method (Figure 22 and 23). It is obvious that the acetylated polyamines or polyamine conjugates account for the major portion of polyamines in urine. Free polyamines in unhydrolyzed urine made up only a small percentage of the total polyamines.

10. CSF Polyamines

Polyamine concentrations in CSF are considerably lower than in tissue or urine. Thus, for CSF analyses the sample is concentrated several fold before derivatization, and the sensitivity of the detector is also increased. An interfering substance was occasionally present in CSF which coeluted with the internal standard. Therefore, an external standardization method was employed in the determination of CSF polyamines.

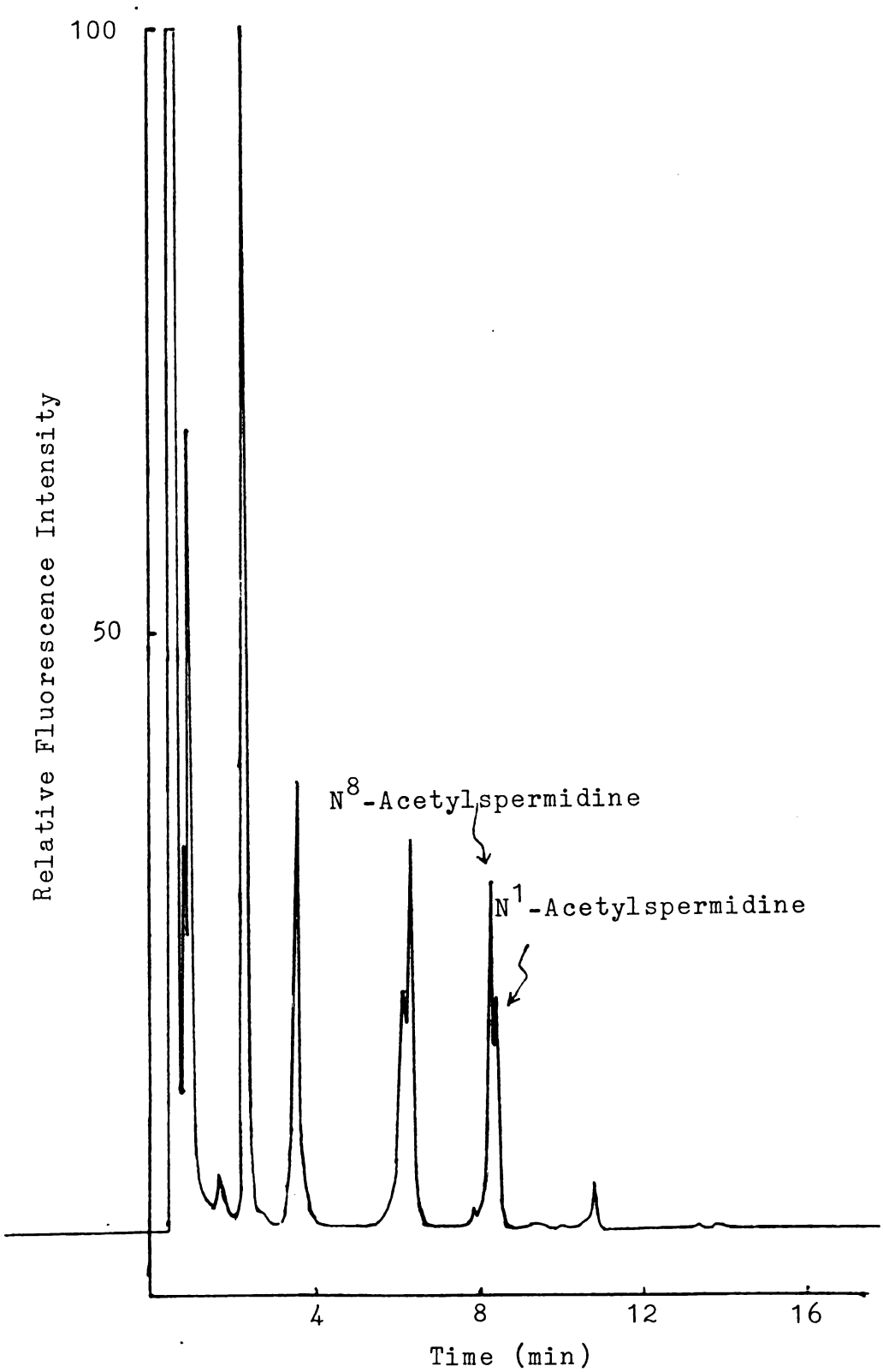


Figure 20. Chromatogram of N^1-acetylspermidine and N^8-acetylspermidine standards.

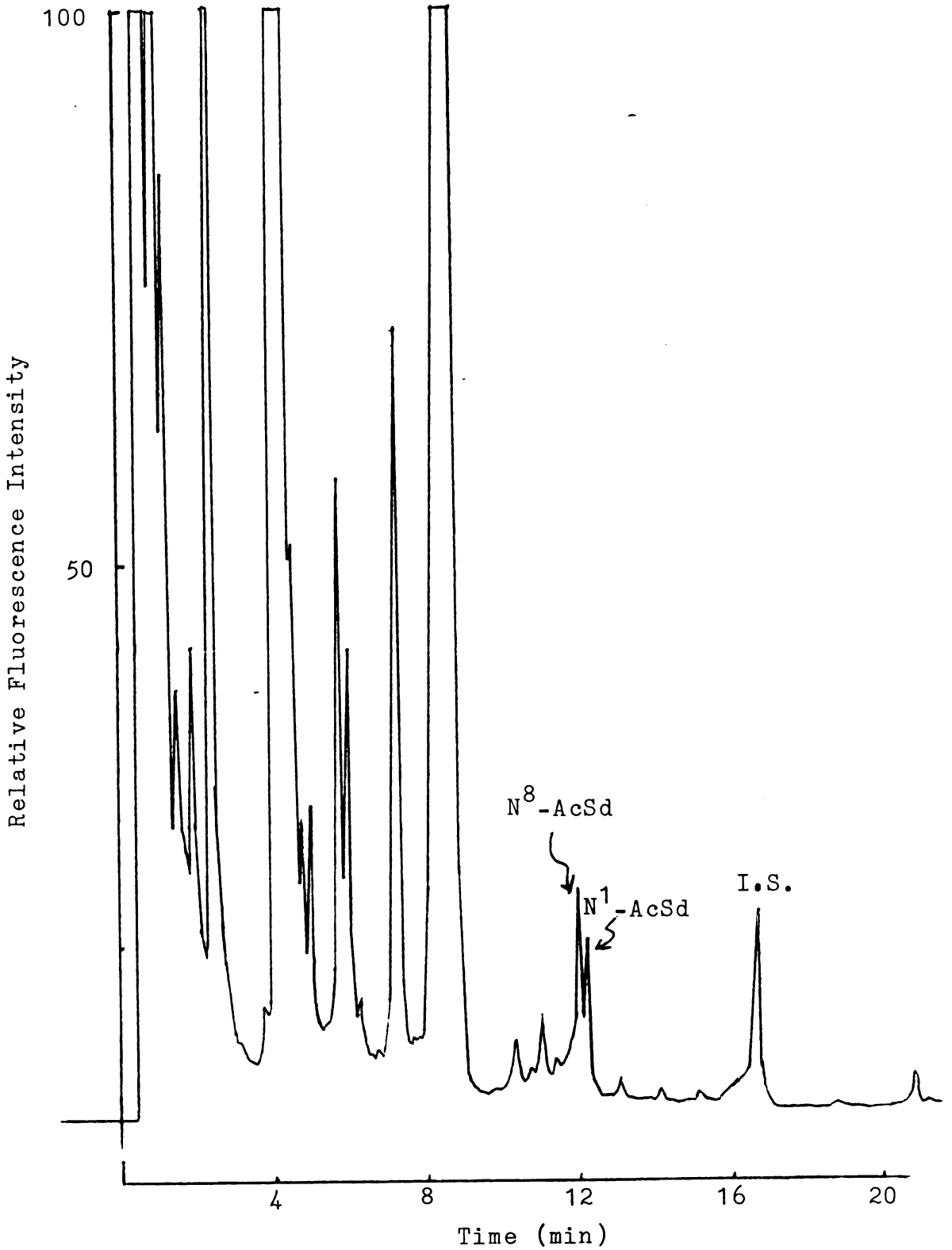


Figure 21. Chromatogram of urine spiked with N^1- and N^8-acetylspermidine. Elution program: 50% to 70% acetonitrile in water in 20 min; 70% to 90% in 2 min; 90% for 3 min.

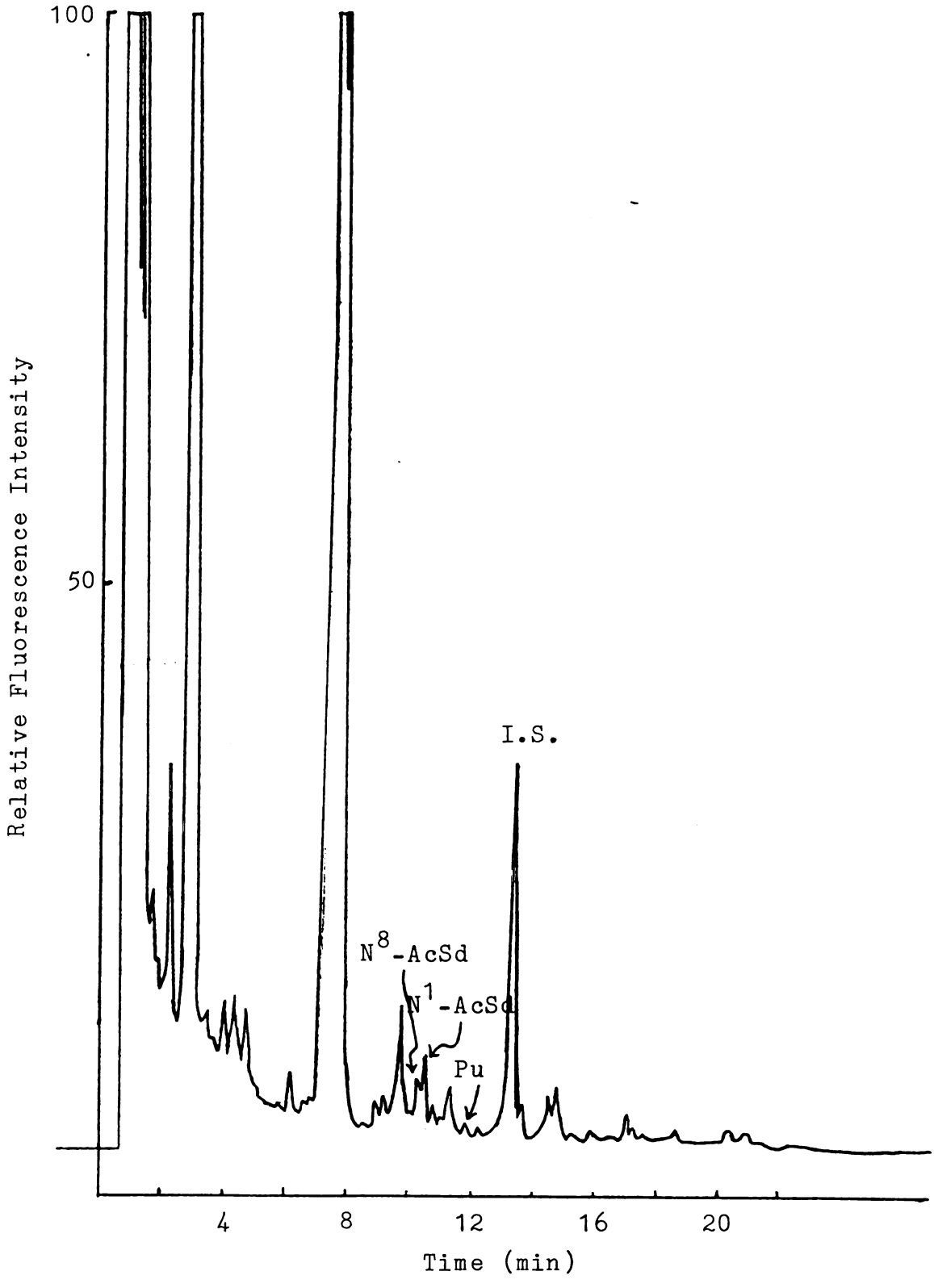


Figure 22. Chromatogram of unhydrolyzed urine.

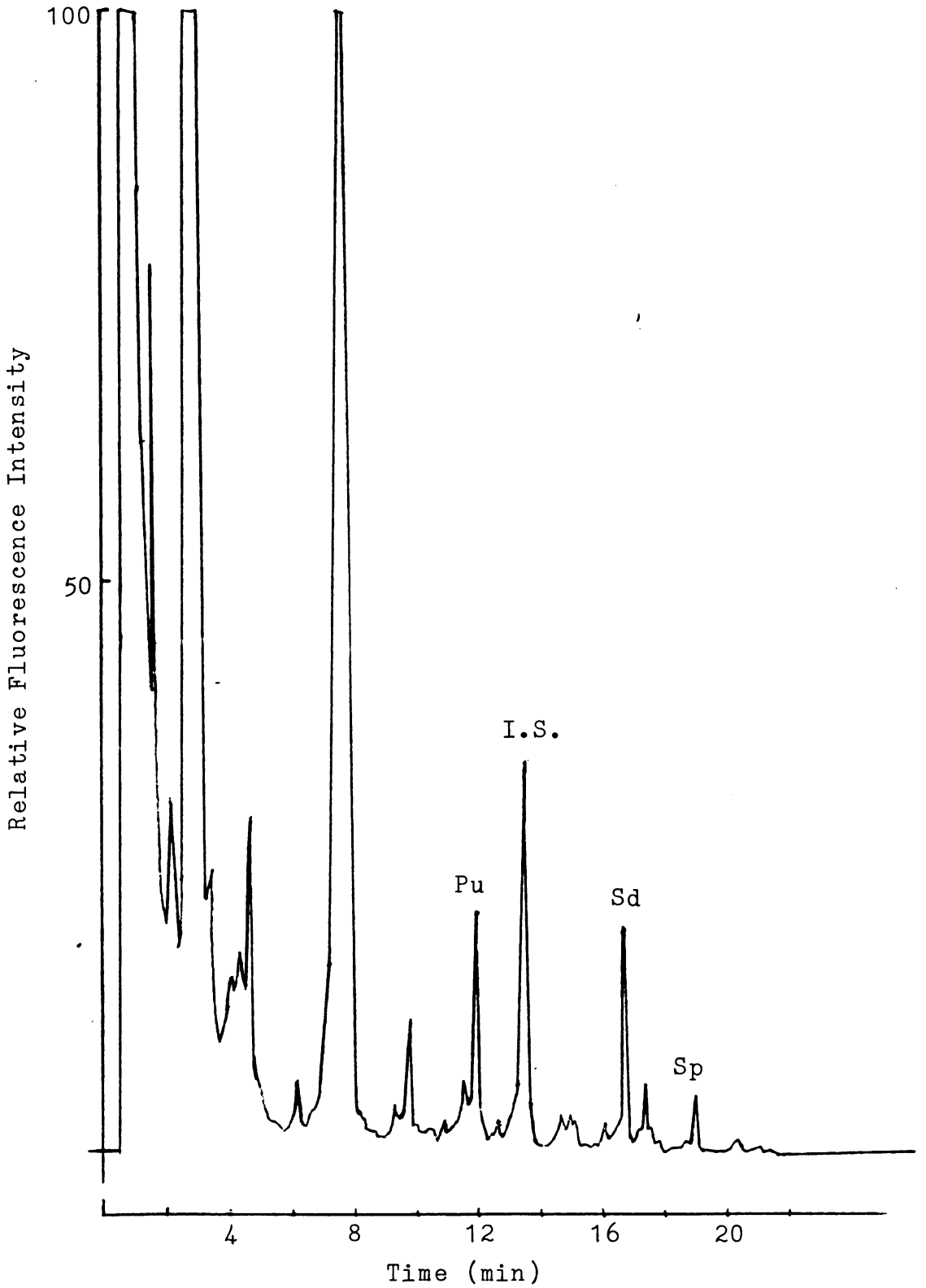


Figure 23. Chromatogram of hydrolyzed urine.

CONCLUSIONS

Monitoring polyamine levels in physiological fluids to study its usefulness in cancer detection and the evaluation of therapy would benefit from a rapid, simple and relatively inexpensive methods. The method should be sensitive and capable of analyzing all polyamines. A variety of methods for the assay of polyamines have been reported. However, most of them are complicated or tedious, and not suitable for routine clinical investigation. Seiler, in his review of assay procedures for polyamines in urine, serum and CSF (122), concluded that none of the methods based on paper chromatography, thin-layer chromatography or paper electrophoresis were appropriate for routine assay of urine polyamines. Although gas-liquid chromatography has proven sensitivity and resolution for polyamine analyses, it requires tedious and time-consuming cleanup steps. Gas chromatography-mass spectrometry is the most specific technique currently available, but the equipment cost for such an assay is high and instrument down-time is an important factor in its practical application. Radioimmunoassay is very sensitive and rapid and seems to be very suitable for screening purposes. However, it has problems of cross-reactivity and is dependent on the availability of specific antibodies for each of the amines; thus for a putrescine assay is not available. Ion-exchange chromatography with amino acid analyzer is presently favored, especially in its most advanced modification, because it requires the least sample preparation. However, the equipment is expensive and the analysis time is relatively long (approximately 1 hr per analysis). In the present

work, we analyze polyamines using pre-column derivatization with dansyl chloride followed by C_{18} column cleanup. Of the available HPLC methods for the simultaneous quantitation of polyamines, it requires the least sample preparation. The method is relatively rapid, requiring only 25 min with little sample preparation. Sensitivity to 0.12, 0.11, 0.08 and 0.06 pmole of putrescine, cadaverine, spermidine and spermine, respectively, is excellent. This procedure compares well with ion-exchange chromatography with amino acid analyzer for urine and tissue extracts, and shows promise for CSF.

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