Determination of Erythrocytic Polyamines by Reversed-Phase Liquid Chromatography
Lucile Gerbault

A simple and rapid semiautomated procedure for determining polyamines in erythrocytes by high-performance liquid chromatography is described. Putrescine, spermidine, and spermine are converted to fluorescent dansyl derivatives, extracted with cyclohexane, and separated in <10 min on a reversed-phase C_{18} ODS column, with an acetonitrile-water gradient as the mobile phase. The method showed a coefficient of variation of 2.73% for spermidine and 3.27% for spermine. The respective reference values, evaluated in 10 healthy patients, were 7.88 (SD 2.09) and 5.42 (SD 1.55) μmol/L of packed erythrocytes. Only negligible amounts of putrescine were found.

Additional Keyphrases: spermidine • spermine

Because circulating free polyamines are principally transported in blood by erythrocytes (I), clinical interest in polyamine determination in this cell compartment for diagnosis and follow-up of cancer patients has been reported by several authors. Increased concentrations of spermidine and spermine have been found in patients with various cancers (2, 3), lung cancer (4), hepatic tumors (5), acute leukemia (6), and prostatic cancer (7).

Moreover, the diagnostic and therapeutic importance of these amines in neuro-oncology (no other tumor marker being available) has been especially emphasized by Moulinoux et al. (6, 9) and confirmed by Elworthy and Hitchcock (10). The polyamines are most often assayed in erythrocytes by a sensitive high-performance liquid chromatographic (HPLC) method, based on their separation as dansyl derivatives and fluorometric detection, as described by Saeki et al. (2).

Some modifications to that method have been developed to reduce the sample preparation and the elution time, while maintaining satisfactory resolution and precision. Here I describe the detailed procedure of a fast and simple semiautomated method suitable for routine analysis.

Materials and Methods
Chemicals. Putrescine, spermidine, cadaverine, tyramine, 3-hydroxytyramine, histamine, noradrenaline, 5-hydroxytryptamine, 1,7-diaminoheptane and 1,6-diaminohexane as hydrochloride salts, aminobutyric acid, and dansyl chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Spectrophotometric-grade acetonitrile, benzene, ethylacetate, hexane, and cyclohexane were purchased from Merck (Darmstadt, F.R.G.). All other chemicals were analytical reagent grade.

Instruments. Analyses were performed with an HPLC (Model 5000; Varian, Walnut Creek, CA) equipped with a solvent-delivery pump. Samples were injected in a syringe-loading sample injector with a 50-μL loop (Model 7125; Rheodyne, Cotati, CA) onto a 25 cm × 4.6 mm prepacked column (Ultrasphere ODS C_{18}, 5-μm particles; Beckman, San Ramon, CA). Eluted dansyl amines were detected with a spectrofluorometer (JY3D; Jobin Yvon, Longjumeau, France) equipped with a xenon lamp and a 9-μL flow cell. Chromatograms were recorded with a Servotrace chart two-channel recorder (Sefram, Paris, France).

Sample preparation. A 5-mL sample of blood was collected into heparinised tubes and was processed within 3 h. After centrifugation (2500 × g for 10 min at 4°C), the plasma was removed, the leukocytes were carefully discarded, and the erythrocytes were resuspended in four volumes of isotonic saline (0.15 mol/L NaCl). After gently mixing them by inversion, I re-centrifuged the suspended erythrocytes as above, then added 1 mL of distilled water to 0.5 mL of packed erythrocytes and hemolyzed the cells by vortex-mixing them for 30 s. Proteins were removed from the hemolysate by adding 1 mL of ice-cold perchloric acid (100 g/L). After shaking the sample and centrifuging it at 3000 × g for 10 min, I removed the supernate and froze it at −30°C.

Standard solutions. To prepare a stock polyamine standard solution, I diluted 100-fold a solution of 120 μmol of putrescine, 100 μmol of spermidine, and 60 μmol of spermine with distilled water.
of spermine already dissolved in 10 mL of 50 g/L perchloric acid reagent. These two solutions are stable indefinitely when frozen at −30 °C. I diluted this stock solution to obtain five working solutions with final concentrations (μmol/L) of 1.2, 2.4, 3.6, 4.8, and 9.6 for putrescine; 1.2, 3, 4, and 8 for spermidine; and 0.6, 1.2, 1.8, 2.4, and 4.8 for spermine. Aliquots (0.5 mL) of each solution were frozen at −30 °C and were stable for three months.

Internal standard. A 10 mmol/L solution of 1,7-diaminoheptane was prepared and diluted 50-fold. The working solution was prepared by diluting 0.2 mL of this solution in 5 mL of water. This solution could be kept for as long as a week at 4 °C without significant changes. All other solutions were diluted in 50 g/L perchloric acid, not water, and kept frozen at −30 °C.

Derivatization and extraction of dansyl derivatives. I added successively 0.12 mL of internal standard working solution, 0.3 mL of saturated sodium carbonate, and 1 mL of dansyl chloride (5 g/L in acetone) to 0.5 mL of supernates (from erythrocytes) or standard solutions. After vortex-mixing, I left the samples to form derivatives overnight at room temperature. The dansyl amides were extracted with 2 mL of cyclohexane. The cyclohexane phase was then removed and evaporated to dryness at 50 °C under a stream of nitrogen.

Quantification of polyamines. The residues were dissolved in 250 μL of acetonitrile in water (70/30 by vol); 50 μL was injected onto a reversed-phase Ultrasphere ODS column previously equilibrated with the same solution. Gradient elution was performed, the concentration of acetonitrile solution increasing linearly to 100% over 10 min at a flow rate of 2 mL/min. The column was then reequilibrated with the initial mobile phase for 2 min. The eluted dansyl derivatives were detected by a spectrofluorometer at 342 and 512 nm (excitation and emission wavelengths). The ratios of fluorescence peak heights of polyamines/internal standard were plotted against the amount of each polyamine added.

Results

As shown in Figure 1, the mixture of dansyl derivatives of polyamines in a standard solution and in packed erythrocytes is well separated in a total analysis time of <10 min. The limit of detection (twice the signal-to-noise ratio) was 15 pmol of spermidine and spermine injected, corresponding to a concentration of <1 μmol/L of erythrocytes. The content of putrescine in erythrocytes was negligible. Linearity of response to concentration was observed up to concentrations of 14, 12, and 7 μmol/L for putrescine, spermidine, and spermine, respectively, corresponding to concentrations of 70, 60, and 35 μmol/L of packed erythrocytes (Figure 2). As shown in Table 1, the reproducibility and the repeatability of the assay were satisfactory.

Analytical recoveries of putrescine, spermidine, and spermine—measured by addition of standard solutions to packed erythrocytes—were 97%, 94%, and 96%, respectively.

The reference values obtained from 10 healthy volunteers were 7.88 (SD 2.09) μmol for spermidine and 5.42 (SD 1.55) μmol for spermine per liter of packed erythrocytes. These results are similar to those of Moulinoux et al. (8) and slightly less than the normal concentrations indicated by Saeki et al. (2).

Discussion

Only a few methods proposed for the determination of polyamines in erythrocytes deal with precolumn dansylation and fluorometric detection. Killian et al. (7) described a modification of the method of Saeki et al. (2), adding an internal standard. The present study represents a simplification of several steps as compared with those two methods.
Blood samples. The blood sample preparation procedures reported describe five washes of erythrocytes with isotonic saline (2) and three washes with plasma, the concentrations of polyamines in plasma being negligible. To determine whether this last step could be avoided, I compared the results obtained with different blood sample preparations. After centrifugation and removal of plasma and leukocytes, I washed three samples of packed cells once with isotonic saline, three times with isotonic saline, or once with human plasma, then quantified the polyamines in each of these nine samples. Because there were no significant differences between each group of samples, I thus recommend a single wash of the erythrocytes, which saves considerable time.

Dansylation. Dansylation was usually performed overnight at room temperature; however, the same results were obtained with dansylation in a water bath at 50 °C for 45 min. In this latter procedure, the remaining acetone must be evaporated under a nitrogen stream.

In both previous procedures (2, 7), L-proline is used to consume the excess dansyl chloride reagent. In the working conditions described here, it was unnecessary to eliminate this excess, because the fluorometer I used was not affected by it.

Extraction of dansyl polyamines. Benzene (2) and toluene (7), used as extraction reagents previously, are toxic solvents and their use is not allowed in this laboratory. Consequently, I tested other reagents for the extraction of dansyl polyamines: hexane, cyclohexane, and ethylacetate. Compared with benzene, the extraction of putrescine and spermidine was similar for all three solvents, but hexane gave less satisfactory results for spermine. A single extraction with 2 mL of cyclohexane was satisfactory; the recovery with a second extraction was <5% for each polyamine.

Internal standard. Addition of an internal standard was considered necessary to avoid the variability of peak heights because of possible losses of polyamines during the extraction step. 1,7-Diaminohexane was preferred to the 1,6-diaminohexane because of a better resolution for putrescine in these working conditions.

Elution. The retention time of spermine obtained with the elution gradient used by Saeki et al. (2) (from 200 to 1000 mL of acetonitrile per liter) was 25 min. To shorten this elution time while maintaining a good resolution, I tested various elution gradients, with volume ratios of 20/80, 40/60, or 70/30 acetonitrile in distilled water to 100% acetonitrile, at different elution times: 5, 10, 15, and 25 min with a flow rate of 1 or 2 mL/min. Satisfactory results were obtained with an elution gradient from

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\( ^{a} \) n = 10 assays of one blood sample. 
\( ^{b} \) n = 10 successive runs of a standard solution.

![Fig. 3. Calibration curve for dansylated derivatives of polyamines](image)

![Fig. 4. Chromatogram of four polyamines and seven amines (retention time in minutes listed in parentheses): 1, aminobutyric acid (2); 2, putrescine (3.5); 3, cadaverine (3.7); 4, histamine (4.1); 5, internal standard (4.9); 6, serotonin (5.1); 7, tyramine (6); 8, spermidine (6.8); 8, norepinephrine (7.5); 10, 3-hydroxytyramine (8.8); 11, spermine (9.5) Some unidentified peaks are cropped for space considerations](image)
acetonitrile in water (70/30 by vol) to 100% acetonitrile at a flow rate of 2 mL/min. The elution time was then 9.5 min, and the time between sample injections was <12 min instead of the <22 min indicated by Killian et al. (7), and the separation time of 20 min reported by Saeki et al. (2). To test the resolution of the technique, I added cadaverine and six biological amines—tyramine, 3-hydroxytyramine, histamine, norepinephrine, 5-hydroxytryptamine (serotonin), and aminobutyric acid—to a standard solution and analyzed with a linear gradient between acetonitrile in water (70/30 by vol) and 100% acetonitrile over a 10-min period at a flow rate of 2 mL/min. Figure 3 shows satisfactory results.

In conclusion, these modifications simplify the analysis of polyamines. The elution time is shortened, allowing the analysis of five chromatograms per hour, and cyclohexane is substituted for benzene, eliminating a toxic solvent and making this technique suitable for routine assays. This method was used to determine polyamines in erythrocytes from patients with brain cancers. The results of the follow-up of those patients will be published later.

References

Considerations When Measuring Urinary Albumin: Precision, Substances That May Interfere, and Conditions for Sample Storage

Mary Louise W. MacNeill, Patricia W. Mueller, Samuel P. Caudill, and Karen K. Steinberg

The measurement of small but abnormal amounts of albumin in urine is important in evaluating kidney disease in people with diabetes mellitus, hypertension, or possible adverse health effects from exposure to nephrotoxins. Routine laboratory methods for measuring albumin are not sensitive enough to measure the amounts that are significant in urine (<30 mg/L). In our laboratory we used three immunoassays for measuring urinary albumin: enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and immunoturbidimetric assay (IT). We calculated the CVs of the three methods, investigated potential interfering substances at three times their normal concentrations, and stored urine under different conditions to find the best way to protect the sample until assay. The potential interferents we checked were transferrin, urea, β2-microglobulin, retinol-binding protein, creatinine, kappa and lambda light chains, IgG, hemoglobin, ketone, and glucose. The stability study involved two study temperatures (−20 and −70°C) and four treatments (centrifuging or filtering, before or after storage). We found the following: the RIA had the lowest CV; the results from the interference study showed no interference from normal physiological concentrations of the substances investigated; storage at −70°C regardless of the treatment should be adequate to prevent loss of albumin immunoactivity.

Additional Keyphrases: radioimmunoassay · immunoturbidimetry · enzyme immunoassay · variation, source of · sample handling · intermethod comparison

Our laboratory is engaged in an interlaboratory assessment program to study among-laboratories precision and bias in urinary albumin values. Participating laboratories measure albumin for studies of diabetes, hypertension, and environmental and occupational

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