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DETERMINATION OF POLYAMINES IN HUMAN ERYTHROCYTES BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN–PHOSPHORUS DETECTION

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SUMMARY

A capillary gas chromatographic method with nitrogen–phosphorus detection is used to determine simultaneously 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine in human erythrocytes. The compounds are isolated by adsorption on silica and converted into their heptafluorobutyryl derivatives. We give quality-control data and (age-dependent) normal values for 48 apparently healthy controls.

INTRODUCTION

Urinary polyamines have been extensively studied as biochemical markers of cancer [1]. Measurements of polyamines in erythrocytes appear to be the most promising area for assessing their levels in the circulation. Anucleated cells, erythrocytes in particular, do not have a mechanism for the synthesis of polyamines. Therefore their polyamine content must either be residual from their nucleated erythropoietic precursors and/or the result of binding to their membrane [2–5]. The majority of the circulating polyamines are associated with erythrocytes [2–4], leading to the hypothesis that they may serve as passive "carriers" from sites of liberation to sites of conjugation, catabolism, excretion and re-uptake [3,6]. In recent years, it has been suggested that erythrocyte polyamine levels have some clinical utility in malignancies [1,4,6–8]. Additionally, altered patterns of erythrocyte polyamines have been established in numerous non-cancerous disease states, such as chronic renal failure, sickle cell disease, cystic fibrosis and liver disease [1,4,6–8].

The most commonly applied methods for the analysis of polyamines in erythrocytes make use of amino acid analysers and high-performance liquid chroma-

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tographs (for a recent review, see ref. 9). Recently, methods based on gas chromatography (GC) with electron-capture [10] and nitrogen–phosphorus detection [11] have been described. In the latter methods packed columns were used.

Here we describe a capillary GC method with nitrogen–phosphorus detection for the simultaneous determination of 1,3-diaminopropane (DAP), putrescine (Pu), cadaverine (Cad), spermidine (Sd), and spermine (Sp) in human erythrocytes.

EXPERIMENTAL

Reagents

DAP, Pu, Cad, Sd, Sp, 1,6-diaminohexane and 1,7-diaminoheptane were from Sigma (St. Louis, MO, U.S.A.) and bis(3-aminopropyl)amine was from Fluka (Buchs, Switzerland). Sep-Pak silica cartridges were from Waters (Milford, MA, U.S.A.), Carbowax 1000M was from Chrompack (Middelburg, The Netherlands) and heptafluorobutyric anhydride from Pierce (Rockford, IL, U.S.A.). All other reagents were from Merck (Darmstadt, F.R.G.).

Syntheses of internal standards

The internal standards N-(3-aminopropyl)-1,5-diaminopentane (non-symmetrical homospermidine) and N,N′-bis(3-aminopropyl)-1,5-diaminopentane (symmetrical homospermine) were prepared as previously described [12].

Samples and preparation of erythrocyte suspension

Blood samples were collected by venipuncture into EDTA-containing Venoject tubes (Terumo Europe, Leuven, Belgium). The plasma was removed by centrifugation at 800 *g* for 10 min at 4 °C. The buffy coat was carefully removed and the erythrocytes were washed three times with two volumes of 0.9% sodium chloride. After each wash the buffy coat was removed by aspiration. Washed cells were resuspended in an equal volume of 0.9% sodium chloride. Red blood cell (RBC) and white blood cell (WBC) counts were performed by a Coulter counter Model S plus IV, and the samples were stored at −20 °C until analysis.

Prepurification

To a 2-ml aliquot of the final erythrocyte suspension was added an internal standard cocktail, containing 12.5 nmol each of 1,6-diaminohexane, 1,7-diaminoheptane, bis(3-aminopropyl)amine, N-(3-aminopropyl)-1,5-diaminopentane and N,N′-bis(3-aminopropyl)-1,5-diaminopentane in 400 μl of 0.1 *M* hydrochloric acid. After equilibration at room temperature for 30 min, the cell suspension was carefully mixed with 2 ml of 0.47 *M* sulphosalicylic acid solution. After standing for 30 min at room temperature, the samples were frozen, thawed and centrifuged at 800 *g* for 10 min. The supernatant was transferred to a polypropylene tube, and 8 ml of borate buffer (50 m*M*, pH 9.0) were added. The pH of the solution was adjusted to 9.0 by adding a few drops of 4 *M* sodium hydroxide, and passed through a Sep-Pak silica disposable column that was previously washed

with 0.1 *M* hydrochloric acid solution and water. The Sep-Pak column was washed with 25 ml of water and the polyamines were eluted into a 14-ml Sovirel tube with 10 ml of 0.1 *M* hydrochloric acid solution. The eluate was evaporated to dryness at 120 °C under a stream of air, and 200 μl each of acetonitrile and heptafluorobutyric anhydride were added. After standing overnight at 40 °C the isolation of the derivatives from the derivative-containing solution was performed by extraction with dichloromethane, as previously described [12]. The derivatives were dissolved in 200 μl of ethyl acetate containing 0.2% (w/v) Carbowax-1000M. Aliquots (1.5 μl) were analysed by GC with nitrogen–phosphorus detection.

Equipment

GC with nitrogen–phosphorus detection was performed with a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672A automated sampler, and interfaced with a Nelson 3000 Series chromatographic data system (Nelson Analytical, Cupertino, CA, U.S.A.). The capillary column and the GC conditions were the same as previously described [12].

Quantification and quality control

Quantification of the polyamines was done by comparing the peak-area ratio of each analyte and its internal standard with that of the standard [12].

For quality control, we analysed in each series 2 ml of a washed erythrocyte suspension and of the same sample enriched with 12.5 nmol of each polyamine per 2 ml of cell suspension.

Stability of polyamines in washed erythrocyte suspensions

The stability of polyamines in 2-ml aliquots of erythrocyte suspensions (see above) from ten normal healthy adults was investigated. Internal standards and sulphosalicylic acid (see above) were added either immediately or after incubating the suspensions for 18 h at 37 °C. In addition, for one set of 2-ml aliquots, the 0.9% sodium chloride supernatant was replaced by an equal volume of the corresponding thrombocyte-free EDTA plasma and incubated as above. Subsequently polyamines were measured after centrifugation and removal of the plasma. Care was taken not to remove any cells. Further processing and analysis of fresh suspensions and suspensions previously incubated in 0.9% sodium chloride and plasma were done in one series.

RESULTS AND DISCUSSION

Methodology

Fig. 1 shows an example of the GC profiling of polyamines in erythrocytes from a normal healthy adult. Although sometimes only small peaks were encountered for DAP, Pu and Cad, these compounds could reliably be quantified with the data system (see below).

The time elapsing from blood sampling till storage of the final suspension at −20 °C should be as short as possible ($\leqslant$3 h), as a significant decrease in erythrocyte polyamine content, especially of Sd and Sp, may occur in the course of

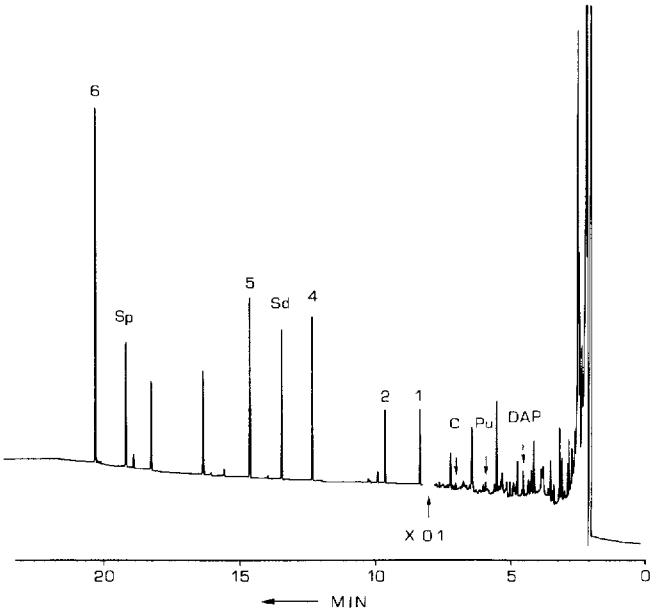

Fig. 1. Capillary gas chromatogram of a heptafluorobutyrated silica extract of erythrocytes from a normal healthy adult. Peaks: 1, 2, 4-6 = added internal standards; DAP = 1,3-diaminopropane; Pu = putrescine; C = cadaverine; Sd = spermidine; Sp = spermine.

time. We found (see Fig. 2) that after 18 h of standing at 37 °C a highly significant decrease of Sp (mean ± S.D., 15.6 ± 7.3%; range 8.7–36.4%) took place for erythrocytes kept in 0.9% sodium chloride, and of Sd (mean ± S.D., 12.0 ± 1.4%; range 9.7–14.4%) and Sp (mean ± S.D., 17.4 ± 3.4%; range 10.6–20.8%) for cells kept in their original EDTA plasma. This observation is most probably caused by

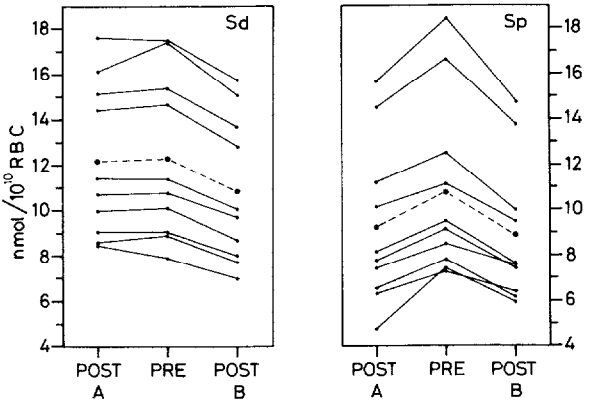

Fig. 2. Erythrocyte content of spermidine (Sd) and spermine (Sp) pre- and post-incubation for 18 h at 37 °C in: (A) 0.9% sodium chloride solution and (B) thrombocyte-free EDTA-plasma, for ten healthy adults (four females and six males). The dashed lines indicate the course of the mean values for the entire population.

TABLE I

WITHIN- AND BETWEEN-SERIES PRECISION AND RECOVERY FOR POLYAMINES IN ERYTHROCYTES FROM NORMAL HEALTHY ADULTS

Between-series quality control was assessed for different samples, and consequently no statistical data on the precision of the endogenous concentration can be given; for each polyamine the enrichment of the erythrocyte suspension amounted to 12.5 nmol per 10^{10} RBCs; n = number of determinations. For abbreviations of analytes, see text; Sd+Sp is the sum of the concentrations of Sd and Sp; PAN is the polyamine nitrogen content equal to three times the Sd content plus four times the Sp content.

Analyte	Within series ($n=6$)				Between series ($n=11$)	
	Concentration (nmol per 10^{10} RBCs)		Recovery (%)		Recovery (%)	
	Mean	C.V.(%)	Mean	C.V.(%)	Mean	C.V.(%)
DAP	0.37	8.1	99.2	2.2	100.6	5.4
Pu	0.22	22.7	101.5	0.9	102.5	6.0
Cad	0.15	20.0	101.2	1.2	100.9	6.2
Sd	12.28	2.0	104.7	2.0	99.0	5.5
Sp	7.91	5.9	112.0	5.0	99.2	7.3
Sd+Sp	20.19	2.8				
PAN	68.50	3.1				

catabolism in the erythrocytes (0.9% sodium chloride) and possibly plasma (incubation in plasma) by amine-oxidases.

In order to obtain a state of equilibrium between endogenous polyamines and their added analogues (internal standards), the samples stood for 30 min at room temperature before and during deproteinization.

The method design, including pretreatment with adsorption on silica gel, derivatization by fluoroacylation and determination by capillary GC with nitrogen–phosphorus detection, was similar to the one previously described [12].

Quality control

Table I gives within-series quality-control data for the erythrocyte polyamine content of a normal healthy adult, together with results of analytical recovery studies for within and between series. The mean recoveries for the various analytes, relative to their respective internal standards, ranged from 99 to 112%.

Normal values

Fig. 3 shows age-dependent normal values for the erythrocyte content of Sd, Sp and Sd plus Sp, expressed in nmol per 10^{10} cells. For some children the erythrocyte levels of Sp and especially Sd were somewhat higher than for adults. This finding is in agreement with data reported for whole blood [13]. For 72% of the population the Sd level was found to be higher than that of Sp. For DAP, Pu and Cad (not depicted) no age dependency was encountered.

Table II summarizes the levels for a group of normal healthy adults (ages 20–66 years). In general, the presented values of Pu, Sd and Sp for the total population

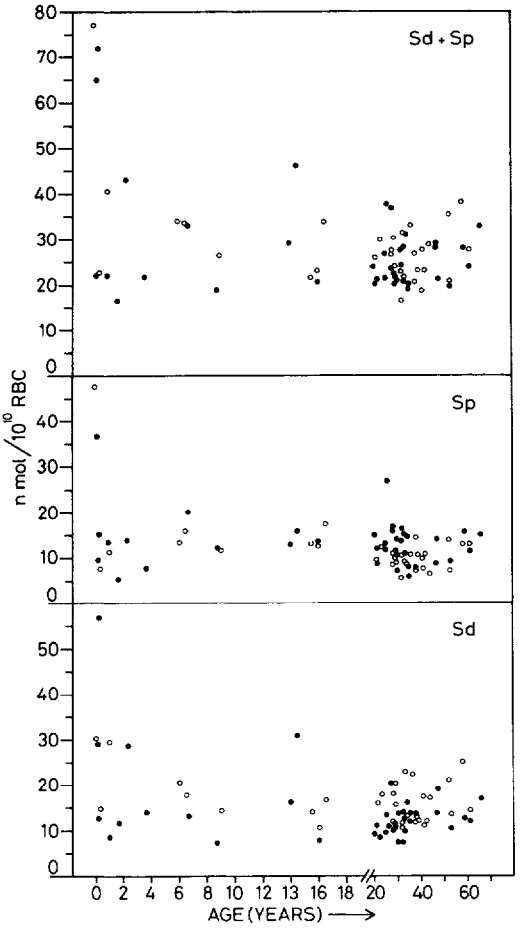

Fig. 3. Age-dependent normal values for erythrocyte content of spermidine (Sd), spermine (Sp) and the sum of Sd and Sp (in nmol per 10^{10} RBCs) from healthy persons (ages 5 days to 66 years). (○) Female; (●) male.

are in reasonable agreement with those reported by others [6,8,13]. Hitherto, no data have been available on the levels of DAP and Cad.

Blood leucocytes contain some 200- to 1000-fold higher amounts of polyamines than erythrocytes [2]. As the mean WBC/RBC ratio for all samples was $0.36 \cdot 10^{-3}$ (range $0.09–0.83 \cdot 10^{-3}$), the mean contribution of leucocyte polyamines (containing ca. 2 nmol of Sd and 4.5 nmol of Sp per 10^7 leucocytes; ref.8) to the final result is estimated to be 5% for Sd and 14% for the Sp content. When only samples with WBC/RBC ratios below $0.5 \cdot 10^{-3}$ were taken into account ($n = 36$; mean WBC/RBC ratio, $0.27 \pm 0.11 \cdot 10^{-3}$) the mean values ± S.D. for Sd and Sp hardly changed. This suggests that, within these limits, the apparent biological variation (Table II) in RBC polyamine content is more pronounced than the variance introduced by the varying contribution of leucocytes. Nevertheless an almost complete removal of the buffy coat after each centrifugation is essential, especially for blood samples with relatively high leucocyte counts.

TABLE II

NORMAL VALUES FOR POLYAMINES IN ERYTHROCYTES OF 48 APPARENTLY HEALTHY ADULTS

Values are in nmol per 10^{10} RBCs The ratio WBC/RBC is the ratio of white blood cells and red blood cells.

Analyte	Females ($n=22$)			Males ($n=26$)			Total population ($n=48$)		
	Mean	C.V. (%)	Range	Mean	C.V (%)	Range	Mean	C V. (%)	Range
DAP	0.96	72 5	0.25- 2.65	0.78	61.8	0.28- 2 17	0 85	71.2	0.25- 2.65
Pu	0 59	72 2	0.17- 1.78	0 42	53 5	0 17- 1 06	0 49	70.5	0.17- 1.78
Cad	0.40	96 2	0.01- 1.98	0.31	58.9	0 06- 0 73	0 35	85.5	0.01- 1.98
Sd	15 80*	27.5	10.75- 25 09	12.34*	26 7	7.14- 20 73	13.93	29.8	7.14- 25.09
Sp	10 04**	24.0	5.73- 14.63	12 65**	34 0	5 80- 26 89	11.46	32 9	5.73- 26.89
Sd + Sp	25.84	21 1	16.48- 38 19	25 00	21.0	19.16- 37.67	25.38	20.9	16.48- 38.19
PAN	87 20	20.9	55.22-127 70	87 62	22 0	63.31-139 93	87.40	21 1	55.22-139 93
WBC/RBC ($\times 10^{-3}$)	0.39	50 4	0.10- 0 83	0.34	51.7	0.09- 0 69	0 36	52 5	0.09- 0.83

*$p=0.0025$.
**$p=0.0075$

For females the erythrocyte content of Sd was found to be significantly higher than for males (Man Whitney U Statistics, one-sided), whereas the Sp content was significantly lower (see also Fig. 3). No differences were found for the other polyamines with respect to sex.

As Sd and Sp are the quantitatively most important erythrocyte polyamines, we also considered the sum of Sd and Sp and the polyamine nitrogen (PAN) content (equal to three times the Sd content plus four times the Sp content). Although there is considerable variation in the erythrocyte Sd and Sp content (see coefficients of variation in Table II), the content of the sum of Sd and Sp, and the PAN were found to vary less. With respect to sex there were no differences for the Sd plus Sp and the PAN contents in erythrocytes. This suggests that there is less variance in the number of erythrocyte polyamine binding sites, while, within certain limits, the identity of the bound polyamine (Sd or Sp) does not seem to be of absolute importance.

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