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DETERMINATION OF FREE AND TOTAL POLYAMINES IN HUMAN SERUM AND URINE BY ION-PAIRING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A RADIAL COMPRESSION MODULE

APPLICATION TO BLOOD POLYAMINE DETERMINATION IN CANCER PATIENTS TREATED OR NOT TREATED WITH AN ORNITHINE DECARBOXYLASE INHIBITOR

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SUMMARY

A sensitive and rapid determination of free and total polyamines (putrescine, cadaverine, spermidine and spermine) in urine and serum is described. The procedure is based on reversed-phase high-performance liquid chromatographic separation using radial compression module (Radialpak C 8). The samples are purified with a silica gel Sep-Pak cartridge. The polyamines are converted to dansyl chloride derivatives and separated using a linear gradient of triethylammonium phosphate—methanol within 15 min. The lower limits of detection are 10 pmoles for spermine and 5 pmoles for other polyamines.

This method is applied to cancer patients treated by cytotoxic chemotherapy with or without difluoromethylornithine (DFMO). All four polyamines are significantly increased in these patients before treatment. On day 8, after onset of treatment, the levels of polyamines in patients not treated with DFMO are more elevated than on day 1, while in patients treated with DFMO the levels are decreased. However, DFMO does not seem to modify the treatment result. The patients which have a low level of putrescine before and during

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treatment, do not respond to treatment. Perhaps this low level is the consequence of conjugation of this polyamine?

INTRODUCTION

The natural polyamines (putrescine, cadaverine, spermidine and spermine) are present in all living cells where they have been particularly implicated in growth processes [1, 2]. They are derivatives of ornithine and methionine. Cadaverine was initially suspected as originating solely via bacterial degradation but it has since been shown to be a product of mammalian cell synthesis [3]. Polyamine synthesis is controlled mainly by the enzyme ornithine decarboxylase. Russell [4] has shown the interest of using these cations in the detection of cancer. It should be noted, however, that the cations are not specific to any particular type of cancer with the possible exception of putrescine in medulloblastoma [5]. Since these first studies, polyamine assay has equally been used to evaluate patient response to therapy and eventually to detect relapse [6]. The assay of polyamines is also interesting in certain other pathological conditions such as growth retardation [7], and in mental illness such as schizophrenia [8]. In this latter case it is cadaverine and its conjugated derivatives which are found in large quantities in serum.

For several years now there has been a need to develop and to improve the techniques of polyamine assay. High-performance liquid chromatography (HPLC) coupled with fluorometric detection presents strong sensitivity and good specificity as a technique for polyamine assay [9, 10]. In the studies concerning cancer, the authors have preferred to determine urinary polyamines which are found in greater quantities than blood polyamines. Also there are few methods permitting the determination of serum cadaverine levels [11].

In this paper we describe a fast and reproducible method which permits the assay of the four cations in their free forms, or freed by hydrolysis, in serum and in urine. The method involves HPLC in reversed phase with a radial compression module and a mobile phase (triethylammonium phosphate—methanol) that is used to separate polyamines already dansylated. This technique has been used particularly in evaluating the effect of an ornithine decarboxylase inhibitor [12], difluoromethylornithine (DFMO), coupled with cytotoxic chemotherapy in cancer patients.

MATERIAL AND METHODS

Apparatus

HPLC was performed on a Waters chromatograph composed of two 6000 A pumps, a U_6K injector and a solvent progammer Model 720, a radial compression module RCM 100 equipped with a cartidge Radialpak C 8 (10 μm) with an internal diameter of 5 mm. Detection was accomplished using a spectrofluometer Aminco SPF 500 equipped with a continuous flow cell of 8 μl and an Aminco SPF 500 recorder (excitation wavelength 350 nm, emission 500 nm).

Reagents

Putrescine, cadaverine, spermidine and spermine, and 1,6-hexanediamine (internal standard) were obtained from Fluka. The standard solution contained 10 mmoles/l of each polyamine and 12 mmoles/l of 1,6-hexanediamine in 0.1 *N* HCl.

Mobile phase

The products were of ultrapure quality. Buffer, triethylammonium phosphate (TEAP) 0.25 *N*, pH 3.5, was filtered through a 0.22-μm Millipore membrane before use; methanol was filtered through a 0.45-μm Millipore membrane. TEAP and methanol mixtures were prepared as follows: solution A was TEAP—methanol (50:50), and solution B TEAP—methanol (20:80). The mixtures were degassed with helium or in an ultrasound bath immediately prior to chromatography. A linear gradient curve (curve no. 6) was realized between solutions A and B going from 0 to 100% of B in 10 min with a flow-rate of 1.5 ml/min. The pressure at the head of the column varied from 40 to 60 bar during the gradient.

Purification of the biological samples

The samples were purified using a Sep-Pak cartridge (Waters) [13].

Urine. Twenty-four-hour urine samples were collected in polyethylene bottles containing 10 ml of 1 *N* HCl and kept at −20° C until assay.

For the free polyamines, a 2-ml aliquot to which were added 100 μl of internal standard, was filtered through a GS Millipore membrane (0.22 μm) and the pH was adjusted to 9 with concentrated NH_4OH. The sample was then loaded onto the Sep-Pak; this was washed with 10 ml of water and the polyamines were eluted with 10 ml of 0.1 *N* HCl. The eluent is lyophilized to prevent any damage resulting from a too slow evaporation. The dried residue was dissolved in 200 μl of 0.1 *N* HCl and was ready to be dansylated.

To determine total polyamine concentration, 2 ml of urine were submitted to hot acidic hydrolysis in 6 *N* HCl at 110° C for 12 h. The hydrolysate was centrifuged and evaporated at 40° C in a rota-vapor. The residue was dissolved in 1 ml of 0.1 *N* HCl and purified with Sep-Pak as described above.

Serum. The blood was collected in polyethylene tubes without anticoagulant and immediately centrifuged; the serum is conserved at −20° C until assay.

For the free polyamines, serum is deproteinized according to the procedure of Kai et al. [11]; 100 μl of internal standard, 1 ml of distilled water and 200 μl of 3 *M* $HClO_4$ were mixed with 1 ml of serum. After centrifugation at 4° C (2000 *g* for 10 min), the supernatant was adjusted to pH 7 with 1.5 *M* KOH and left at 4° C for 30 min, then centrifuged again. The supernatant was adjusted to pH 9 with concentrated NH_4OH and was purified as described above using Sep-Pak.

Determination of the total polyamine content was carried out using hydrolysis in the same manner as described for urine.

Formation of fluorescent derivatives

The purified sample was mixed with 200 μl of 0.1 *N* HCl and placed in a siliconized glass tube; 100 μl of a saturated solution of sodium carbonate and

400 μl of dansyl chloride (Dns-Cl) (a 20-mg/ml solution in acetone of 5-dimethylaminonaphthalene-1-sulfochloride) were added. The tubes were stoppered and placed in darkness at 54°C for 1 h. Excess free Dns-Cl was removed by the addition of 100 μl of a proline solution (150 mg/ml) and the tubes were left in darkness for an additional hour. The dansylated derivatives were extracted using 500 μl of ethyl acetate. After centrifugation, 20 μl of extract were directly injected for urine determinations. For serum, 20—80 μl could be injected depending on polyamine concentration.

Calculations

Polyamine level was automatically calculated by the Waters Model 730 data module using the ratio of the area of the polyamine peak to the area of the internal standard peak.

Normal subjects

The method was applied to both serum and urine samples from healthy subjects.

Cancer patients

Twenty-three men with neck and head cancers (mean age 54.5 years ± 7) and 15 women with breast cancers (mean age 59 years ± 11). All these patients had advanced cancers. One group of patients was treated with cytotoxic chemotherapy and difluoromethylornithine (DFMO, Merrel Laboratory) 10 g per 24 h given four times a day, the other group received chemotherapy only. During treatment, patients with neck and head cancers received chemotherapy (cis-platinum + vincristine + adriamycin + bleomycin) 6 days a month. Patients with breast cancers received chemotherapy (adriamycin + vindesine) one day a month.

Treatment was considered as being positive if after a period of two months there was a verifiable amelioration of the patient's general condition and a regression of $\geqslant$ 50% of metastases. The treatment was considered as having failed if during the two months there was no change or there was an increase of tumor mass.

All of the patients had one blood polyamine determination prior to treatment (day 1) and several determinations were performed during the follow-up of their disease (eight dosages were performed for each patient).

Statistical evaluation

We used the non-parametric Mann—Whitney *U* test [14].

RESULTS

Fig. 1 shows a chromatogram of a standard polyamine solution. The dansylated derivatives are well separated in less than 15 min with a linear gradient of TEAP—methanol, pH 3.5, in 10 min and isocratic conditions until 15 min. At pH 4 the capacity factor is less (Fig. 2) but the separation of putrescine and cadaverine is not satisfactory.

Table I gives the retention times measured on ten different days for a

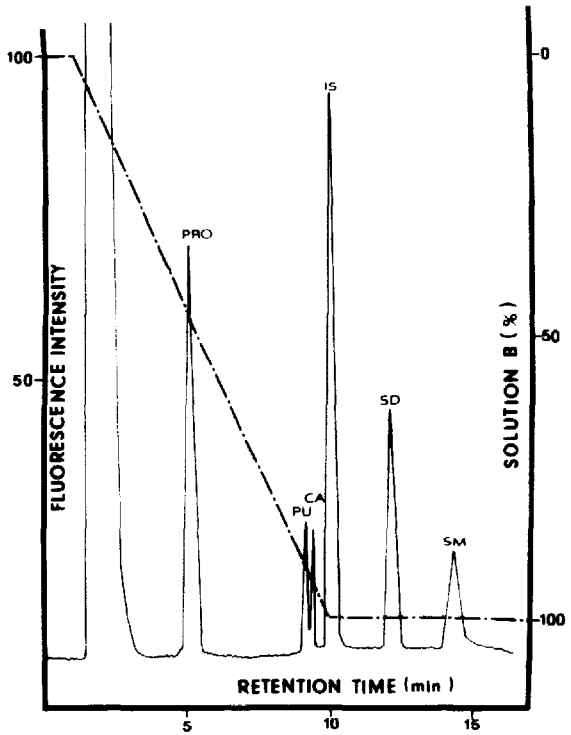

Fig. 1. Reversed-phase HPLC separation of the dansylated derivatives of a standard polyamine solution (100 pmoles per 20 μl), using a Radialpak C 8 column. Mobile phase: 0.25 N TEAP—methanol pH 3.5 (solution A = TEAP—methanol, 50:50; solution B = TEAP—methanol, 20:80). Flow-rate = 1.5 ml/min. PRO = proline, PU = putrescine, CA = cadaverine, IS = internal standard, SD = spermidine, SM = spermine.

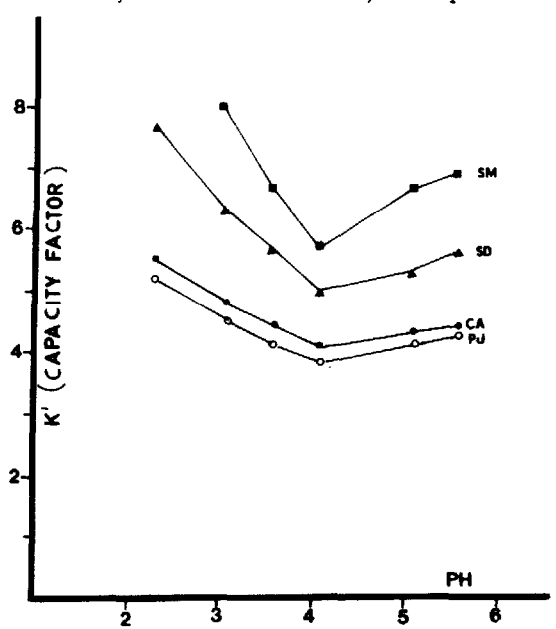

Fig. 2. Variations in capacity factor (k') versus pH with a Radialpak C 8 column.

TABLE I

RETENTION TIMES (MIN) OF PUTRESCINE, CADAVERINE, SPERMIDINE AND SPERMINE

Injection of a standard solution (100 pmoles per 20 μl) on ten different days using a Radialpak C 8 column. The internal standard has a retention time of 9.95 ± 0.18 min. $n = 10$.

	Putrescine	Cadaverine	Spermidine	Spermine
Mean	9.25	9.56	12.08	14.26
S.D.	±0.06	±0.15	±0.17	±0.19
C.V. (%)	0.64	1.65	1.40	1.33

TABLE II

PERCENTAGE RECOVERY OF STANDARD POLYAMINES ADDED TO SERUM AND URINE

The values represent the difference between the levels of polyamines in the biological fluid with and without load (each result is the mean of two assays).

Polyamine	Quantity (pmoles) of polyamine added to 2 ml of urine			Mean recovery (%)
	40	80	160	
Putrescine	36	78	162	96
Cadaverine	39	84	182	101
Spermidine	42	79	158	101
Spermine	30	62	145	81
	Quantity (pmoles) polyamine added to 2 ml of serum			
	40	80	160	
Putrescine	42	83	149	100
Cadaverine	25	77	168	88
Spermidine	25	71	154	82.5
Spermine	20	72	125	72.6

standard polyamine solution. A linear response is obtained for each polyamine for concentrations of 10—250 pmoles injected. This range corresponds to the values that were found in normal and pathological blood samples.

Chromatographic reproducibility is good because a standard solution containing 100 pmoles of each polyamine injected on ten different days ($n = 10$) gives for putrescine, cadaverine, spermidine and spermine, respectively, a coefficient of variation of 2, 3, 4 and 7%.

The limit of detection, which is defined as the minimum quantity of injected polyamine which gives a signal ratio to the background noise superior to 2, is 10 pmoles for spermine and 5 pmoles for the other polyamines. After purifica-

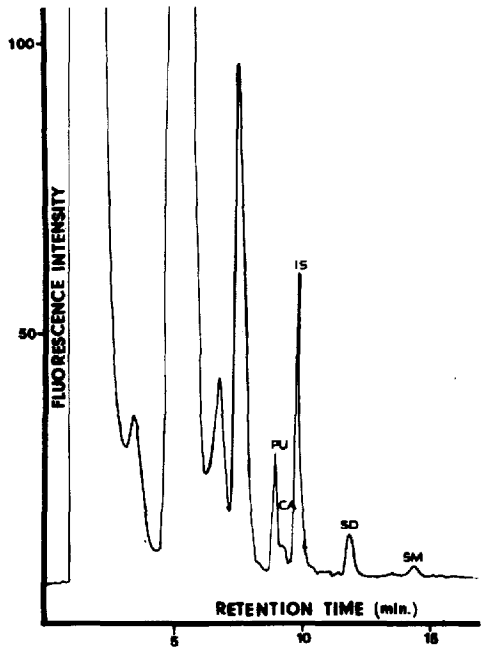

Fig. 3. Chromatogram of the free polyamines obtained from normal human plasma. Experimental conditions are defined in Materials and methods.

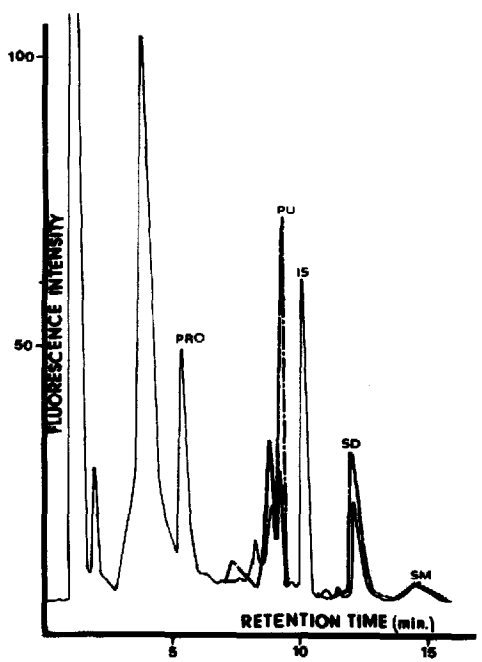

Fig. 4. Chromatogram of the free (——) and total (— · —) polyamines obtained from a 24-h urine sample (normal subject).

TABLE III

FREE POLYAMINE LEVELS FOUND IN NORMAL HUMAN SERUM, AND FREE AND TOTAL POLYAMINE LEVELS FOUND IN NORMAL HUMAN URINE

	Concentration in serum (nmoles/l)			
	Putrescine	Cadaverine	Spermidine	Spermine
Mean	155	7.72	45	17
S.D.	±122	±15	±35	±16
Range	42—388	0—45	19—104	0—52
(n = 11)				
	Urinary concentration (mmoles/mg creatinine)			
	Putrescine	Cadaverine	Spermidine	Spermine
Free polyamines				
Mean	1.49	0.015	0.87	0.46
S.D.	±0.56	±0.04	±0.71	±1
Range	0.83—2.43	0—0.12	0.22—2.06	0—2.91
(n = 12)				
Total polyamines				
Mean	4.86	0.54	3.31	0.99
S.D.	±0.88	±1.05	±2.37	±1.35
Range	3.31—5.81	0—2.64	0.83—6.97	0—3.62
(n = 12)				

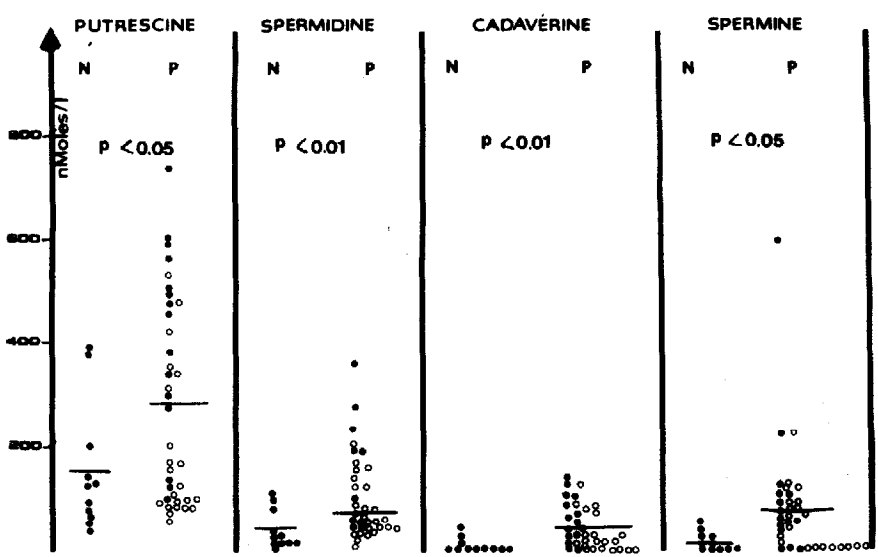

Fig. 5. Pre-treatment serum polyamine levels in cancer patients (P). (•), Breast cancers; (○), neck and head cancers. Comparison with normal subjects (N).

tion on Sep-Pak and extraction of the dansylated derivatives, the percentage recovery is 81—100% for the polyamines added to urine and 72—100% for the polyamines added to serum (Table II).

This technique has been applied to the urine and serum of normal subjects (Figs. 3 and 4, and Table III).

TABLE IV

SERUM POLYAMINE LEVELS IN PATIENTS WITH BREAST OR HEAD AND NECK CANCERS

The differences between the two groups were not significant.

	Polyamine concentration (nmoles/l)			
	Putrescine	Cadaverine	Spermidine	Spermine
Breast cancers (n = 15)				
Mean	412	59.8	133	106
S.D.	±193	±48	±105	±160
Head and neck cancers (n = 22)				
Mean	176	32	75	52
S.D.	±144	±36	±22	±59

TABLE V

VARIATION IN POLYAMINE* LEVELS ON DAYS 8 AND 30 IN PATIENTS RECEIVING OR NOT RECEIVING DFMO WITH TREATMENT

The results are expressed as percentages compared to day 1.

	With DFMO				Without DFMO			
	Pu	Ca	Sd	Sm	Pu	Ca	Sd	Sm
Day 8	−36	−27	−81	−89	+54	+19	+5	+10
	−30		−22		+42	−8	+140	+141
	−57	−45	+50	−100	+11	+400	+59	+400
	+110	+462	+22	+109	+128	0	−31	0
	−40	0	−69	−44	+35	+69	+108	+9
	+307	+400	−22	−13	+13	+166	+690	0
	−59	−13	−2	0	+22	0	+24	0
	−59	−100	−150		+17	−11	−37	+11
Day 30	−79	−100	−89	−100	−16	−58	−54	−89
	−39	0	0		−66	−100	−16	−100
	−74	−100	+14	−24	−49	0	−35	
	−86		−41	−38	−73	−95	−39	
	−17	−22	−16	0	−25	−23	+61	+158
	−79	−60	−12.5	−100	−91	−61	−16	−100
	−15	−100	−69	0	−38	−25	−51	−100
	−8	0	+400	+400				

*Pu = putrescine, Ca = cadaverine, Sd = spermidine, Sm = spermine.

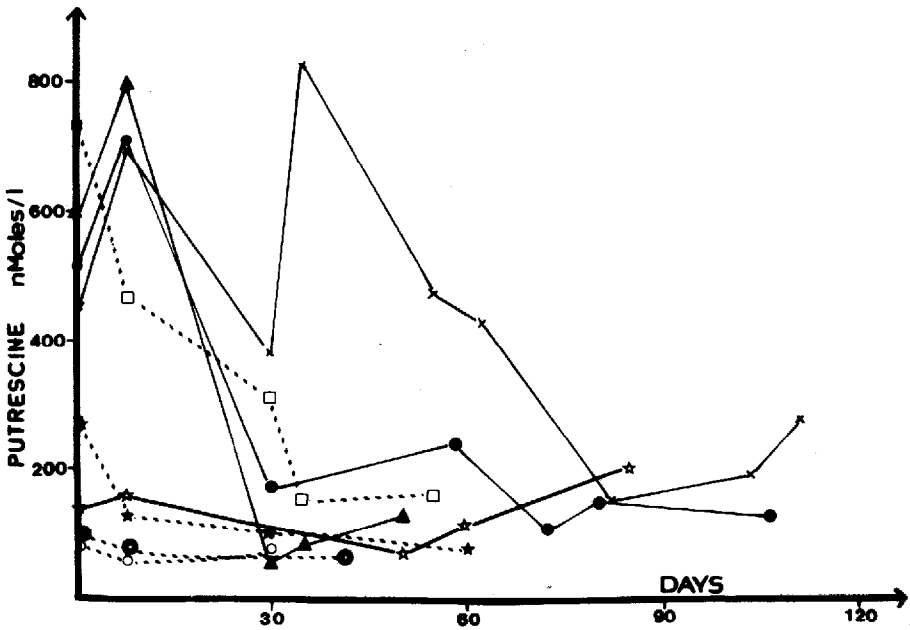

Fig. 6. Serum putrescine levels in eight cancer patients during induction treatment. (——), Without DFMO; (●●●), with DFMO. Patients in which treatment brings about no remission (○, ★, ☆).

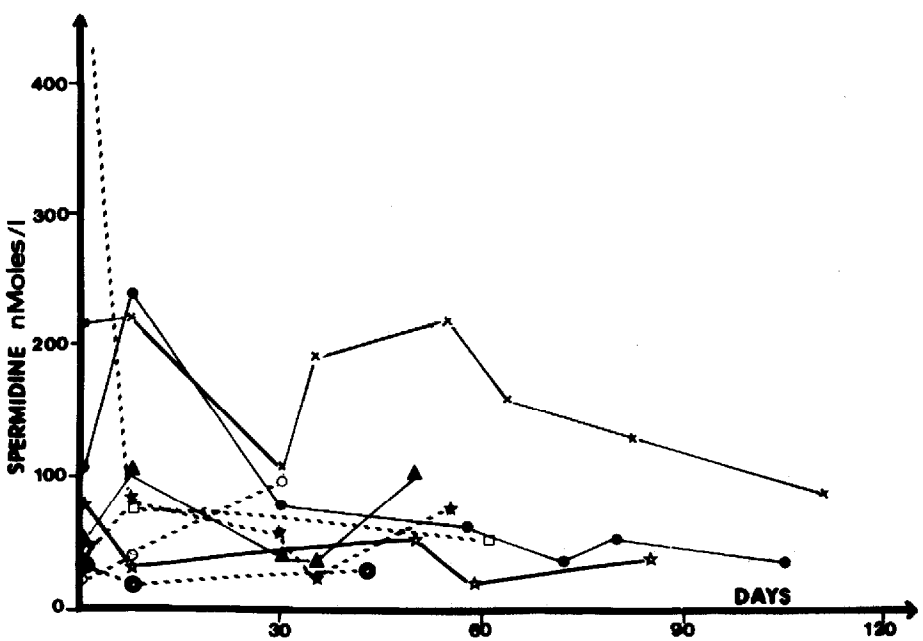

Fig. 7. Serum spermidine levels in eight cancer patients during induction treatment. (——), Without DFMO; (●●●), with DFMO. Patients in which treatment brings about no remission (○, ★, ☆).

The results showed that levels in cancer patients before treatment were significantly higher than those in normal subjects (Fig. 5). Although, one must note, an important overlap exists between these two groups. The average values found in the breast cancer patients were higher for each polyamine than those found in patients with neck and head cancers (Table IV); however, the difference between these two groups was not significant. Table V shows polyamine variations, days 8 and 13, in patients who were treated or were not treated with DFMO. On day 8, patients treated with DFMO have, in general, a lower level of polyamines when compared to the first day. On the other hand, patients who were not treated with the inhibitor presented an increasing polyamine level.

On day 30 the polyamines are lowered in both cases, and in general the four polyamines vary in the same direction. Figs. 6 and 7 show the evolution of putrescine and spermidine in some patients during several months. The two polyamines evolve in a comparable manner.

Patients who have a low level of putrescine prior to any treatment and show no modification in their putrescine level during the course of treatment, are patients in which treatment brings about no remission. These patients have also a low spermidine level. For this second polyamine there is more overlap between patients presenting a remission and those in which the disease evolves.

DISCUSSION

In the different methods of polyamine assay using HPLC already discussed, classic columns were used, i.e. Bondapak C_{18} [15] and LiChrosorb RP-18 [11]. We chose a radial compression module because its small cartridge size permits an increase in flow-rate without an increase in pressure. Thus the equilibration time of the column, as well as the lapse time for the assay are shortened; therefore there is a gain of time in comparison to classic C_{18} columns. With daily use the Radialpak C 8 column has an effective lifetime of at least six months; however retention time varies from one column to another and each column must be standardized.

Stationary phases which are more polar such as CN [13] have also been recommended by certain authors. However, with the mobile phase which we used (TEAP—methanol) a CN Radialpak column does not efficiently separate the dansylated polyamine derivatives while a Radialpak C 8 column gives a good resolution in less than 15 min.

After having tested the mobile phases that are habitually used (distilled water—methanol; acetic acid—methanol; Pic B7 (Waters)—methanol) with the Radialpak C 8 column and having no success, we chose a technique using ion-pairing TEAP—methanol, ion-pairing having previously been applied to peptides [16]. In effect, phosphate ions at acid pH form a system of ion-pairing with the cationic polyamines. This diminishes the hydrophobic dansylated derivatives' retention time and thus assures a good separation. In addition, it is possible that the triethylamine recovers the free polar groups of the stationary phase, therefore giving better reproducibility.

Orthophthalaldehyde (OPA) has also often been retained in the formation of fluorescent polyamine derivatives [17]. The results using a Radialpak C 8

column are more reproducible with Dns-Cl than with OPA. In addition, the dansylated derivatives are stable, preparation is faster and they can be stored in ethyl acetate in darkness at 4°C for up to a week. The glass tubes used for this step and for hydrolysis must be siliconized because the polyamines are easily adsorbed onto glass.

The values we found for both the blood and urine of normal subjects in good health are comparable to those found by other authors [6, 18, 19]. However, some authors seemed to have found higher urinary polyamine values [20].

This polyamine assay technique was applied to blood from cancer patients. Most studies on polyamine variations in cancer patients were on urinary polyamines, in particular the study of putrescine and spermidine [6, 21, 22]. Our study on blood polyamines has shown that, as found with the urinary polyamines in cancer patients, blood polyamine levels are significantly elevated. We have also determined that this elevation was sustained by all four polyamines, confirming that cadaverine is also synthesized in man.

In our experience, blood polyamine levels of putrescine are more strongly and regularly elevated than those of the other polyamines. This fact was not demonstrated by the results of Bakowski et al. [19] because their polyamine assay technique shows interference at the level of putrescine. It seems, however, that the type of cancer has an influence on the level of blood polyamines found. Analogous results have been shown with urinary polyamine levels [23].

During treatment of cancer using chemotherapy, different authors have observed that urinary polyamines show a peak in the 24—48 h following onset of treatment [24]. We have ascertained that 8 days after the onset of chemotherapy, the plasma level of the four polyamines assayed remains elevated in the blood while the level of blood polyamines in patients treated with DFMO falls rapidly. This diminution generally concerns all four polyamines. However, this rapid drop has no prognostic significance in that there was no more remission in patients who were treated with DFMO than in those patients who received chemotherapy alone. We have also determined that those patients with low blood levels of putrescine and spermidine prior to any treatment and during treatment, are, contrary to any results found in the urine level [21], patients who do not respond to treatment. This difference can in part be explained by the fact that in the blood we measured the free polyamines, whereas in urine it is the total polyamines that are measured and are found to be elevated. It seems that in these patients there could be a modification in the conjugation of these polyamines [25] or in their clearance.

These results are not in accordance with the hypothesis of Russell who supposes that putrescine might reflect recruitment of tumor growth and spermidine reflect tumor cell death. Also one must certainly consider other difficult accessible parameters such as the speed of polyamine renewal, their cellular compartmentalization and the cell cycle [26].

In conclusion, the proposed assay for putrescine, cadaverine, spermidine and spermine permits the measurement of these cations in serum. Blood is often easier to obtain than 24-h urine samples, whose collection often poses problems. The results obtained show that the levels of these compounds are elevated in cancer patients and that these elevations concern all four cations.

It should be noted, however, that the elevations are variable from one type of cancer to another and that chemotherapy involves an increase of all the serum polyamines which lasts longer than the increase observed in the urine. DFMO, an ornithine decarboxylase inhibitor, masks the elevation of polyamines in subjects undergoing chemotherapy without, it seems, modifying the underlying evolution of their disease.

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