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Determination of polyamines in human saliva by high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatographic method for the determination of polyamines (spermine, spermidine and putrescine) in human saliva was developed. This method is based on pre-column derivatization with *o*-phthaldialdehyde (OPA). The derivatives were separated on a Nucleosil ODS column (250×4.6 mm I.D.; 5 μm). The gradient elution was performed with two mobile phases A (water) and B (methanol) at a flow rate of 0.8 ml/min. The column eluate was monitored by fluorescence detection (excitation, 360 nm; emission, 510 nm). The within- and between-assay coefficients of variation for all the compounds were below 5%. The detection limits for spermine, spermidine and putrescine were 0.04, 0.05 and 0.06 nmol/ml, respectively. The recovery was greater than 90%. Our analytical technique requires neither preliminary extraction with an organic solvent, nor long multi-step procedures. For saliva samples, this is a simple, rapid and highly reproducible method that can be easily applied to the routine determination of salivary polyamines, whose levels increase early in several pathological conditions.

Keywords: Polyamines; Spermine; Spermidine; Putrescine

1. Introduction

The aliphatic polyamines spermine (SP), spermidine (SPD) and putrescine (PUT) play an important role as cofactors in several biochemical processes associated with cellular activities and proliferation. They act as regulatory molecules in several steps of DNA, RNA and protein synthesis which are themselves intimately connected with the growth rate [1,2]. Since the presence of polyamines in buccodental fluids has been well established [3], it is conceivable that their levels could increase in several pathological proliferative processes. The analytical determination in human saliva of these mediators may be of particular interest in some pathologies of oral cavity tissues associated with an intense metabolic activity, such as gingival hypertrophy or tumors of the maxillofacial area. Therefore, the availability of a rapid and convenient procedure for routine analysis of these compounds in saliva allows the diagnosis and prognosis of these pathological processes to be early and correctly performed.

To our knowledge, until now it has not yet been

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reported any method about SP, SPD and PUT measurement in human saliva. In fact, though numerous HPLC techniques concerning the assay of polyamines have been published, they were applied to other biological fluids such as plasma [4], urine [5,6], seminal liquid [7], cerebrospinal fluid [8,9], bile [10], colostrum [11].

The present work bears evidence of a new method developed by us for the determination of salivary polyamines using *o*-phthaldialdehyde (OPA) as fluorogenic derivatizing agent. This analytical technique offers advantages of great simplicity and rapidity, high degree of versatility and adequate sensitivity and specificity.

The validity of this method was tested by analysis of 50 human saliva samples.

2. Experimental

2.1. Apparatus and chromatographic conditions

The HPLC system consisted of two pumps model LC-10 A*i* (Shimadzu) coupled to a high pressure mixer, a Rheodyne injection valve fitted with a 20-μl loop. Separation was achieved on a Nucleosil ODS column (250×4.6 mm I.D., 5 μm).

The elution procedure was performed with two mobile phases, A (water) and B (methanol), at a flow rate of 0.8 ml/min. It first consisted of an isocratic elution with 80% solvent B for 2 min, then of a linear gradient elution built up to 85% B within 15 min.

Detection was accomplished using a spectrofluorimeter (RF-10A*xl*, Shimadzu) at excitation wavelength of 360 nm and emission wavelength of 510 nm.

2.2. Reagents

SP free base, SPD free base and PUT dihydrochloride (Sigma–Aldrich, Milano, Italy), were dissolved in water (10 μg/ml).

The *o*-phthaldialdehyde (OPA) reagent solution (Sigma–Aldrich, Milano, Italy), containing OPA (1 mg/ml), Brij 35, methanol, potassium hydroxide, boric acid and 2-mercaptoethanol as reducing agent, was diluted in methanol (1:10). The organic solvents used (LC grade) were purchased from Sigma–Aldrich (Milano, Italy).

2.3. Saliva collection and storage

Fifty healthy volunteers of different sex and age were enrolled for this study. None of them suffered from systemic or salivary gland diseases that could affect saliva secretion nor had they been administered drugs. Subjects were also requested to refrain from eating 2 h prior to saliva withdrawal.

Saliva pH ranged from 7.5 to 8.0 in all the subjects. The collected saliva, drawn from the sublingual region using a polyethylene transfer pipette, was centrifuged at 3000 rpm for 30 min to remove cellular elements. The clear supernatant fluid was mixed with 5% perchloric acid (1:1) to remove proteins and centrifuged at 1500 rpm for 10 min. The acidic extract was decanted and stored at −20°C until analysis**.**

2.4. o-Phthaldialdehyde derivatization

To 30 μl of saliva extract or standard mixture 50 μl of borate buffer (0.01 *M*, pH=9.0) and 30 μl of the diluted OPA reagent solution were added. The derivatization mixture was shaken for 4 min and 50 μl were injected into the HPLC system.

3. Results and discussion

3.1. Retention time reproducibility

In Fig. 1(A) the chromatogram of a standard mixture containing the three polyamines at a concentration of 0.5 μg/ml is reported. It can be seen that satisfactory resolution and symmetrical peaks were obtained. Under the selected HPLC conditions the retention times were 6 min for SPD, 9 min for PUT and 11 min for SP. These values recurred in repeated analysis and showed a coefficient of variation ranging from 0.13 to 0.71. These data indicate that polyamine retention times were reliable and reproducible. Fig. 1(B) shows a blank chromatogram of a solution containing only the reagents employed in the derivatization reaction. It is clearly demonstrated that no interfering peaks could be detected at

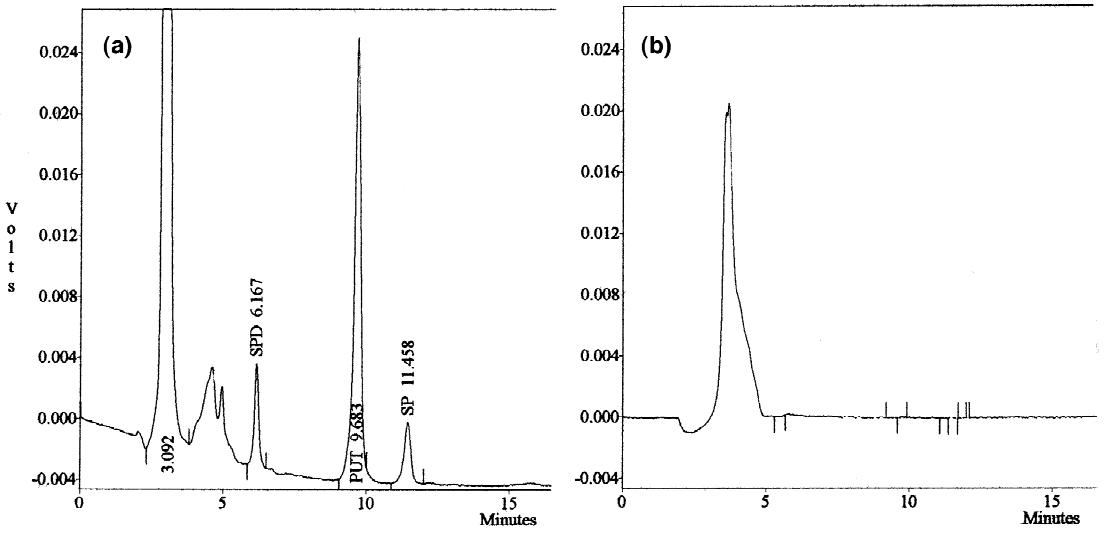


Fig. 1. (A) Chromatogram of a standard mixture (0.5 μg/ml) containing the three OPA derivatized polyamines SPD, PUT and SP. Retention time values were 6.167 min for SPD, 9.683 min for PUT and 11.458 min for SP. (B) Blank chromatogram. For chromatographic conditions, see Section 2.1.

the retention times of SP, SPD and PUT. The chromatogram of a human saliva sample confirmed the values of the three polyamine retention times obtained from standard mixture (Fig. 2).

3.2. Polyamine derivatization conditions

Chemical structures of polyamines SP, SPD and PUT present two terminal amino groups, as evidenced in Scheme 1.

Therefore, the derivatization reaction between OPA and polyamines led to the formation of bis-isoindol-derivatives, as shown in Scheme 2.

Optimized conditions of pH, OPA-reagent volume and reaction time were achieved testing different experimental procedures. The optimum reaction pH was determined by derivatizing each of the three polyamines at pH values ranging from 5.0 to 10.0 and measuring the development of fluorescence intensity as function of pH. As shown in Fig. 3(A), the optimum pH was found to be 8.0. The volume of OPA-reagent solution needed for the derivatization of SP, SPD and PUT was optimized by adding various OPA-reagent volumes (5–100 μl) to 30 μl of an aqueous polyamine solution (0.5 μg/ml) alcalinized with 50 μl 0.01 *M* borate buffer. The fluorescence response was a function of the amount of OPA-reagent employed. As evidenced in Fig. 3(B), the optimal OPA-reagent volume was found to be 30 μl for 30 μl of polyamine solution. Optimal time was

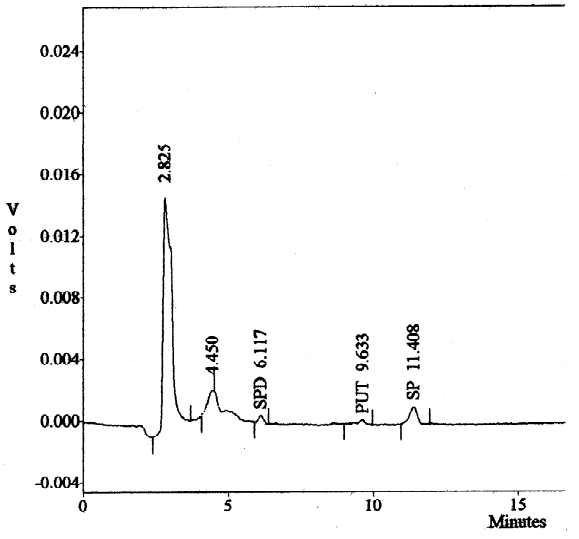
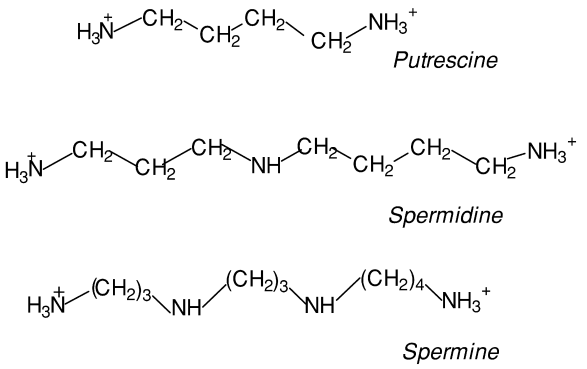


Fig. 2. Chromatogram of OPA-derivatized polyamines from a human saliva sample. The estimated concentrations of SP, SPD and PUT were 0.132, 0.105 and 0.025 μg/ml, respectively. Retention time values were 6.117 min for SPD, 9.633 min for PUT and 11.408 min for SP. For chromatographic conditions, see Section 2.1.



Scheme 1.

tested by evaluating the maximum intensity of fluorescence in relation to the time of reaction, ranging from 2 to 10 min. Optimum reaction time was 4 min, as illustrated in Fig. 3(C).

A gradient run was necessary to ensure an optimal resolution of symmetrical peaks.

3.3. Linearity

A calibration curve was obtained for SP, SPD and PUT by plotting the peak-areas versus known concentrations of each OPA-derivatized polyamine. Table 1 shows the linear regression analysis in a concentration range from 0.01 to 10 μg/ml. The typical equations were: SP, $y = 0.013 + 0.48x$; SPD, $y = 0.008 + 0.61x$; PUT, $y = 0.016 + 0.53x$. The correlation coefficients (0.9997 for SP; 0.9998 for SPD; 0.9995 for PUT) were high and exhibited low standard deviations of the residuals (0.003 for SP, 0.002 for SPD and 0.008 for PUT) demonstrating the linearity of the assay.

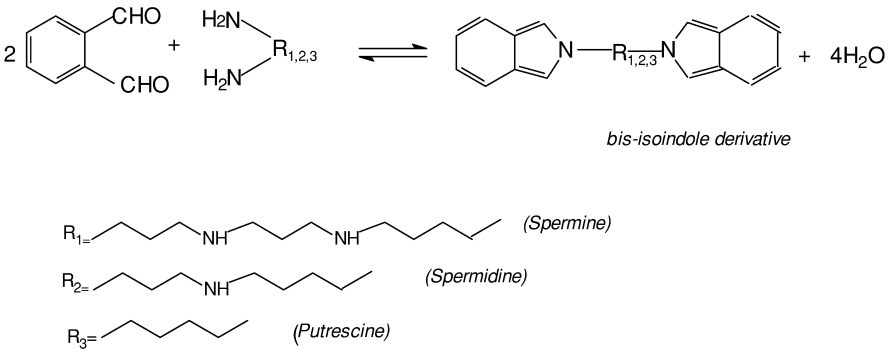
y-*Y* values for each of the ten points considered in the regression analysis were used to calculate SD of the residuals from the interpolated straight line.

3.4. Precision

The intra- and inter-assay coefficients of variation (C.V.) obtained are summarized in Table 2. Within-run reproducibility was tested by performing ten replicate analyses of a saliva sample. Between-run reproducibility was checked by analysing aliquots of a saliva sample stored at −20°C over a period of 6 weeks ($n = 12$). For all the compounds, both between-run and within-run coefficients of variation were below 5%.

3.5. Sensitivity

The minimum detection limits were calculated for each of the three considered polyamines. It was achieved by setting the detector sensitivity at the maximum range and successively injecting decreasing quantities of each of the OPA-derivatized polyamines. Minimum detection limits were found to be 0.04, 0.05 and 0.06 nmol/ml for SP, SPD and PUT, respectively, at a signal-to-noise ratio (S/N) of 3. Below this ratio none of the three polyamines could be revealed.



Scheme 2.

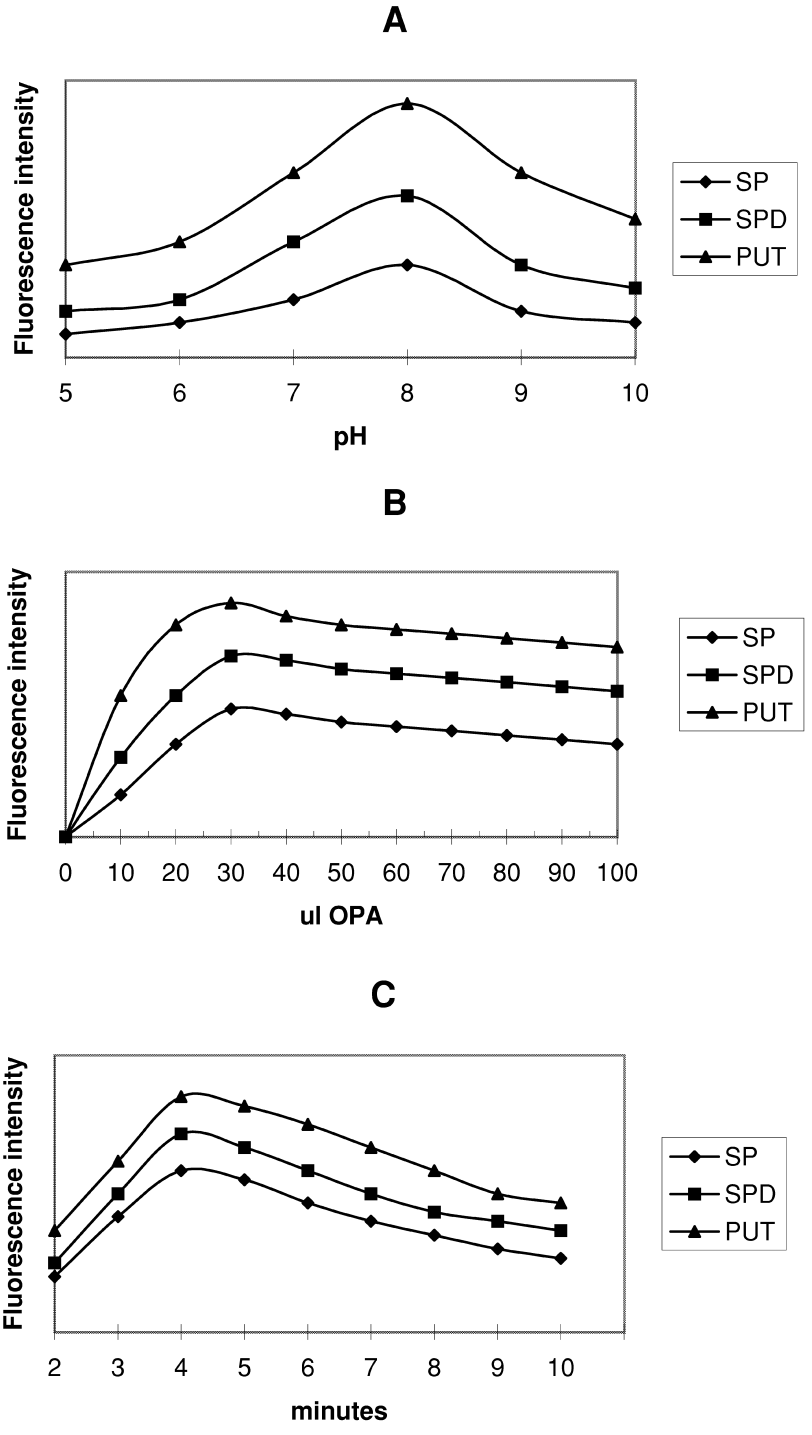


Fig. 3. Fluorescence intensity of polyamine-OPA complex as function of pH (A), OPA-reagent volume (B) and reaction time (C).

Table 1
Linear regression analysis for the relationship between concentration and peak area of SP, SPD and PUT[a]

Compound	r	SD of the residuals	Slope (b)±SD	X-intercept (a)±SD
SP	0.9997	0.003	0.48±0.002	0.013±0.0005
SPD	0.9998	0.002	0.61±0.001	0.008±0.0003
PUT	0.9995	0.008	0.53±0.005	0.016±0.0005

[a] $n=10$ for all determinations; r is the correlation coefficient.

3.6. Recovery

For recovery studies, ten saliva samples were spiked with known amounts of polyamine standards and analyzed. The recoveries were: SP, 95%, SPD, 97% and PUT 92%.

3.7. Application

Saliva samples collected from 50 healthy donors of different sex and age were analysed. The subjects were divided into five groups as follows: the first and the second ones were composed of 10 women (30–40 years old) and 10 men (30–40 years old), respectively. In the third, fourth and fifth groups were enrolled ten subjects whose age ranged from 10 to 25, from 25 to 40 and from 40 to 65 years, respectively. Results reported in Table 3 represent the first ever quantitative analysis of polyamines in saliva from healthy donors. In order to relate the concentrations of SP, SPD and PUT to both age and sex, data obtained by us were analyzed by Multiple Linear Regression. The levels of polyamines appeared not to be associated with any of the two above-mentioned independent variables (multiple $r=$ 0.0002 for SP, 0.0003 for SPD and 0.0005 for PUT), as shown in Table 4. Therefore, our results indicate that the salivary polyamine content was age- and sex-independent and that the values reported here may be used as a reference index of oral well-being in all the subjects.

Table 2
Precision of the assay

Compound	Concentrations (mean±SD) (μg/ml)	C.V. (%)
Intra-assay ($n=10$)		
SP	0.116±0.003	2.93
SPD	0.107±0.003	2.42
PUT	0.024±0.001	3.33
Inter-assay ($n=12$)		
SP	0.118±0.005	4.41
SPD	0.110±0.003	2.81
PUT	0.027±0.001	4.07

Table 3
Salivary polyamine concentrations in healthy subjects (M±SD, μg/ml)[a]

Groups	M±SD (μg/ml)		
	SP	SPD	PUT
I°	0.119±0.004	0.107±0.005	0.025±0.001
II°	0.118±0.003	0.109±0.005	0.029±0.002
III°	0.120±0.004	0.108±0.003	0.029±0.002
IV°	0.121±0.002	0.107±0.004	0.024±0.001
V°	0.119±0.006	0.110±0.005	0.025±0.003

[a] I° group: 10 women (30–40 years old); II°group: 10 men (30–40 years old); III° group: 10 subjects from 10 to 25 years old; IV° group; 10 subjects from 25 to 40 years old; V° group 10 subjects from 40 to 65 years old.

Table 4
Multiple linear regression analysis of polyamine concentrations in terms of age and sex[a]

Polyamines	Multiple linear regression equation[a]			Multiple r
	(intercept) a	(slope$_1$) b_1	(slope$_2$) b_2	
SP	0.118	1.5×10^{-5}	2.8×10^{-4}	0.0002
SPD	0.107	1.08×10^{-5}	2.4×10^{-4}	0.0003
PUT	0.025	2.6×10^{-5}	5.5×10^{-4}	0.0005

[a] $y=a+b_1x_1+b_2x_2$, where y=polyamine concentration; x_1=age; x_2=sex (♂=1; ♀=2).

4. Conclusions

The analytical procedure developed by us, besides its innovative feature, allows several advantages to

be achieved: (a) high degree of reproducibility, precision and sensitivity; (b) rapidity and easiness in the execution; (c) good versatility of employment.

Therefore, because of the considerable simplicity and satisfactory accuracy, it can be considered as a valid routine method for the determination of salivary polyamines, suitable for biomedical studies.

The availability of a procedure able to detect salivary polyamine concentrations opens new perspectives in the diagnosis and the monitoring of phlogistic and proliferative processes of oral cavity, in view of the stimulatory role of polyamines in cellular growth and differentiation.

Experiments are under way in our laboratory to establish if the administration of common drugs, such as analgesics, paracetamol or salicylic acid and others, may interfere with salivary polyamine biosynthesis.

References

[1] M.H. Goyns, The role of polyamines in animal cell physiology, J. Theor. Biol. 97 (1982) 577.
[2] C.W. Tabor, H. Tabor, Polyamines, Annu. Rev. Biochem. 53 (1984) 749.
[3] R. Perez, Presence and function of polyamines in oral tissue and fluid, Pract. Odontol. 11 (1990) 59.
[4] N. Seiler, in: H. Tabor, C. Tabor (Eds.), Methods in Enzymology, Vol. 94, Academic Press, New York, 1983, p. 10.
[5] E. Brandsteterova, S. Hatrik, I. Blanarik, K. Marcincinova, HPLC determination of polyamines in urine, Neoplasma 38 (1991) 165.
[6] C. Molins-Legua, P. Campins-Falco, A. Sevillano-Cabeza, M. Pedron-Pons, Urine polyamines determination using dansyl chloride derivatization in solid-phase extraction cartridges and HPLC, Analyst 124 (1999) 477.
[7] P.J. Oefner, S. Wongyai, G. Bonn, High-performance liquid chromatographic determination of free polyamines in human seminal plasma, Clin. Chem. Acta 205 (1992) 11.
[8] N. Seiler, Assay procedures for polyamines in urine, serum and cerebrospinal fluid, Clin. Chem. 23 (1977) 1519.
[9] P.M. Kabra, H.K. Lee, W.P. Lubich, L.J. Marton, Solid phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue, J. Chromatogr. 380 (1986) 19.
[10] A. Hallak, R. Rosenberg, T. Gilat, G.J. Somjen, Determination of free polyamines in human bile by high-performance liquid chromatography, Clin. Sci (Colch) 85 (1983) 451.
[11] K. Dunchen, L. Thorell, Nucleotide and polyamine levels in colostrum and mature milk in relation to maternal atopy and atopic development in the children, Acta Paediatr. 88 (1999) 1338.