[Frontiers in Bioscience E4, 1795-1801, January 1, 2012]

Polyamines analysis by HPLC and their application as tumor markers

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1. ABSTRACT

Cancers are one of the main causes of human deaths globally. Great effort has been dedicated to the search for sensitive and specific markers of cancer. Polyamines including mainly putrescine (PUT), spermidine (SPD), and spermine (SP), are promising tumor markers since their excretion is frequently elevated in patients with various types of cancers. In the present study, we developed an efficient high performance liquid chromatographic (HPLC) method for the determination of polyamines in human serum using dansyl chloride for pre-column derivatization. All polyamines were separated within 10 min. The analytical method is simple, rapid, and highly reproducible. We applied of 11 cancers as objects of study, and made comparisons between polyamines and other 24 common tumor markers with six indexes: specificity, sensitivity, negative predictive value, positive predictive value, total effective, and the mean value. We drew the conclusion that polyamines are promising tumor markers, and they might be of great value in diagnosing cancers, predicting therapeutic success, or indicating the relapse of tumors.

2. INTRODUCTION

Cancerous malignancies are one of the major issues of present medicine and one of the main causes of human deaths globally. To fight cancer effectively, early diagnosis and timely treatment are of great importance. Thus, much attention is being paid to look for new, selective, and inexpensive tumor diagnostic techniques. Great effort has been dedicated to the search of sensitive and specific markers of cancer. Tumor markers are substances related to the malignancy process. Their elevated concentrations or presence indicate tumor occurrence (1).

Polyamines, including mainly putrescine (PUT), spermidine (SPD), and spermine (SP), are one group of such substances. They are low-molecular-mass aliphatic compounds, widespread in all organisms. Studies in the past few years have shown that polyamines play a significant role in the regulation of eukaryotic cell growth and proliferation (2). A variety of research has revealed that polyamine levels in various body fluids related closely with cancer. It is a promising tumor marker, since its excretion is

frequently elevated in patients with various types of tumor (3).

Contrary to other tumor markers, which are usually proteins, polyamines are small molecules that cannot be sensitively and specifically analyzed by means of immunoenzymatic techniques, popular in biological laboratories (4). Thus, they need to be determined using classical assay methods like electrophoretic techniques (5) or chromatography (6). Unfortunately, polyamines can not be detected directly for because they do not possess any structural feature that would allow their sensitive detection without derivatization. They lack fluorophor absorption and electrochemical properties. In the present study, we have made great efforts to develope a new method of determining polyamines in human serum, using the well-known Dansyl chloride (Dns-Cl) as a fluorogenic derivatizing agent. We then compared the values of polyamines with the other 24 common tumor markers in clinical application. In addition, we applied of 11 cancerous malignancies as objects of study, determined the polyamine concentrations and put the tumors in sequences according to the value of three polyamines.

3. MATERIALS AND METHODS

3.1. Materials

The polyamines, putrescine (MW, 161.1), spermidine (MW, 584.3), and spermine (MW, 398.3), the internal standard, hexamethylenediamine (HDA, MW, 116), and the hydrochloride salts were purchased from Sigma (St. Louis, MO.USA). Dns-Cl was the product of Fluka (St. Louis, MO. USA). Other grade reagents used for HPLC were all obtained from China mainland. We prepared distilled water by ourselves.

The instruments used for the experiment contained a Hewlett-Packard (HP) 1090M liquid chromatograph, an HP 106309 solvent delivery system, an HP#044 automatic sample injector, an HP 1046A fluorescence detector (Excitation 370 nm, Emission 506 nm), an HP 9153A integrator, an HP Think Jet printer, an HP Color Pro plotter, a Corning 250 automatic pH meter (Switzerland), a Mether AE 240 electronic balance (Mater, Switzerland), and a μ Bondapak C18 column (250 mm×4.6 mm I.D., 10μm).

3.2. Methods

3.2.1. Polyamines determination by HPLC

3.2.1.1. Sample preparation

The standard polyamines and internal standards were dissolved in distilled water at final concentrations of 6 μmol/ml for PUT, 3 μmol/ml for SPD, 3 μmol/ml for SP, and 6 μmol/ml for HDA, respectively. Hhydrochloric acid was added in SP to promote dissolving. Dns-Cl was dissolved in acetone. All those solutions were kept at 4^0C.

Human blood samples were collected in blood collection tubes with serum separator gel. After centrifugation, 500 μl serum samples were mixed thoroughly with 300 μl trichloroacetic acid (10%). After precipitation by centrifugation at 3000g for 10min at room temperature, the pH of the supernatants were neutralized by saturated sodium carbonate (pH=11.12) prior to derivatizing with Dns-Cl.

3.2.1.2. Derivatization

600μl standard polyamines or trichloroacetic acid pretreated serum were mixed thoroughly with 75μl HAD, 150μl Dns-Cl dissolved in acetone (4mg/ml), and 75μl saturated sodium carbonate into a tube, and then incubated in water bath for 30 min at 50^0C. After centrifugation at 3500g for 5 min, the supernatants were analyzed using an automatic injection.

3.2.1.3. Chromatographic conditions

Derivative polyamines were separated on a μ Bondapak C18 column (250mm×4.6mm I.D, 10 μm) held at 50^0C. Samples were separated at a flow-rate of 1.0 ml/min with a gradient mixture of methanol and distilled water, manipulated by a computer-controlled elution procedure, ran with 80% methanol, then linearly increasing the proportion of methanol and reaching 100% in the 11^{th} min. Fluorimetric detection at 370 nm for excitation and 506 nm for emission was employed.

3.2.2. Determination of other tumor markers except polyamines

Academia has invented several indexes to describe the characteristics of tumor markers, such as sensitivity, specificity, positive predictive value, negative predictive value, and total effective. Sensitivity is the ratio of true positives to the sum of true positives and false negatives. It relates to the test's ability to identify positive results. Specificity is the ratio of true negatives to the sum of true negatives and false positives. It relates to the ability of the test to identify negative results. Positive predictive value is the ratio of true positives to combined true and false positives. It reflects how likely someone is to have the characteristic if the test is positive. Negative predictive value is the ratio of true negatives to combined true and false negatives. It reflects how likely someone is not to have the characteristic if the test is negative.

Sensitivity and positive predictive value are basal indexes; some tumor markers have ideal sensitivity and positive predictive value, but the specificity is unfavorable. This situation weakens their value of application. Similarly, high specificity and positive predictive value but low sensitivity is also not so good. In addition, the total effective could be affected by the ratio between the human number of the experimental group and the control group. So we introduced the index of mean value, which is the average of the five common indexes above. It has the same variation tendency with total effective but can preferably depict the characteristics of tumor markers.

The tumor markers, except polyamines, were determined by our lab previously (7). AKP, LDH, CK, GGT, and FU were determined by automatic biochemical analyzer; SA, GPI, CU-P, and ADA were determined manually; the others were used enzyme-linked immunosorbent assay (ELISA)

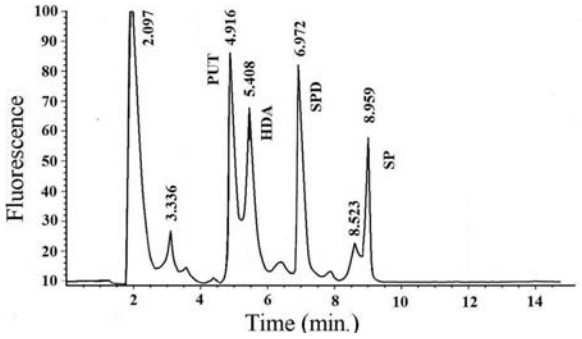

Figure 1. Chromatogram of the standard polyamines with gradient elution. From the chromatogram we can see that PUT, SP, SPD, and HDA were separated thoroughly in only 9 min. The retention times were 4.916 min for PUT, 5.408 min for HDA, 6.972 min for SPD, and 8.959 min for SP, respectively.

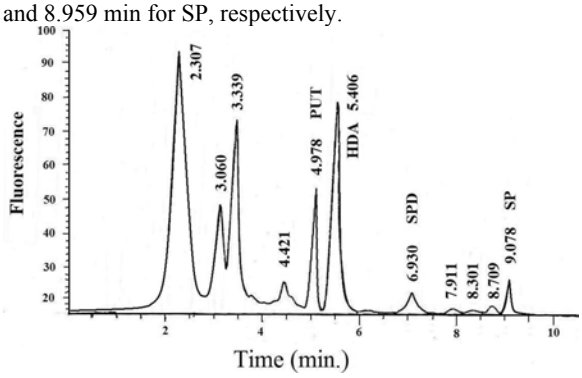

Figure 2. Chromatogram of the polyamines in human serum. The retention times were 4.978 min for PUT, 5.406 min for HDA, 6.930 min for SPD, and 9.078 min for SP, respectively.

4. RESULTS AND DISCUSSION

4.1. Results of polyamines determination

Figure 1 and Figure 2 showed the chromatogram of the standard polyamines and polyamines in human serum, respectively. Figure 3 showed the chromatogram of standard polyamines without gradient elution.

4.2. Validation of the method

4.2.1. Linearity

Excellent linearities of the three derivatives were obtained from the calibration curves, which were completed by plotting the peak area ratios of the standard polyamines relative to the internal standard HDA (responses ratio), against the concentration ratios of the standard polyamines relative to HDA (concentration ratio).

The linear regression equations were as follows: (y: responses ratio; x: concentration ratio)

Putrescine (PUT): y = 18.7x+2.34; r=0.997 (n=6)

Spermidine (SPD): y = 64.3x-12.2; r=1.000 (n=6)

Spermine (SP): y = 25.1x-10.7; r=1.000 (n=6)

4.2.2 Precision

In order to evaluate the precision of the method, in term of repeatability, we firstly prepared eight copies of human serum. After deproteinization and derivatization, continuously injected the serum eight times in the same day and got the within-day precision. Likewise, the between-day precision test was assessed by injecting the pretreatment serum eight different days. The within-day (n=8) concentrations of polyamines were 7.435±0.196 nmol/ml for PUT, 14.737±0.404 nmol/ml for SPD, and 14.128±0.161 nmol/ml for SP. The within-day (n=8) coefficients of variation (CV) percentages of polyamines were 2.64% for PUT, 2.74% for SPD, and 1.14% for SP. The between-day (n=8) concentrations of polyamines were 7.433±0.199 nmol/ml for PUT, 14.735±0.408 nmol/ml for SPD, and 14.123±0.161 nmol/ml for SP. The between-day (n=8) coefficients of variation (CV) percentages of polyamines were 2.68% for PUT, 2.77% for SPD, and 1.14% for SP.

4.2.3. Recovery

Took 200 μl serum sample that had been deproteined and neutralized, determined the polyamines concentration (A) as above. Then, added known amounts of standard polyamines (B) into the serum and analyzed (C) again. Calculate polyamine recovery as follows:

Polyamines recovery = (C-A)/B×100%

In our study, the recoveries of polyamines were 97.4-98.2% for PUT, 98.0-98.6% for SPD, and 102.9-103.2% for SP.

4.2.4. Detection limits

Set the detector to the most sensitive state and injected each derivatized polyamine at varying concentrations from 0.01 to 1 nmol/ml successively. It was accomplished by recording the lowest concentration level that can be determined to be statistically different from a blank. The detection limits were 0.06, 0.07, and 0.05 nmol/ml for PUT, SPD, and SP, respectively.

4.3. Comparison between polyamines and other tumor markers

We compared the value of polyamines and the other 24 common tumor markers in clinical application (Table 1). The data were obtained by determining the concentrations of every tumor marker of the inpatients of our hospital. From the data we can see that, in terms of sensitivity, putrescine (77.71%) and spermidine (59.50%) got the first and the second place. But the specificity of putrescine (77.92%) was the lowest. Spermine had comparatively excellent specificity (92.86%) although the sensitivity (27.33%) did not fulfill our expectations. We had put the 27 tumor markers in sequence according to the mean values. Took an overall view of this table, polyamines, particularly putrescine and spermidine, were well ahead of the rest of tumor markers. These data were propitious for clinicians to select appropriate markers in the diagnosis of cancers.

Table 1. Comparison between polyamines and other tumor markers

	Tum	Mea	Tot	Sensitiv	Neg	Specific	Pos
1							
2							
3							
4							
5							
6							
7							
8							
9							
1							
0							
1							
2							
3							
4							
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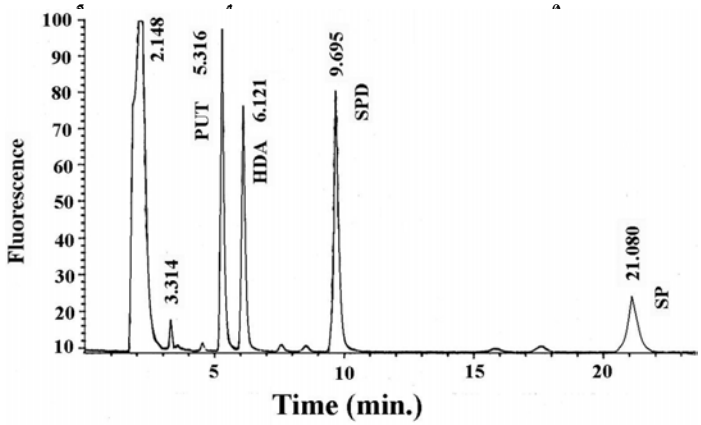

Figure 3. Chromatogram of the standard polyamines without gradient elution. Compared with Figure 1, the whole analyzed time (21.080 min) was longer obviously without gradient elution.

4.4. The value of polyamines as tumor markers in 11 cancers

We had also compared the value of SP, SPD, and PUT as tumor markers in 11cancers, respectively (Table 2). In term of SP, the negative predictive value (94.08%) and total effective (87.95%) of pancreas cancer were the highest in 11 cancers. Positive predictive value of breast cancer came out first. Took a comprehensive view of all the cancers, SP was much appropriate for the application of ovarian cancer because of its high sensitivity (48.00%) and mean value (74.26%). In term of SPD, the negative predictive value (99.23%) and sensitivity (94.44%) of pharyngeal cancer were the best of all cancers. SPD was suitable for the use of esophageal cancer and pharyngeal cancer. Considering PUT, the negative predictive value and sensitivity were so excellent that they had reached 100%, but the mean value it was fourth in 11 cancers. Esophageal cancer was the first with the mean value up to 81.92%.

We also determined the mean values of 27 tumor markers in 11 cancers, respectively. We compared mean values between polyamines and the leader of each cancer (Table 3). The leader marker was the one whose mean value was the highest compared to the other 26 tumor markers in the same cancer. Although SPD and PUT both adequately reflected the value in esophageal cancer compared with that in other cancers, SPD (83.08%) had higher mean value than PUT (81.92%). The mean values of PUT were higher than SPD and SP in most cancers

except for esophageal cancer, lymphoma, and pharyngeal cancer. SPD was the best in these three cancers (Figure 4). PUT was the

Table 2. The value of three major polyamines as tumor markers in 11 different cancers

Ca	Po	Sp	Neg	Se	Pos	To	Me
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 3. The comparison between polyamines and the mean value leaders of 11 cancers

Cancers	Mean value (%) The leader			
esophageal cancer	CA242	[illegible]	S	P
lung cancer	PUT	[illegible]	[illegible]	[illegible]
gastric cancer	PUT	[illegible]	[illegible]	[illegible]
breast cancer	PUT	[illegible]	[illegible]	[illegible]
ovarian cancer	TSGF	[illegible]	[illegible]	[illegible]
liver cancer	TPA	[illegible]	[illegible]	[illegible]
lymphoma	SPD	[illegible]	[illegible]	[illegible]
colorectal cancer	CA242	[illegible]	[illegible]	[illegible]
pancreas cancer	AKP	[illegible]	[illegible]	[illegible]
pharyngeal cancer	SPD	[illegible]	[illegible]	[illegible]
glioma	TSGF	[illegible]	[illegible]	[illegible]

leader of mean values in lung cancer, gastric cancers, and breast cancer, similarly, SPD was the leader in lymphoma and pharyngeal cancer.

5. DISCUSSION

The index of mean value had the same variation tendency with total effective but could preferably describe the characteristics of tumor markers as a whole. Data in these tables highlight polyamines as valuable tumor markers. They might be of great value in diagnosing cancers, predicting therapeutic success, or indicating the relapse of tumors.

The analytical method we used is novel, unique, and creative in many aspects, it is simple, rapid, and highly reproducible and can be employed to the routine detection of other biological and biomedical samples. The method

pioneered a computer-controlled elution procedure worldwide. Its derivatization is also excellent, which is quite different from traditional methods. Sample or standard polyamine derivatives do not need organic solvent (such as ethanol) extraction, evaporation under vacuum, and other condensation procedures. It was so rapid that only 9 min were needed to complete the whole assay procedure.

Samples were separated at a flow-rate of 1.0 ml/min with a gradient mixture of methanol and distilled water, manipulated by a computer-controlled elution procedure, ran with 80% methanol, then linearly increasing the proportion of methanol and reaching 100% in the 11th min. (Figure 3) showed the result of polyamines analysis with isocratic elution, in which the mobile phase composition remained constant (methanol: water= 80: 20) throughout the procedure. To all appearance, gradient elution had superiority in the determination of polyamines.

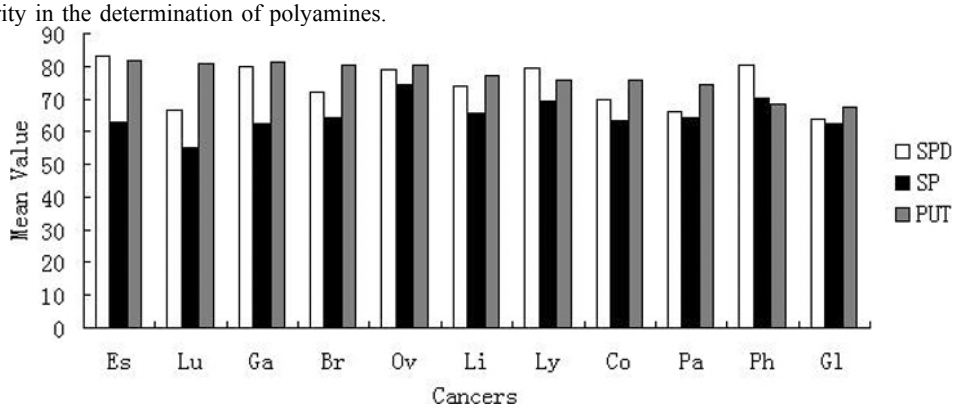

Figure 4. Comparison of three polyamines in 11cancers. Although SPD and PUT both adequately reflected the value in esophageal cancer compared with that in other cancers, SPD (83.08%) had higher mean value than PUT (81.92%). The mean values of PUT were higher than SPD and SP in most cancers except for esophageal cancer, lymphoma, and pharyngeal cancer. SPD was the best in these three cancers. Abbreviations: Es, Esophageal cancer; Lu, Lung cancer; Ga, Gastric cancer; Br, Breast cancer; Ov, Ovarian cancer; Li, Liver cancer; Ly, Lymphoma; Co, Colorectal cancer; Pa, Pancreas cancer; Ph, Pharyngeal cancer; Gl, Glioma.

It decreased the retention of the later-eluting components so that they eluted faster, gave narrower peaks for most components. This also improved the peak shape for tailed peaks, as the increasing concentration of methanol pushed the tailing part of a peak forward. This also increased the peak height, which was important in trace analysis.

Precipitation is of great importance in polyamines analysis with HPLC. We tried heating, salting out and so on to remove protein in the serum. Heating could disrupt hydrogen bonds and non-polar hydrophobic interactions to denature proteins thoroughly. But too high a temperature (we found with temperatures above 60□) can induce polyamines into decomposition. Salting out is another method of deproteinization, which cannot remove proteins completely. Proteins are so deleterious for the chromatographic column that they could shorten the column life span. Trichloroacetic acid is an outstanding precipitation reagent; it forms nonsoluble salts with protein and precipitates entirely and rapidly, without destroying polyamines.

The derivative reaction of Dns-Cl and polyamine was found to be pH and temperature dependent (8). Higher temperature was essential for derivatization, but too high a temperature could lead polyamines into decomposition. The optimum reaction pH and temperature was found to be 9.0 and 50□, respectively. In addition, we centrifuged the derivant solution at 3500g for 5 min, we found that if the centrifugal pull above 4000g, the derivant could be precipitated, which would affect the concentration of polyamines.

Acetonitrile and methanol used to be reagent for the dissolving of Dns-Cl, but we found that the solubility of Dns-Cl in them is very low and the result of derivatization is poor. So, large amount of work was devoted to the searching for ideal reagent to resolve Dns-Cl. Finally we found acetone is nearly perfect for dissolving Dns-Cl. We adopted acetone to dissolve Dns-Cl into the concentration of 4mg/ml, which we found to be the best concentration for the derivative reaction. If Dns-Cl concentration is lower, much

more derivative solution is needed which would dilute the sample and if the concentration of Dns-Cl is too high, L-proline (150 mg/ml in water) were needed to remove the excrescent Dns-Cl (9). Furthermore, in our study, we used 600µl standard polyamines and 150µl Dns-Cl, and found the optimum volume ratio of polyamines to Dns-Cl was 4:1.

We also tried o-Phthalaldehyde (OPA), which is widely used as pre-column derivative agent, but found that OPA solution and OPA derivatives have the disadvantage of limited stability (10). It seems suitable for on-line derivatization.

5. ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NSFC) (30870121) and Shandong Provincial Foundation (y2007c001). Data in Table.1 to Table.3 were gotten from the program "Tumor markers screening" which was accomplished by professor Xiong Zou, professor Shanji Fu, and so on. We thank Mr. Gao Yang, Mr. Xia Hongyin, Liu Yanhong, and other colleagues who contributed to this work.

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Abbreviations: PUT: Putrescine; SPD: Spermidine; SP: Spermine; HPLC: High Performance Liquid Chromatography; ELISA: Enzyme-Linked Immunosorbent Assay; Dns-Cl: Dansyl Chloride; HAD: Hexamethylenediamine; TSGF: Tumor Supplied Group of Factors; SA: Sialic Acid; CA: Carbohydrate Antigen; HER2: Human Epidermal growth factor Receptor 2; CEA: Carcinoembryonic Antigen; IL: Interleukin; CYFRA: Cytokeratin 19 Fragment; GST: Glutathione S-Transferase; FU: Fucosidase; GPI: Glucose Phosphate Isomerase; AKP: Alkaline Phosphatase; CU-P: Caeruloplasmin; TPA: Tissue Polypeptide Antigen; NSE: Neuron-Specific Enolase; AFP: Alpha-Fetal Protein; GGT: Gamma-Glutamyl Transpeptidase; LDH: Lactate Dehydrogenase; PSA: Prostate Specific Antigen; CK: Creatine Kinase; ADA: Adenosine Deaminase; HPV: Human Papillomavirus; CRAds: Conditionally Replicating Adenoviruses; OPA: o-Phthalaldehyde

Key Words: Polyamines, Cancer, Tumor Marker, HPV, HPLC, Putrescine, Spermidine, Spermine

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