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Separation and Determination of Benzoylated Polyamines (Spermidine Andspermine) using HPLC Techniqe

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Abstract
A simple and sensitive reversed-phase high performance liquid chromatography (Rp -HPLC) was developed for separation and determination of polyamines (Spermidine and Spermine). Derivatization of polyamines was improved by dissolving benzoyl chloride in methanol to increase the detection of UV-Visible detector at λ max. 254 nm. The benzoylated polyamines eluted by Rp- HPLC using methanol : water (52 : 48) as mobile phase. The detection limited of this method is (50ng/ml) .The separation was performed on BDS-DB (250 x 4.6 mm I.D) column at flow rate 1 ml/ min. using standard solutions. The retention time of two compounds (Spermidine and Spermine) were (7.7 , 8.5 min.) respectively, this method suggested that monitoring the Spermidine and Spermine compounds could play an important role in diagnosis of different types of carcinoma and fallow up chemo and radiotherapy treatments.

1. Introduction

The polyamines(putrescine, cadaverine, spermidine, and Spermine) which show in (Figure 1) are simple aliphatic primary amines that are fully protonated under physiological conditions and are essential constituents of eukaryotic and prokaryotic cells [1,2] .

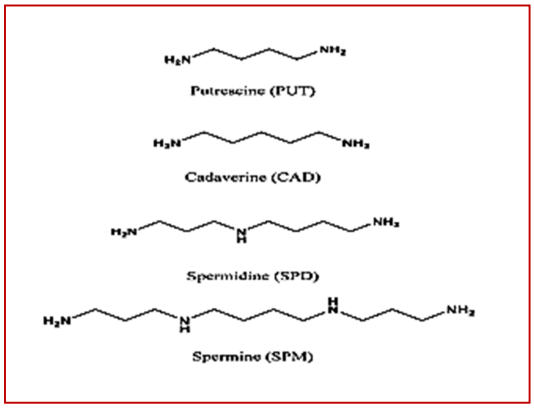

Figure 1. Chemical structures and abbreviations of the polyamines [1]

They have been implicated in a variety of cell functions involving cell growth and differentiation and receptor function [3]. They also impact DNA replication, gene expression, protein synthesis, stabilization of lipids, brain development, nerve growth and regeneration. Over production or over intake of these polyamines is toxic to the cells and facilitates cell death by oxidative mechanism [4] or may cause headaches, nausea, hypo- or hypertension, and cardiac palpitations [5]. These compounds have also been proposed as possible tumor markers [6, 7, 8, and 9]. Polyamine molecules show neither UV absorption nor fluorescence properties [10 - 13]. Many techniques have been developed to determine polyamines, such as thin layer chromatography [14], enzymatic assay [15], high-performance liquid chromatography (HPLC) with derivatization methods in order to increase the sensitivity of the method when using UV or fluorescence detection forming chromophores withbenzoyl chloride [16,17], dabsyl chloride, [18] and forming fluorophores with dansylchloride [19], fluorescamine [20], and o-phthaldialdehyde(OPA) [21,22], liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) [23] or high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (HPLC / Q-TOFMS) methods [24] .The HPLC methods have been used widely due to their high sensitivity and reproducibility, as well as ease of automation , whereas our target is to develop the method as a simple, rapid, sensitive, accuret and precise porcedure for separation and quantitation of polyamines using Rp-HPLC technique .

2. Materials and Method

2.1. Chemicals:

Spermidine and Spermine tetrachloride were purchased from Sigma Chimica (USA), Benzoyl chloride, sodium hydroxide and sodium chloride were obtained from BDH (Chemicals Ltd. Pool. England) , acetonitril and methanol HPLC grade from Flukca (Chime , AG), BDS-C_{18}-DB (250x4.6 mm I.D) column prepacked with 5µm particle size obtained from Supelco-chem (UK,Lid. ,Shine , Hill , England).

2.2. HPLC Instrumentation

The chromatographic system was composed of two solvents reservoirs (500ml) fitted with (0.22Um) stainless still filter at the end of polytriflouroethylene (PTEE) tubes, transferred the mobile phase from reservoir to the pumps, Two groups of pumps model-Lc-6A Shemadizu which delivered the mobile-phase A and B from solvent reservoirs to the mixing cell to create the gradient system program already controlled by SI-6A system controller , the system also involved an injector with 20Ul sample loop model (Rcheadyne-7125) , BDS-C_{18}- DB column (250x4.6 mm I.D), UV-visible detector model SPD- chromatopace Shimadizu model R4A and system controller units (model 6A) Shimadizu.

2.3. Standard solutions:

Spermidine and Spermine (5mg) of each was dissolved in (100ml) of freshly deionized-distilled water, the solutions were filtered through 0.22 µm Millipore filter, degassed and stored at (--20^{o}C) for further uses . The standard solutions were prepared freshly every two weeks.

2.4. Derivatization

Standard solution (1 ml) of polyamines in water were added to (2 ml) of 2% of benzoyl chloride in methanol, (1 ml) of 2N sodium hydroxidewas added and mixed for half minute, then incubated at 37^{o}C for 20 minute. The reaction was stopped by adding of (4ml) of saturated sodium chloride. 3 ml of diethyl ether was added, the solution was vortex for two minutes, then centrifuged at 2500 rpm for 15 minute to separate the organic layer which is containing the benzoylated polyamines. Evaporate to dryness by flow of nitrogen gas, the residue was washed with diethyl ether and evaporated to dryness, to remove any traces of water. Benzoylated polyamines were dissolved in 500Ul of 52% methanol and vortexes for 15 minutes to completely dissolved, this solution was filtered through 0.45 µm Millipore filter to remove any particles. Benzoylated polyamines stored for three weeks at (--20^{o}C).

2.5.Optimization studies

The optimal separation of polyamines (Spermidine and Spermine) was derived by studying the influence of percentage of modifier, column temperature and flow rate of mobile-phase. Methanol was used as modifier which was mixed at different percentages of water and the flow rate of mobile-phase were tested with other variable to select the best retention time of each component of polyamines under study at various column temperatures.

2.6. HPLC optimal condition

For separation and quantitation of two components of polyamines were performed by using HPLC technique on BDS-C_{18}- DB column. The following condition was used on standard solutions: The mobile-phase is 52% methanol: water, flow rate 1 ml/ min., column temperature 35 ^{o}C and UV-visible detection at 254 nm. The typical chromatogram of standard solutions of the two components of polyamines is shown in (Figure 2).

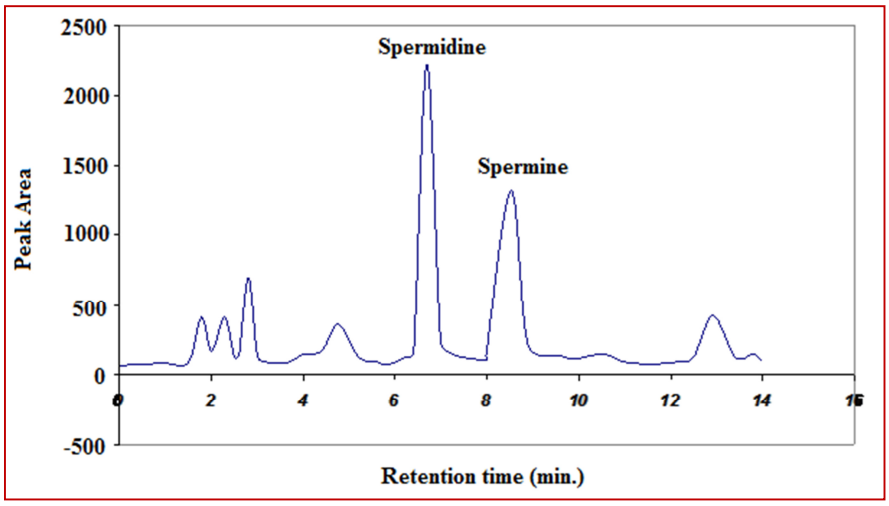

Figure 2. Typical Chromatograme of Spermidine and Spermine using BDS-C_{18}- DB column on HPLC system.

2.7. Linearity:

Linearity was assessed for Spermidine and Spermine using Suplco BDS-C_{18}- DB column on standard solution. Which were submitted to entire excretion procedure at different concentrations of two components in the range of 50 to 1000 ng/ml under optimum conditions (Figure 3).

3. Results and discussion

The availability of sensitive and accurate analysis for the simultaneous of polyamines is important to detect the fluctuation of the concentration levels of these compounds related to regulation of cell proliferation and differentiation. The procedure for benzoyl chloride derivation is quick, stable and permit detection of moderate concentration of polyamines. Benzoyl chloride is decomposed by water to benzoic acid and the excess of this compound was reacted with other molecules to form benzoic anhydride which is an contaminated compound which is extracted by diethyl ether because benzoic acid is instable in organic phase . To reduce the amount of benzoic anhydride, the diethyl ether was washed with diluted sodium hydroxide to enhance the transformation of benzoic anhydride to benzoic acid. The absorbance was measured at 254 nm and this detection proved to be advantages because the absorbance of benzoic acid and benzoic anhydride is much lower than Spermidine and Spermine.

The results of the influence of methanol as modifier used in different percentages and mixed with water are presented in (Table 1).

Table 1: Influence of different percentage of modifier on retention time of Spermidine and Spermine using BDS-DB column, flow rate (1ml/min.), column tem.(35^{o}C) and UV-visible at 254 nm.

Compounds	Retention time in different percentage of methanol (min)						
	100%	90%	80%	70%	65%	52%	45%
Spermidine	5.1	5.4	6.0	6.1	6.3	6.7	6.9
Spermine	5.5	6.0	6.8	7.3	8.0	8.5	8.8

The results indicate that the retention times of two components were decreased when the percentage of methanol was raised from 52% to100%.The eluents which are used in Rp-HPLC are generally polar solvents or mixture of polar solvents such as methanol: water. The best resolution and highly symmetrical peaks were obtained when the percentage of methanol was 52%.The results in (Table 2) show the influence of column temperature on retention time of Spermidine and Spermine. The data was indicated that the retention time decrease when the column temperature is raised from 30 ^{0}C to 50^{o}C. These results indicate that the optimal separation conditions of two components of polyamines mixture solution could be achieved at 35^{o}C. At this temperature a good resolution of two peaks could be obtained. The results also indicate that the retention time of two components were considerably affect by column temperature.

Table 2 : Influence of column temperature on retention time of Spermidine and Spermine using BDS-DB column, flow Rate (1ml/min.), mobile-phase (52% methanol: water) and UV-visible detector at 254 nm .

Compounds	Retention time (min.) in different temperature (^{0}C)			
	30	35	40	45
Spermidine	6.9	6.7	6.2	6.0
Spermine	8.8	8.5	8.1	7.3

The results in (Figure 3) show a linear concentration response for Spermidine and Spermine, the correlation coefficient was 0.99 as shown in equation obtained from plot of each component. Better results were obtained using concentrations between 50 ng/ml to 1000 ng/ml of two components of polyamines.

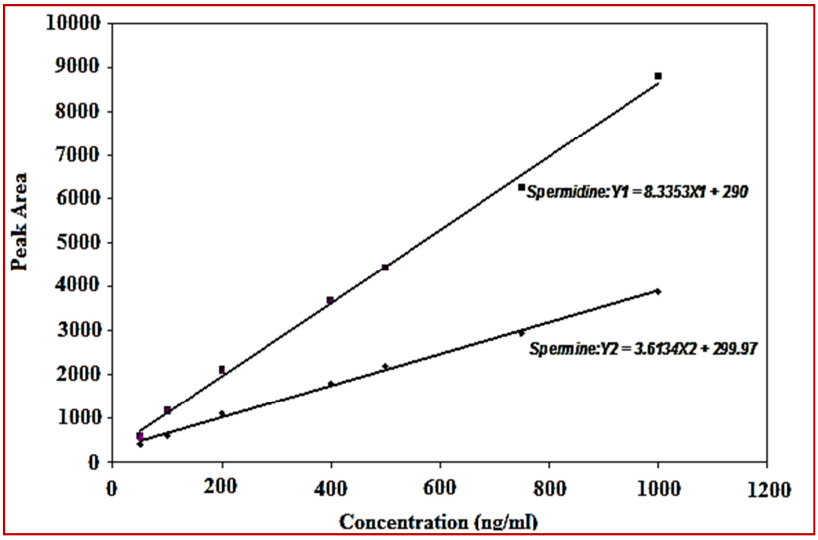

Figure 3. Linearity of different concentrations of Spermidine and Spermine using. HPLC technique at optimal condition.

(Table 3) shows a significant increasing in retention time with decrease of mobile-phase flow rate. Results also indicate best separation was obtained when the flow rate was 1ml/ min.

Table 3 : Influence of flow rate of eluent on retention time of Spermidine and Spermine Using BDS-DB column, Mobile–phase (52% methanol: water), Column temperature (35^{o}C)and UV-visible detector at 254nm.

	Retention time in different flow rate of eluent (1ml/min.)			
Compounds	0.6	0.8	1.0	1.2
Spermidine	7.9	7.2	6.7	6.0
Spermine	9.0	8.5	8.5	7.5

Many substances could be separated by HPLC at flow rate ranging from 0.3 to 0.5 ml/min. Many column available carry manufacture suggestion to use a flow rate around 1ml/ min. to avoid excessive pressure , however , a lower flow rate shows less dilution and better resolution. Finally it is recommended the use of the following optimal conditions for the best separation results of the two components of polyamines (Spermidine and Spermine) with BDS-C_{18}- DB column (250X4.6 mm I.D), 52% methanol: water, column temperature (35^{o}C), flow rate 1ml / min. and UV-visible detection at 254 nm.

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