HPLC Determination of Putrescine and Cadaverine in Serum of Cancer Patients Using Acetylacetone as Derivatizing Reagent

[1]MUHAMMAD YAR KHUHAWAR, [2]ROSHAN ALI GILAL, [2]FAQEER MAHBOOB ALI RIND*, [2]ABDUL HAKEEM MEMON AND [1]MUHAMMAD USMAN MEMON

[1]*Dr. M. A. Kazi, Institute of Chemistry, University of Sindh, Jamshoro, Sindh, Pakistan.*
[2]*Faculty of Pharmacy, University of Sindh, Jamshoro, Sindh, Pakistan.*

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Summary: Liquid chromatographic procedure has been developed for the determination of putrescine (Put) and cadaverine (Cad) after derivatization with acetylacetone in aqueous-methanolic solution at pH 8.2. The derivatives were eluted and separated from μ Bondapak C-18, 10 um (3.9×300 mm id) with water:methanol:acetonitrile (73:22:05 v/v/v) with a flow rate 1.2 ml/min. The UV detection was at 310 nm. Linear calibration curves were obtained 25-200 ng Put and Cad / injection (10 μl). The method was used for determination of Put and Cad in the blood serum of cancer patients before, during and after radiotherapy and were in the range of Put 0.622-3.674 ± S.D 0.017-0.153 μg/mL and Cad 0.229-1.854 ± S.D 0.002-0.046 μg/mL. The limits of detection were observed 2.5 ng Put and Cad / injection (10 μL). the recoveries of Put and Cad from serum were calculated to 94.2-97.3 %. The concentration of Put and Cad observed were lower after radiotherapy.

Introduction

Aliphatic diamines putrescine (Put) and cadaverine (Cad) are biological active and have a definite role in cell multiplication and their regulation [1, 2]. Rapidly growing tissues usually have higher amount of Put and has a stimulating effect on DNA, RNA, and protein synthesis. A significant increase in the concentration of Put in serum of cancer patients is reported [3]. A number of analytical methods are available for the determination of Put and Cad but chromatographic procedures are more reported mainly gas, liquid, and planer chromatography [4]. Recent advances in the field of capillary electrophoresis (CE) have enabled the use of CE for the determination of Put and Cad [5]. Among chromatographic methods, more procedures are available based on high performance liquid chromatography (HPLC) using both pre and post column derivatization. Recently separation methods for the determination of Put and Cad have been reviewed [4]. The reagents for precolumn derivatization are mainly orthophthaldehyde [6], fluorescamine [7], benzoyl chloride [8], dansyl chloride [9, 10], 9-fluorenylmethyl chloroformate [11], 4-(1-pyrene) butanyl chloride [12], 1-pyrene butanoic acid succinnidyl ester [13], 6-aminoquinoyl-*N*-hydroxy succinimidyl carbamate and related compounds [14-15]. Acetylacetone (AA) has been used as precolumn derivatizing reagent for the separation and determination of Put and Cad from water samples [16-17]. In the present work AA has been used for the HPLC determination of Put and Cad from serum of cancer patients.

Results and Discussion

Put and Cad react with AA in 1:2 molar ratio to form characteristic derivatives, which are extractable in organic solvent from slightly alkaline medium. The derivatives formed eluted and separated from μ Bondapak column with methanol-water, but a better peak shape was observed by including acetonitrile in the eluent (Fig. 1), with resolution factor Rs between Put and Cad derivatives was calculated 2.1.

The reproducibility in terms of retention time and peak height (n = 4) was observed with coefficient of variation (C.V) for Put 0.5 % and 1.9 % & for Cad 0.4 % and 2.3 %, respectively.

Linear calibration curves by plotting average peak height (n = 3) versus concentration were obtained with 25-200 ng Put and Cad / injection (10 μL) with coefficient of determination (r^2) 0.9981 and

*To whom all correspondence should be addressed.

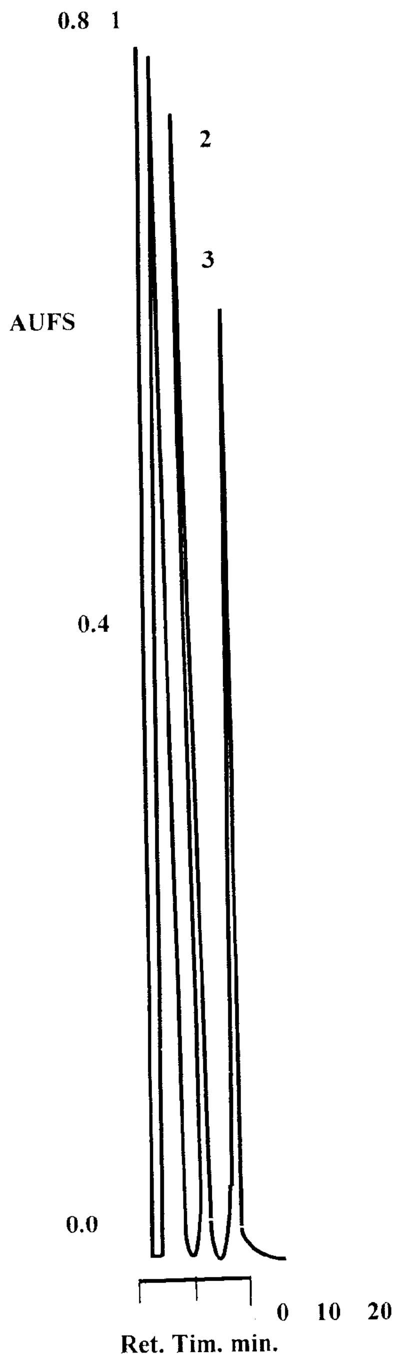

Fig. 1: HPLC separation (1) AA and (2) Put (3) Cad derivatives. Column μ Bondapak C-18, 10 μm (3.9×300 mm id). Elution water:methanol:acetonitrile (73:22:05 v/v/v). Flow rate: 1.2 mL/min. UV detection at 310 nm.

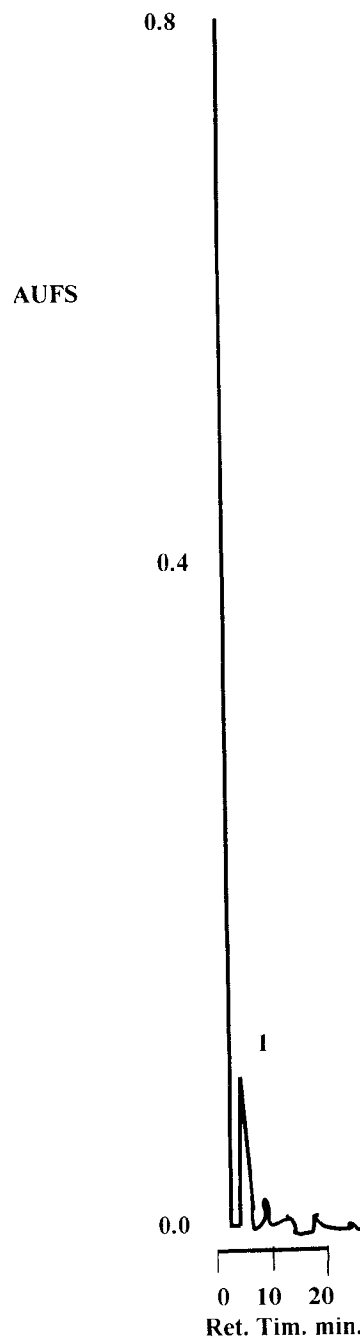

Fig. 2: Blank determination (1) chloroform. Column μ Bondapak C-18, 10 μm (3.9×300 mm id). Elution water:methanol:acetonitrile (73:22:05 v/v/v). Flow rate: 1.2 mL/min. UV detection at 310 nm.

0.9989. The detection limits measured as three times the background noise were calculated to 2.5 ng Put and Cad / injection (10 μL).

The chromatographic separation was examined for the determination of Put and Cad from the serum. Deproteinization was carried out with methanol, followed by derivatization in aqueous-methanolic solution and extraction in chloroform. Blood samples from the diagnosed cancer patients were collected and Put and Cad contents in the serum were analyzed from three different groups; (1) before (2) during and (3) after radiotherapy. Three patients in each of the group were selected. The analysis was carried after pre concentration by the factor of 5. The results of the analyses (Tables-1 and 2) indicate the concentration of Put 0.622-3.674 ± S.D 0.017-0.153 μg/mL and Cad 0.229-1.854 ±S.D 0.002-0.046 μg/mL. A serum sample of cancer patient during radiotherapy was also analyzed by standard addition technique. The amounts found were 3.6 μg/mL Put and 0.65 μg/mL Cad as compared to 3.7 μg/mL Put and 0.69 μg/mL Cad obtained by calibration. The % recovery was calculated to 97.3-94.2 %.

Experimental

Putrescine dihydrochloride, cadaverine dihydrochloride (Sigma, USA), methanol, acetonitrile, acetylacetone (Merck, Germany) were used as received. Freshly prepared double distilled water from all glass was used for HPLC studies. HPLC studies were carried out with Shimadzu LC-5A liquid chromatograph connected with variable wavelength UV SPD-2A detector, Rheodyne 7125 injector and Shimadzu C-R1B Chromatopac. Column μ Bondapak C-18, 10 μm (3.9 × 300 mm id) (Waters, U.S.A.) was used throughout the study. The buffer solutions at unit pH interval within 1-10 were prepared from the following: hydrochloric acid (0.1M) and potassium chloride (1M) pH 1-2, acetic acid (1M) and sodium acetate (1M) pH 3-6, ammonium acetate (1M) pH 7, dipotassium hydrogenphosphate (15 %) adjusted to pH 8-10 with hydrochloric acid (0.1M) or sodium hydroxide (0.1M).

Determination of Put and Cad from serum

Blood samples of cancer patients before, after 48 to 72 hrs (250 centigray/day) radiotherapy, and at the end of radiotherapy (5500 centigray) were collected from Atomic Energy Medical Centre Liaquat University of Medical and Health Sciences, Jamshoro by venipuncture. The blood sample (4-5 mL) was centrifuged at 4500 rpm for 15 min and 1mL from the supernatant layer was transferred to another test tube. Methanol (10 mL) was added and

Table-1: Analysis of serum of cancer patients for the concentration of putrescine before during and after treatment by radiotherapy, using acetylacetone as derivatizing reagent.

S. No.	Organs Affcted by the Cancer	Pre Treatment (μg/mL)	During Treatment (μg/mL)	After Treatment (μg/mL)
1.	Cheek	1.31 (0.061)		
2.	Cheek	1.31 (0.061)		
3.	Brain	2.30 (0.153)		
4.	Cheek		3.68 (0.153)	
5.	Urinary bladder		2.91 (0.153)	
6.	Tonsils		2.81 (0.088)	
7.	Lung			1.11 (0.038)
8.	Cervix			0.84 (0.045)
9.	Larynx			0.62 (0.017)

Values in Parentheses are standard deviation (± S.D)

Table-2: Analysis of serum of cancer patients for the concentration of cadaverine before, during and after treatment by radiotherapy, using acetylacetone as derivatizing reagent.

S. No.	Organs Affcted by the Cancer	Pre Treatment (μg/mL)	During Treatment (μg/mL)	After Treatment (μg/mL)
1.	Cheek	0.66 (0.035)		
2.	Cheek	1.16 (0.046)		
3.	Brain	1.86 (0.046)		
4.	Cheek		0.69 (0.035)	
5.	Urinary bladder		0.48 (0.026)	
6.	Tonsils		1.84 (0.026)	
7.	Lung			0.61 (0.035)
8.	Cervix			0.42 (0.023)
9.	Larynx			0.23 (0.002)

Values in Parentheses are standard deviation (± S.D)

contents were mixed well and centrifuged for 20 min. Supernatant layer was separated and was warmed on water bath at 90 °C to reduce the volume to about 3-4 ml. AA (0.5 mL, 1.5 % v/v in water) and dipotassium hydrogenphosphate (0.2 mL, 15 % w/v in water pH 8.2) were added and contents were heated at 90 °C for 15 min. The volume was adjusted with methanol:water (1:10) to 5 mL. Chloroform (1 mL) was then added and contents were mixed well. The layers were allowed to separate and exactly 0.5 mL of organic layer was transferred to screw cap sample vial. The solvent was evaporated and residue redissolved in methanol (0.1 mL). An aliquot (10 µL) was injected on µ Bondapak C-18, 10 µm column (3.9×300 mm id) and eluted with water:methanol:acetonitrile (73: 22:05 v/v/v) with a flow rate of 1.2 mL/min. The UV detection was at 310 nm. The amount of Put and Cad from serum was calculated from the external calibration curve prepared with 2.5-20 µg/mL Put and Cad following the above procedure.

Analysis of Serum by Standard Addition Technique

Serum (1mL) in duplicate from a patient under radiotherapy was collected. A sample was treated as "Determination of Put and Cad from serum" and another after deproteinization with methanol was added Put and Cad 5 µg each (0.5 mL of 10 µg/mL). Remaining procedure "Determination of Put and Cad from serum" was followed. The quantitation was carried out from linear calibration curve and an increase in response with added standard.

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