Determination of Urinary Putrescine and Cadaverine of Cancer Patients by Gas Chromatography

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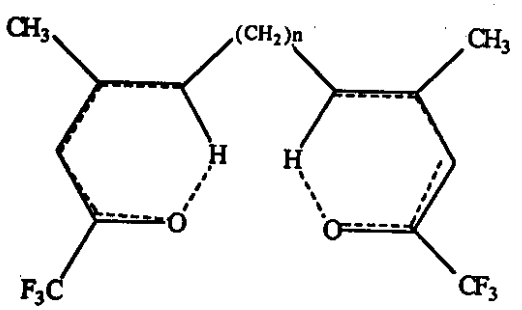
Summary: Capillary gas chromatography (CGC) was examined from the determination of urinary putrescine(Pu) and cadaverine (CA) based on off line precolumn derivatization with trifluoroacetylacetone (FAA), GC was carried out on BP5 (25m x 0.22 mm id) column with a layer thickness of 0.25 μm at 230°C with a heating rate of 2°C/min upto 250°C, a nitrogen flow rate of 3.5 ml/min, a split ratio of 1:15 and detection by FID. The method was used for determination of Pu and CA in urine of cancer patients before and after radiotherapy and healthy volunteers. The amount found was in the range of 0.04-3.89 μg/ml with a coefficient of variation (C.V) of 1.25-5.56%.

Introduction

Putrescine(Pu) and cadaverine (CA) are biological active and they have a role in the cell development [1,2]. The interest in the determination of Pu and CA increased since Russell observed a high concentration of Pu and CA in the urine of cancer patients [3]. A number of analytical methods have been proposed for the determination of Pu and CA in urine, which include liquid and gas chromatographic procedures [4-10]. GC of Pu and CA is carried out as underivatized diamines or after derivatization with different fluorinated and non-fluorinated acid anhydrides [4,7,8,11-15]. Recently trifluoroacetylacetone (FAA) has been used for GC determination of Pu and CA in human blood serum [16] but some difficulties were observed in getting blood samples from advanced stage cancer patients. Therefore the work reports GC method for determination of Pu and CA in urine using FAA as derivatizing reagent.

Results and Discussion

ß-Diketone trifluoroacetylacetone (FAA) reacts with diamines Pu and CA in 2:1 molar ratio to form H_2FAA_2Pu and H_2FAA_2CA which elute from capillary GC column BP5 (25m x 0.22 mm id) and gave visual symmetrical peaks. The diamines 1,2-ethylenediamine(en) 1,3-propylenediamine (Pn) and 1,7-diaminoheptane (HP) also react with FAA to form derivatives (Fig. 1) when added together with Pu and CA and eluted from the chromatographic column. A complete separation was obtained (Fig. 2) for quantitative determinations.



H_2FAA_2en	n = 2
H_2FAA_2Pn	n = 3
H_2FAA_2Pu	n = 4
H_2FAA_2CA	n = 5
H_2FAA_2HP	n = 7

Fig. 1: Structural diagram of diamine derivatives with FAA.

The effect of pH on the derivatization, solvent extraction and GC determination of Pu and CA was examined in pH range 3 to 10 using different buffer solutions. It was observed that a reasonable extraction was observed within pH 6 to 9 with maximum at 6.75 and was selected.

The calibration curves were constructed for Pu and CA by plotting average peak height (n=3)

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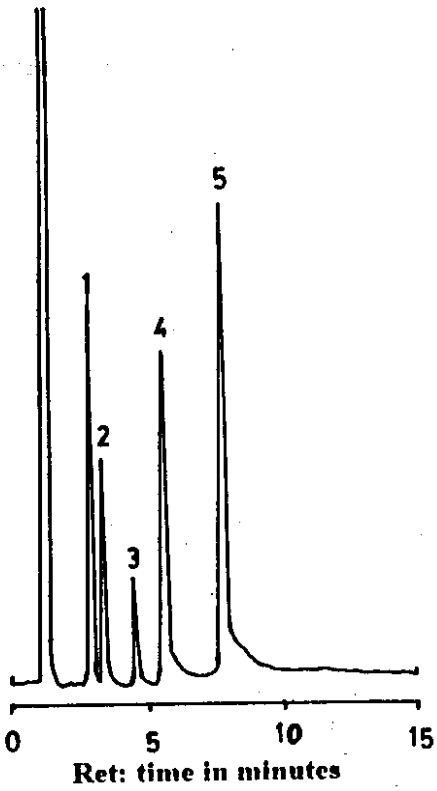

Fig. 2: Gas chromatographic separation of (1) H_2FAA_2en (2) H_2FAA_2Pn (3) H_2FAA_2Pu (4) H_2FAA_2CA (5) H_2FAA_2HP GC conditions: Column BP5 (25m x0.22mm id) with layer thickness 0.25 μm;initial temperature 230°C with programmed heating rate 2°C/min upto 250°C and stay at maximum for 5 min.Injection and detector temperatures were 280 and 285°C respectively. Nitrogen flow rate 3.5 ml/min. Split ratio 1:15 and FID detection.

versus concentration of diamines μg/ml in final extract, The linear calibrations were prepared from five standards (n=5) within 0-100 μg/ml with coefficient of correlation (r) 0.998 and 0.989 for Pu and CA respectively. The detection limits measured as three times the background noise were 0.66 μg/ml and 0.53 μg/ml for Pu and CA, corresponding to 0.66 ng and 0.53 ng for Pu and CA, per injection (1 μl). The method was used for the determination of Pu and CA in the urine sample of cancer patients and 2 healthy persons using external calibration. The determination required to hydrolyse urinary diamine derivatives by acid digestion. The digestion period of 12 hrs was sufficient to get reproducible results. The results are summarized in (Table 1) and indicate that the concentration of Pu and CA in the urine of cancer patients before radiotherapy were 1.43-3.89 μg/ml and 1.04-2.16 μg/ml with C.V within 1.43-5.98% and 1.25-5.0 % respectively, with highest concentration from the patients suffering from lungs carcinoma (Fig. 3a). The amount of Pu and CA in the cancer patients after the radiotherapy for 1 month decreased to 0.81-2.41 μg/ml and 0.29-1.28 μg/ml with C.V within 1.07-2.29% and 1.46-5.56% respectively (Fig. 3b), with concentration still highest in the patients suffering from lungs cancer. The urine samples of healthy persons from the laboratory were also analysed for the contents of Pu and CA. The amounts of Pu and CA were much lower in the range of 0.095-0.116 μg/ml and 0.048-0.062 μg/ml respectively. Therefore it may be suggested that urinary Pu and CA are higher in cancer patients and some decrease in their concentrations were observed with radiotherapy, but are still much higher than healthy subjects (Table 1).

Table-1: Capillary Gas Chromatographic Determination Of Urinary Putrescine And Cadaverine In Cancer Patients

		Before Radiotherapy		After Radiotherapy	
No.	Cancer Type	Putreacine (Pu) μg/ml (C.V%)	Cadaverine (CA) μg/ml(C.V%)	Putreacine (Pu) μg/ml(C.V%)	Cadaverine (CA) μg/ml/ (C.V%)
1.	Head & Neck	1.69(5.98)	0.42(1.92)	0.81(2.21)	0.29(5.56)
2	Head & Neck	1.43(2.72)	0.74(4.08)	1.11(1.07)	0.37(1.46)
3	Head & Neck	1.67(4.32)	0.58(0.86)	1.26(1.62)	0.23(5.26)
4.	Right Lung	3.26(4.16)	1.04(3.19)	2.32(1.78)	0.68(1.78)
5.	Left Breast	1.89(1.41)	0.63(1.25)	1.68(2.29)	0.45(2.24)
6.	Left Breast	2.38(2.19)	0.97(2.46)	1.73(1.88)	0.51(2.33)
7.	Tongue	2.23(3.26)	0.48(5.00)	1.61(1.26)	0.31(3.52)
8.	Lungs	3.89(2.57)	2.16(1.58)	2.41(1.41)	1.28(2.15)

- In Normal Persons Pu and CA in μg/ml/ with C.V.% in Paranthesis i) 0.116(3.00) and 0.062 (2.83) ii) 0.095(2.63) and 0.0481(1.49)

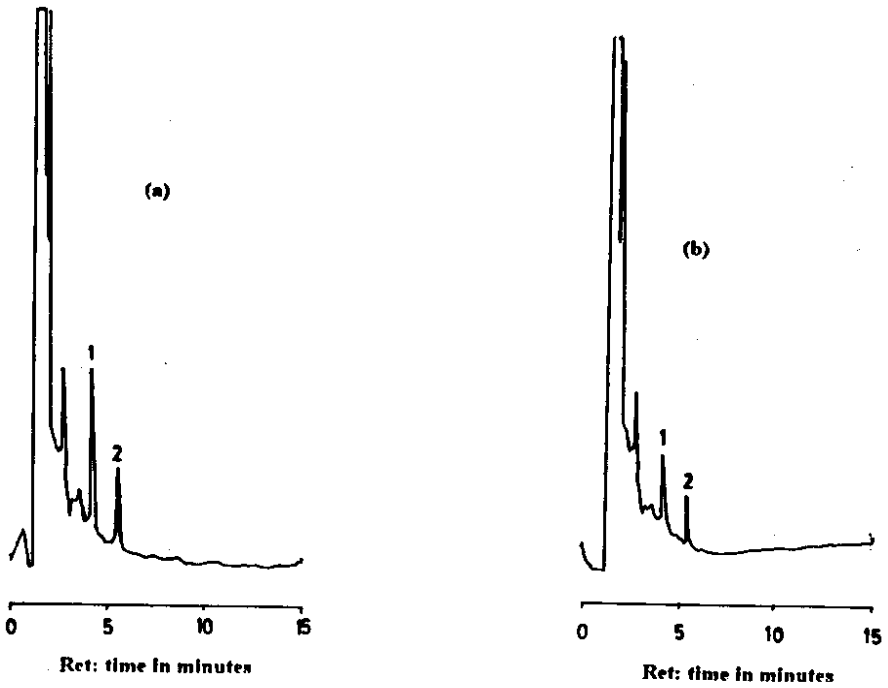

Fig.3: Gas chromatographic determination of (1) putrescine (Pu) (2) Cadaverine (CA) from urine of cancer patients with lungs carcinoma (a) before radiotherapy (b) after radiotherapy. GC conditions same as in Fig. 2.

Experimental

1. Material and Method

Trifluoroacetylacetone(FAA) (Fluka, Switzerland) 1,2-ethylenediamine, 1,3-propylenediamine, 1,7-diaminoheptane (E. Merck Darmsladt), putrescine dihydrochloride and cadaverine dihydrochloride (Sigma, ST Louis MO, USA) were used. Perkin Elmer 8700 gas chromatograph (Beaconsfied, Buckinghamshire, England) connected with FID detection, pure nitrogen (POC, Karachi) as carrier gas, OPGS 1500(s) a hydrogen generator (Shimadzu, Japan) for FID and an LX-800 printer were used.

A capillary column BP5 (25m x 0.22 mm id) with layer thickness of 0.25 μm (SGE. Ring wood Victoria, Australia) was used. Buffer solutions in pH range 3-10 at unit interval were prepared from acetic acid (1 M), sodium acetate (1 M), sodium bicarbonate (1 M), sodium carbonate (Saturated), ammonium chloride (1M), sodium carbonate (Saturated), ammonium chloride (1 M) and ammonia (1 M). Double distilled water from all glasses was used for preparation of solutions. pH measurements were made with Orion 420A (Boston MA USA). combined with glass electrode and reference electrode.

2. Analytical procedure

Diamines a Pu (4-20 μg) and CA (2-10 μg) solution (1-5 ml) was added to FAA (3 ml, 3% V/V in methanol), sodium acetate buffer pH 6.75 (2 ml) and heated on water bath for 15 min. The mixture was cooled and chloroform (3 ml) was added to it. The contents were mixed thoroughly and layers were allowed to separate. The organic layer was collected and extraction was repeated with chloroform (2 ml). The solvent (1 μl) was injected on GC column BP5 (25m x 0.22 mm id) with layer thickness 0.25 μm at 230°C with programmed heating rate of 2°C/min. upto 250°C and maximum temperatures was maintained for 5 min. The injection and detector temperatures were 280°C and 285°C respectively. The nitrogen flow rate was 3.5 ml/min with split ratio 1:15. The detection was by FID.

3. Analysis of Putrescine and Cadaverine in Urine

The urine samples were collected from cancer patients before and after radiotherapy. The urine

samples from healthy volunteers were also collected. The samples were collected in clean and dry glass bottles. The urine sample (5 ml) was transferred to sterilized screw cap tube and was added 10% trichloroacetic acid in water (2 ml). The ice cooled sample was centrifuged at 650x g for 15 min. The clear solution was separated and added hydrochloric acid (5 ml, 6N). The contents were heated on water bath for 12 hr and centrifuged for 15 min at 650x g. The clear solution was separated and neutralized to pH 7-8 with sodium hydroxide (1 M). The reagent FAA (3 ml, 3% V/V in methanol) was added and pH was adjusted to 6.75 with sodium acetate/acetic acid. The contents were heated at water bath for 15 min. and cooled. Chloroform (3 ml) was added and shaken vigorously. The organic layer was separated and extraction was repeated with chloroform (2 ml). The remaining procedure was followed as above.

Urine samples were collected from Atomic Energy Medical Centre, Liaquat Medical College Hospital, Jamshoro. Eight samples were collected from cancer patients, three suffering from head and neck, two left breast, two lungs and one tongue. The urine samples were collected before receiving radiotherapy and after receiving doses of 5000-5500 centigray during 1 month. The urine samples of two healthy volunteers from the laboratory were also collected.

Conclusion

Simple GC method has been suggested for the determination of urinary Pu and CA after acid hydrolysis of urine samples and derivatization with FAA. The determination limits were within 0.048 to 3.87 μg/ml.

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