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Capillary gas chromatographic determination of putrescine and cadaverine in serum of cancer patients using trifluoroacetylacetone as derivatizing reagent

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Abstract

Trifluoroacetylacetone (FAA) derivatives of 1,4-diaminobutane (putrescine) (Pu) and 1,5-diaminopentane (cadaverine) (CA) were prepared and characterized by elemental microanalysis, IR, and mass spectrometry. Diamine derivatives were eluted from capillary gas chromatographic (CGC) column BP1 (12 m×0.22 mm I.D.) or BP5 (50 m×0.22 mm) with layer thickness 0.25 μm, using nitrogen as a carrier gas and flame ionization detection (FID). A solvent extraction procedure was developed for the extraction of Pu and CA from aqueous solution with a linear calibration range 0–20 μg/0.2 ml of extract with a detection limit of 0.5–0.6 ng/injection. The method was applied for the determination of Pu and CA in the serum of five cancer patients before and after radiotherapy. The serum of two healthy persons was also analyzed for Pu and CA contents. Pu and CA concentrations were found within the range 1.16–3.96 μg/ml and 0.88–1.46 μg/ml in cancer patients as compared to 0.11–0.16 μg/ml and 0.06–0.075 μg/ml respectively in healthy persons with a coefficient of variation (CV) within 0.62–5.47%. Pu and CA concentrations decreased on radiotherapy in cancer patients, but were much higher than in healthy persons. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, GC; Putrescine; Cadaverine

1. Introduction

The biologically active diamines putrescine (Pu) and cadaverine (CA) have a definitive role in nucleic acid metabolism, protein synthesis and cell growth [1,2]. Pu and CA could act as cancer markers as their concentrations are reported to be higher in the serum of cancer patients as compared to healthy volunteers [3]. A large number of analytical methods are reported for their quantitative determination based on high-performance liquid chromatography (HPLC) using both pre-column and post column derivatization [4–16]. Different derivatizing reagents have been used for spectrophotometric, spectrofluorimetric and electrochemical detection [13–18]. However a few gas chromatographic (GC) methods have been reported for the determination of Pu and CA. The GC methods involve mostly isobutyloxycarbonyl, pentafluoropropionyl, trifluoroacetyl and heptafluorobutionyl derivatives using packed and capillary column GC [19–27]. The derivatization is mostly

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carried out using their corresponding acid anhydrides.

Nishikawa has used acetylacetone for the HPLC determination of Pu and CA [11]. The introduction of the trifluoromethyl group is reported to enhance the volatility of derivatized molecules [28,29]. The trifluoroacetylacetone (FAA) is easily available and the presence of five carbon atoms in a straight chain is expected to enhance the FID sensitivity . Therefore, in the present work FAA has been examined as a derivatizing reagent for the capillary GC separation of diamines and determination of Pu and CA in the serum of cancer patients.

2. Experimental

2.1. Preparation of Bis(trifluoroacetylacetone)1,4-diiminobutane (H_2FAA_2Pu) and Bis(trifluoroacetylacetone) 1,5-diiminopentane (H_2FAA_2CA)

A solution of FAA (0.77 g, 0.005 *M*) in ethanol (20 ml) was added to a clear solution of putrescine dihydrochloride (0.4 g, 0.0025 *M*) or cadaverine dihydrochloride (0.43, 0.0025 *M*) in ethanol (20 ml) and sodium hydroxide (0.2 g). The mixture was refluxed for 30 min on a water bath and was kept at 5 °C for 2 days. The solid material obtained was filtered and recrystallized from ethanol. H_2FAA_2Pu, m.p. 191°C, calculated for $C_{14}H_{18}N_2O_6F_6$; expected %: C=46.67, H=5.04, N=7.78; found %: C=47.35, H=5.21, N=7.85. IR in KBr indicated main bands at 3190(w), 1625(s), 1595(s), 1540(w), 1250(s), 1190(s), 1140(s). Mass spectrum indicated peaks at *m/z* (Rel. intensity %) 360(10), 341(8), 291.1(8) 263(0.1), 250(0.1), 208(30), 207(95), 194(10), 180(10), 166(15), 152(10), 138(100), 111(143), 110(68), 97(12), 69(17). H_2FAA_2CA, m.p. = 74, calculated for $C_{15}H_{20}N_2O_6F_6$; expected %: C=48.13, H=5.38, N=7.48; found %; C=47.91, H=5.38, N=7.56. IR in KBr indicated main bands at 3190(w), 1625(s), 1595(s), 1500(w), 1250(s), 1195(m). Mass spectrum indicated peak, at *m/z* (Rel. intensity %) 373.9(35), 355(0.4), 304.9(33), 277(0.1), 222(33), 221(34), 220(58), 208(20), 194(12), 180(20), 166(30), 152(100), 138(5), 110(13), 97(27), 69(28).

2.2. Methods and materials

Trifluoroacetylacetone (Fluka, Buchs, Switzerland), 1,3-propylenediamine, 1,7-diaminoheptane (E.Merck, Darmstadt, Germany), putrescine dihydrochloride and cadaverine dihydrochloride (Sigma, St. Louis, MO, USA) were used. Elemental micro analyses and mass spectra were recorded at HEJ Research Institute of Chemistry, University of Karachi. IR in KBr were carried out on a Perkin Elmer 1430 IR spectrophotometer within the range 4000–250 cm^{-1}. A Perkin Elmer 8700 gas chromatograph (Beaconsfield, UK) connected with FID detection, pure nitrogen (POC, Karachi) as carrier gas, OPGS 1500(s) hydrogen generator (Shimadzu, Tokyo, Japan) for FID and an LX-800 printer were used.

Capillary columns BP1(12 m×0.22 mm I.D.), BP5 (25 m×0.22 mm I.D.) and BP5 (50 m×0.22 mm I.D.) with layer thickness 0.25 μm (Ringwood, Victoria, Australia) were used.

Buffer solutions in the pH range 3–10 at unit intervals were prepared from acetic acid (1 *M*), sodium acetate (1 *M*), sodium bicarbonate (1 *M*) sodium carbonate (saturated), ammonium chloride (1 *M*) and ammonia (1 *M*). Double distilled water from all glass equipment was used for the preparation of solutions. pH measurements were made with an Orion 420A (Boston, MA, USA) pH meter combined with glass electrode and reference electrode.

2.3. Solvent extraction procedure of diamines

A solution (1–5 ml) of diamines, Pu (4–20 μg) and CA (2–10 μg), separately or in a mixture was added to FAA (1.5 ml, 3% v/v in methanol), sodium acetate buffer pH 6.75 (2 ml) and heated on a water bath for 15 min. The mixture was cooled and chloroform (3 ml) was added The contents were mixed well and the layers were allowed to separate. The organic layer was collected and the extraction was repeated with chloroform (2 ml). The solvent from the combined extract was evaporated under a stream of nitrogen and the residue was dissolved in ethanol (0.2 ml). Solution (1 μl) was injected on to the column BP1 (12 m×0.22 mm I.D.) at a column temperature of 200°C with a programmed heating

rate 2°C/min up to 220°C and the temperature was held at 220°C for 2 min. The nitrogen flow-rate was 3.5 ml/min with split ratio 1:10. Alternatively the solution (1μl) was injected on to column BP5 (50 m×0.22 mm I.D.) at a column temperature 240°C with a programmed heating rate of 2°C /min up to 260°C and held at the maximum temperature for 10 min. The nitrogen flow-rate was 4.5 ml/min with split ratio 1:20.

2.4. Analyses of putrescine and cadaverine in serum

Blood samples, collected from cancer patients before radiotherapy, after radiotherapy, and from healthy persons by venipuncture (5 ml) in a sterilized screw cap tube, were placed in an ice bath. Separation was carried out within 15 min of collection by centrifugation at 650 *g* for 10 min.The supernatant layer was added to 10% (w/v) trichloroacetic acid in water (2 ml). The contents were thoroughly mixed and cooled in an ice box for 30 min. The contents were centrifuged for 15 min and the supernatant layer was collected. The residue was again added to 10% (w/v) trichloroacetic acid in water (2 ml), thoroughly mixed and centrifuged for 10–15 min. The combined supernatant liquid was added to diethyl ether (5 ml) and the contents were well mixed. The organic layer was separated and the extraction was repeated twice with 5 ml portions of diethyl ether. The aqueous layer was collected, FAA (1.5 ml, 3%, v/v in methanol) and sodium acetate (2 ml, 1 *M*) were added and the pH was adjusted to 6.75. The remaining procedure was carried out as described in Section 2.3. The amounts of Pu and CA from blood samples were calculated from external calibration curves prepared from known standards ($n=5$) of Pu and CA as dihydrochlorides.

2.5. Recovery of Pu and CA from serum

A blood sample from a normal volunteer (15 ml) was collected and a portion (5 ml) was processed as described in Section 2.4 and two portions (5 ml each) were added to 10 μg and 20 μg of Pu and CA and the solutions were again processed as described in Section 2.4. The elution was carried out from the column BP5 (50 m×0.22 mm I.D.) with layer thickness 0.25 μm, initial temperature column 240°C with an increase of 2°C/min up to 260°C, and held at maximum temperature for 10 min. Detection, FID; nitrogen flow-rate, 4.5 ml/min; split ratio, 1:20. Injection port and detector temperatures were 280°C and 285°C, respectively.

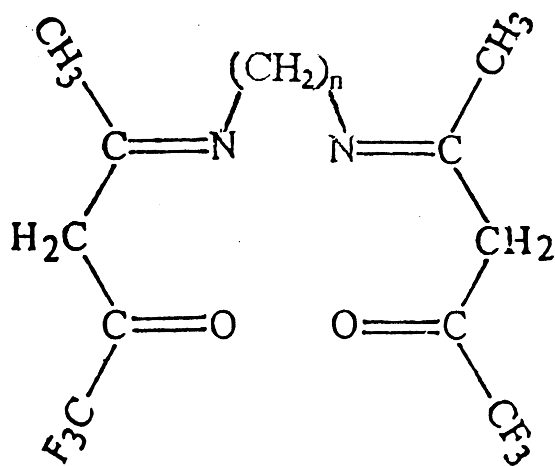
The blood samples were collected from Atomic Energy Medical Centre, Liaquat Medical College Hospital, Jamshoro. Three male and two female cancer patients with (1) urinary bladder (2) left breast (3) head and neck (4) left breast and (5) lung carcinoma. The blood samples were collected before the patients received any radiotherapy and 1 month after receiving doses of 5000–5500 centigray. Blood samples of two healthy volunteers from the laboratory were also collected.

3. Results and discussion

FAA reacts with diamines Pu and CA in 2:1 molar ratio to form H_2FAA_2Pu and H_2FAA_2CA. The compounds H_2FAA_2Pu and H_2FAA_2CA were characterized by elemental micro-analyses, IR and mass spectrometry. The observed results of elemental micro-analyses closely corresponded to the expected values. The IR of FAA derivatives indicated a band at 3190−5 cm^{-1}due to hydrogen bonded NH vibration. The derivatives demonstrated a strong band in the region 1620−5 cm^{-1} because of hydrogen-bonded carbonyl stretching vibration. The compounds demonstrated one to three bands of strong to medium intensities between 1600–1500 cm^{-1}, which may be assigned to C=C, NH and C=N vibrations. The strong intensity absorption bands around 1250 cm^{-1} and 1190–1140 cm^{-1} could be assigned to CF_3 group [28] (Fig.1).

The mass spectra of FAA derivatives H_2FAA_2Pu and H_2FAA_2CA indicated prominent molecular ion peaks [M]+ at m/z 360.1 (10%) and 373.9(35%), respectively, and a number of fragment peaks were observed as could be expected from their structures (Fig. 1).

The solutions of pure compounds H_2FAA_2Pu and H_2FAA_2CA containing 1 mg/ml in ethanol, after appropriate dilution were injected on to the capillary



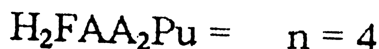


Fig. 1. Structural diagram of diamine derivatives with FAA.

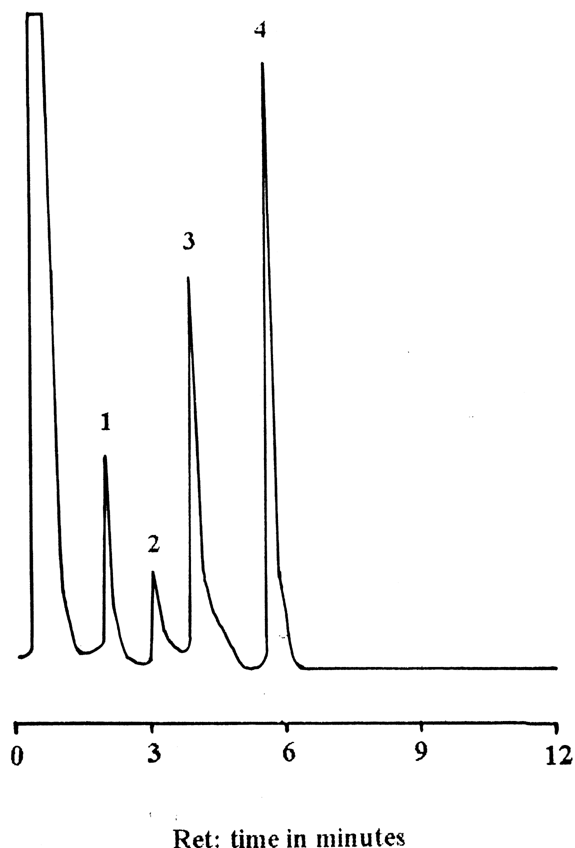


Fig. 2. Gas chromatographic separation of (1) H_2FAA_2Pn, (2) H_2FAA_2Pu, (3) H_2FAA_2CA (4) H_2FAA_2HP. Conditions : Column BP1 (12 m×0.22 mm I.D.) with layer thickness 0.25 μm; initial temperature 200°C with a rise in temperature of 2°C/min up to 220°C for 2 min, injection port 280°C and detector 285°C. Nitrogen flow-rate 3.5 ml/min split ratio 1:10 and FID detection.

column BP1 (12 m×0.22 mm I.D.) at an initial column temperature of 200°C with a heating rate of 2°C/min up to 220°C and the temperature was held at 220°C for 2 min. Temperatures of injection port and detector were 280°C and 285°C, respectively, with split ratio 1:10. The compounds eluted easily and gave visually symmetrical peaks. The diamines 1,3-propylenediamine (Pn) and 1,7-heptanediamine (HP) also react with FAA to form H_2FAA_2Pn and H_2FAA_2HP, when added to with Pu and CA, eluted from the chromatographic column. The compound H_2FAA_2Pn eluted first followed by H_2FAA_2Pu, H_2FAA_2CA and H_2FAA_2HP. The retention time of the compounds increased with an increase in the carbon number at the bridge position. However a complete separation between the compounds was obtained (Fig. 2).

For the quantitative determination of diamines in samples using capillary GC, a precolumn derivatization procedure, followed by solvent extraction was examined. The effect of variations in pH, concentration of derivatization reagent, heating time and choice of extracting solvent on the quantitative transfer of Pu and CA from the aqueous to organic phase was investigated. Equal aliquots were injected at capillary GC on the optimized conditions and average peak height were recorded. The conditions which gave maximum response were considered as an optimum. The pH was varied between 3–10, the diamines Pu and CA could be extracted between 5–9 with maximum extraction at pH 6.75. The concentration of the derivatization reagent FAA was varied between 0.5–3.0 ml of 3% (v/v) in methanol at intervals of 0.5 ml. Constant response was ob-

served with 1.0 to 3.0 ml and an amount of 1.5 ml was selected. Heating time at 95°C was varied from 5–30 min at intervals of 5 min. The same response was observed for heating for more than 5 min and a heating time of 15 min was selected for the quantitative transfer of Pu and CA to H_2FAA_2Pu and H_2FAA_2CA, respectively. For solvent extraction, chloroform, *n*-hexane, cyclohexane and carbon tetrachloride were checked; chloroform demonstrated better extraction and was selected.

Using these conditions, the calibration curves were constructed for Pu and CA by plotting average peak height ($n=3$) versus concentration of diamines in μg/ml in the final extract. The linear calibration curves were obtained with 0–100 μg/ml with 1 μl injected and split ratio 1:10, which corresponded to 0–10 ng reaching to the detector. Correlation coefficients (r) of 0.996 and 0.998 for Pu and CA, respectively, were obtained. The validity of calibration was examined by the analysis of test solutions of Pu and CA and relative error was found within ±3%. The detection limits, measured as three

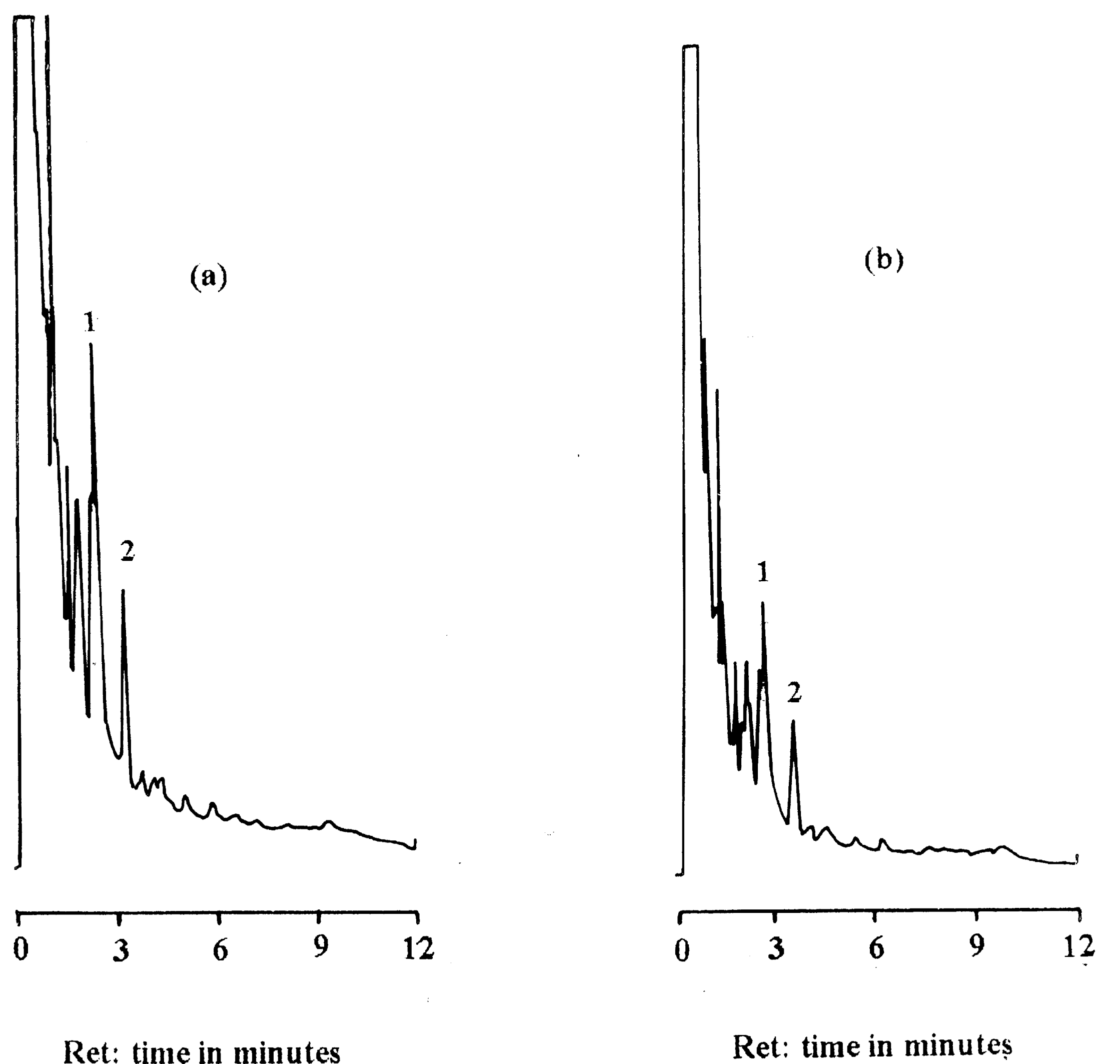


Fig. 3. Gas chromatographic determination of (1) putrescine (2) cadaverine from serum of a cancer patient with carcinoma of the lungs (a) before radiotherapy and (b) after radiotherapy doses. GC conditions as in Fig. 2

times the background noise, were 0.6 μg/ml and 0.5 μg/ml Pu and CA, corresponding to 0.6 ng/injection and 0.5 ng/injection (1 μl), respectively. The lower limit of detection (LOD) based on the blood sample was also calculated. The blood sample from a healthy person (5 ml) was processed as described in Section 2.4 to remove the Pu and CA present in the blood sample. The serum was spiked with Pu and CA and the derivatization and extraction procedure was repeated. Solution (1 μl) was injected to ascertain LOD based on the preconcentration of 25, LOD in the blood sample of Pu and CA was 7 ng/ml and 6ng/ml, respectively. The percent recovery of Pu and CA from the blood sample of the healthy person was checked. The blood sample was spiked with 10 μg and 20 μg and was processed as described in Section 2.5 (Fig. 3). The increase in the response in the signal due to Pu and CA was evaluated from standard calibration curves prepared from pure Pu and CA at the column conditions for blood samples. It was observed that percent recovery of Pu and CA was 87.5 and 90.12 with coefficients of variation 3.26% and 2.68%, respectively. Replicate analyses of 10 μg each of Pu and CA were examined ($n=5$) and CVs of 2.4% and 2.5%, respectively, were obtained. The GC method was applied for determination of Pu and CA in the serum of cancer patients. Calibration curves for Pu and CA were prepared followed by the analysis of Pu and CA on the same day. The average peak height obtained on different days from same amount of Pu and CA (10 μg each) were examined. The observed CVs were 2.9% and 3.6% for Pu and CA ($n=5$), respectively. The results in Table 1 indicate that Pu and CA found before radiotherapy were 1.92–3.96 μg/ml and 1.01–1.46 μg/ml with coefficients of variation (CV%) within 2.86–5.47% and 1.12–1.97%, respectively. The lowest amounts of Pu and CA among the series were observed in a patient with left breast carcinoma. The concentrations of Pu and CA in the patients decreased within 1.16–2.68 μg/ml and 0.88–1.21 μg/ml with CVs 1.18–2.29% and 0.62–1.43%, respectively, after radiotherapy (Fig. 4). The blood samples of two healthy persons were also analyzed for the concentrations of Pu and CA. The amounts found were much lower in the range of 0.11–0.16 μg/ml and 0.06–0.075 μg/ml, respectively. Thus it may be suggested that the concentrations of Pu and CA in the serum of cancer patients are high and some decrease in their concentrations were observed with radiotherapy for 1 month, but still their observed concentrations were much higher than in healthy persons within the range of 0.88–2.68 μg/ml with CV 0.62–2.29%.

4. Conclusion

A capillary GC method has been developed for the quantitative determination of Pu and CA in human serum, based on precolumn derivatization with trifluoroacetylacetone and solvent extraction. The detection was by FID. The method has been applied for the determination of Pu and CA in the serum of cancer patients and healthy persons.

Table 1
Analyses of serum of cancer patients for the concentrations of putrescine and cadaverine before and after radiotherapy using trifluoroacetylacetone as derivatizing reagent[a,b]

No.	Cancer type	Before radiotherapy		After radiotherapy	
		Putrescine (μg/ml)	Cadaverine (μg/ml)	Putrescine (μg/ml)	Cadaverine (μg/ml)
1.	Urinary bladder	3.96 (3.72)	1.40 (1.27)	1.75 (1.48)	1.06 (0.67)
2.	Left breast	2.52 (4.17)	1.46 (1.87)	1.84 (0.79)	1.08 (1.34)
3.	Head and neck	3.44 (4.64)	1.36 (1.62)	2.68 (1.62)	1.21 (0.62)
4.	Left breast	1.92 (5.49)	1.01 (1.12)	1.16 (2.29)	0.88 (1.75)
5.	Lungs	3.68 (2.86)	1.45 (1.79)	2.04 (1.18)	1.04 (1.43)

[a] In healthy persons Pu and CA were (I) 0.11 (3.12) and 0.06 (1.34). (II) 0.16 (2.74) and 0.075 (2.73).
[b] Values in parentheses are coefficients of variation (%).

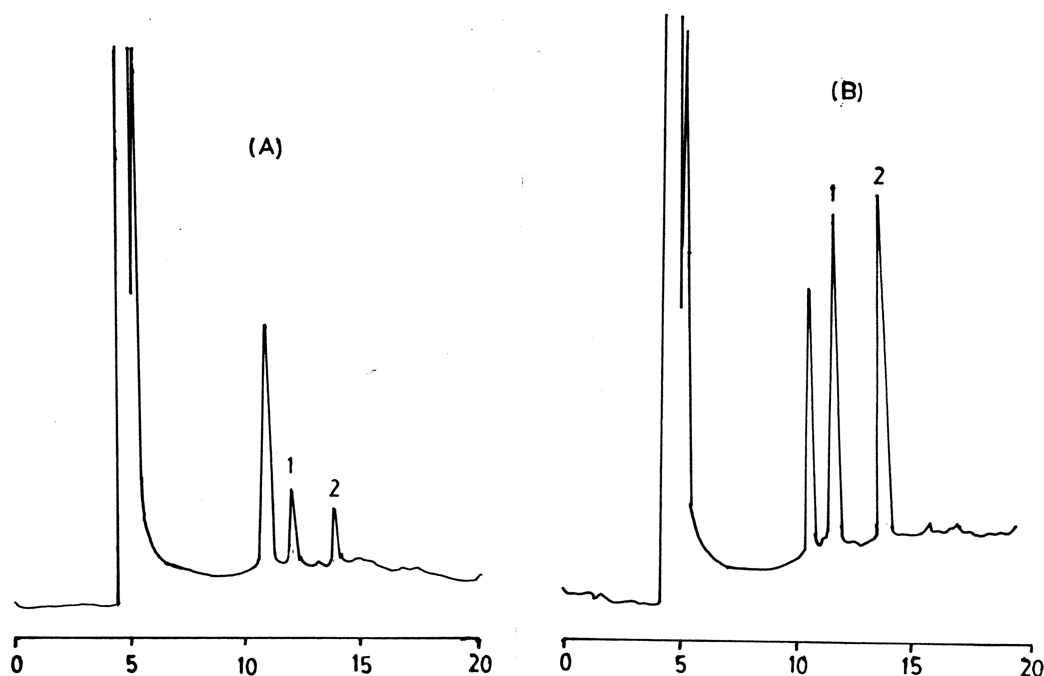


Fig. 4. Recovery of Pu and CA from the serum of a healthy person (blank serum sample) (A) serum sample (B) serum sample spiked with 20 μg of each of Pu and CA. Conditions: column BP 5 (50 m×0.22 mm I.D.) with layer thickness 0.25 μm; initial temperature 240°C with a rise in temperature of 2°C/min up to 260°C and held at maximum temperature for 10 min, injection port temperature 280°C and detector temperature 285°C. Nitrogen flow-rate 4.5 ml/min. Detection FID..

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