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DETERMINATION OF POLYAMINES IN HUMAN BLOOD BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

The present work was undertaken to develop a sensitive and selective method for the estimation of putrescine, spermidine and spermine in human blood employing electron-capture gas—liquid chromatography. Polyamines were derivatized with heptafluorobutyric anhydride. The heptafluorobutyric derivatives of polyamines could be well resolved within 15 min under a temperature programme. The detection limit was 0.1 pmol for putrescine and cadaverine, and 0.02 pmol for spermidine and spermine. The method was applied to polyamine determinations in erythrocytes from human blood. For pre-separation of the polyamines from other compounds, a simple clean-up method utilizing an activated Permutit has been devised. Major interfering substances could be removed by the batchwise Permutit treatment. The mean values of spermidine and spermine concentrations, and the spermidine/spermine ratio in erythrocytes obtained from normal subjects (n = 11) were similar to reported values. The analytical procedure is thought to be applicable to various biological materials.

INTRODUCTION

Over the past decade, numerous analytical methods have been devised for the quantitative determination of the naturally occurring di- and polyamines putrescine, spermidine and spermine [1—3]. Among these methods gas—liquid chromatography (GLC) has been shown to be a useful tool for the routine analysis of polyamines in biological materials. The method is fast, convenient, selective and shows good resolution. An additional advantage of GLC seems to be in the application to a structural analysis of unusual natural polyamines [4—7] or identification of metabolic products of polyamines [8, 9] in combination with mass spectrometry. Since putrescine, spermidine and spermine were successfully separated as their trifluoroacetic derivatives [10], trifluoroacetic anhydride has been extensively used as an acylating reagent for polyamine assay by flame ionization GLC. The method has been adopted for

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the estimation of the polyamine contents of urine from normal and cancer patients [11, 12]. However, due to the lack of sensitivity, the application of this method has been limited to the analysis of urine, which usually contains much higher levels of polyamines than other physiological fluids such as blood and cerebrospinal fluid.

Electron-capture detection (ECD) is a highly sensitive detection system for fluorine-containing compounds. Makita et al. [13] first applied this technique to the quantitative assay of polyamines using pentafluorobenzyl chloride for derivatization, but determination of spermine was unsuccessful. More recently Rattenbury et al. [14] developed an ECD method for measuring urinary polyamines as their pentafluoropropionyl derivatives. In spite of its excellent sensitivity which enables the measurement of polyamines at picomole level, little has been reported so far on the analysis of blood polyamines by ECD—GLC. The major difficulty appears to be in the purification step for separating polyamines from other compounds, mainly amino acids, which readily react with acylating reagents and produce serious interfering peaks. With the rapid and accurate determination of polyamines in human blood in mind, we devised a simple method using an activated Permutit for the clean-up of samples containing large quantities of amino compounds.

In our method, heptafluorobutyric anhydride (HFBA) was selected as an excellent acylating reagent for the conversion of the polyamines into volatile and highly ECD-sensitive derivatives. This paper describes the optimal conditions for derivatization and separations of the polyamines. The applicability of the ECD—GLC technique to the quantitative assay of these amines in erythrocytes from human blood is demonstrated.

EXPERIMENTAL

Chemicals

Putrescine, cadaverine, spermidine and spermine were purchased from Nakarai Chemicals (Kyoto, Japan) in the form of their hydrochlorides. Heptafluorobutyric anhydride (HFBA), activated Permutit, and 1,5-diamino-3-azapentane were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dimethylamine was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); it was redistilled before use. Other organic and inorganic chemicals were obtained from commercial sources.

Preparation of erythrocytes and extraction of polyamines

Human blood was obtained from healthy volunteers (11 males; age 22—43 years). The blood was collected in glass centrifuge tubes containing 1/10 vol. (v/v) of 3.8% sodium citrate solution and was immediately centrifuged at 1500 *g* for 15 min to pellet the cells. The plasma was removed, the buffy coat aspirated and the cellular pellet was resuspended in 0.9% NaCl and centrifuged again at 1500 *g* for 15 min. Extraction of free polyamines from the erythrocyte fraction was carried out with 10% (w/v) trichloroacetic acid (TCA). Generally, 0.5 ml of packed erythrocytes was used for polyamine analysis. The samples were mixed with 3 ml of 10% TCA, shaken mechanically for 5 min and centrifuged at 1500 *g* for 15 min. The supernatant fluid was placed into a glass

centrifuge tube equipped with a glass stopper and extracted twice with 5 ml of diethyl ether to remove the excess TCA.

Sample pre-separation

The polyamine-containing extracts of erythrocytes were pre-separated by means of activated Permutit. To the sample solution, 1/10 vol. (w/v) of activated Permutit was added and mechanically shaken for 5 min. The supernatant was discarded and the Permutit was washed once with 5 ml of 2% NH_4OH and twice with 5 ml of distilled water. The polyamines were eluted from the Permutit particles by shaking with 5 ml of a 40% solution (w/v) of dimethylamine in methanol for 5 min. This step was repeated two times in order to complete the elution of polyamines. After sedimentation of the Permutit particles, the supernatant was collected and evaporated to dryness in order to remove dimethylamine. The residue was dissolved in 1 ml of 1 *N* HCl. Distilled dimethylamine should be used for the extraction of polyamines, since the commercial reagent always contained significant amounts of interfering materials.

Reaction of polyamines with HFBA

An aliquot of the sample solution was transferred to 3-ml reaction vials equipped with a Teflon-lined screw cap, and an exact amount of 1,5-diamino-3-azapentane was added as an internal standard. The mixture was evaporated to dryness under a stream of pure nitrogen at 90°C. After cooling, 200 µl of acetonitrile and 50 µl of HFBA were added to the dryed residue. The vials were capped and heated at 65°C for 10 min. The reaction mixture was evaporated to dryness under a stream of nitrogen and then redissolved in 1 ml of diethyl ether. The ether solution was washed once with an equal volume of saturated Na_2CO_3 solution. After centrifugation, the aqueous phase was discarded and 1 µl of ether phase was taken for the GLC analysis.

Separation of HFB polyamine derivatives

An Hitachi 163 gas chromatograph fitted with a ^{63}Ni EC detector (pulse interval = 50 µsec) was employed in this study. The GLC column of Pyrex glass (150 cm × 3 mm I.D.) was packed with 3% silicon OV-17 on 80—100 mesh Chromosorb W HP. The glass column was silanized before use with a 10% solution of hexamethylenedisilazane in toluene and washed with methanol and acetone. The initial column temperature of 120°C was maintained for 2.5 min. The temperature was then increased at a rate of 15°C/min to a final temperature of 280°C. The temperature of the EC detector was 300°C. Highly pure nitrogen was used as carrier gas at a flow-rate of 60 ml/min. Mass spectra of the HFB derivatives of the polyamines were obtained with a double-focussing Hitachi M-80 mass spectrometer.

RESULTS AND DISCUSSION

Preliminary experiments were carried out to examine the optimal conditions for derivatization of the polyamines with HFBA. For preparing derivatives at the nanogram level, 50 µl of HFBA were sufficient to complete the reaction.

With the use of acetonitrile as solvent, acylation of putrescine, cadaverine, spermidine and spermine was quantitative within 10 min at 65° C. The reaction at room temperature lowered the yield of HFB derivatives and sometimes resulted in additional peaks on the gas chromatogram, possibly due to partial acylation of the amines. Diethyl ether was a good solvent for dissolving HFB polyamine derivatives. Other organic solvents such as benzene, dioxane, pentane, hexane and heptane did not completely dissolve the polyamine derivatives, especially HFB spermine. Washing the ether phase with saturated Na_2CO_3 solution was effectively removing impurities which interfered in the gas chromatogram. HFB polyamines were stable in ether solution at least for several weeks, no decomposition occurred even in the presence of water.

From the structural analysis of the HFB polyamines by gas chromatography—mass spectrometry, it was confirmed that HFB groups were transferred into both primary and secondary amino groups in each polyamine molecule. Molecular ions (M^+) were obtained at *m/e* 480 for HFB putrescine, *m/e* 494 for HFB cadaverine, *m/e* 733 for HFB spermidine, and *m/e* 986 for HFB spermine. Excess acylation of the primary amino groups did not occur under the experimental conditions.

For the conversion of the polyamines to volatile and EC-sensitive derivatives, other fluorinated acylating reagents have also been studied. Derivatization with trifluoroacetic anhydride was unsuitable for the ECD technique since trifluoroacetic derivatives of putrescine and cadaverine showed extremely low sensitivity to the EC detector. In the case of pentafluoropropionic anhydride, the by-product pentafluoropropionic acid was a serious source of interference as pointed out by Rattenbury et al. [14], because this acid eluted close to spermidine. Pentafluoropropionic imidazole has the advantage of not releasing pentafluoropropionic acid into the reaction mixture, but it was not practical for the purpose of routine analyses of polyamines, since special care is needed in the handling and storage of this reagent due to its sensitivity to moisture. In the case of HFBA, HFB acid was produced by hydrolysis during the reaction, but did not interfere with the elution of the polyamine derivatives under the conditions described in this paper and the HFB polyamine derivatives showed the most excellent EC sensitivity among the acylating reagents.

Fig. 1 shows an elution profile of a standard mixture of the HFB derivatives of putrescine, cadaverine, spermidine and spermine. Each peak represents 1 pmol of polyamine. As might be expected from the numbers of HFB groups incorporated into each polyamine molecule, HFB spermidine and HFB spermine gave higher detector responses than those of the diamines. Symmetrical peaks and good separations of the HFB polyamine derivatives were obtained on a column of 3% silicon OV-17 coated on Chromosorb W HP. Other liquid phases tested, including 2% OV-25, 3% OV-225, 5% SE-30 and 10% Apiezon grease M, were found to be unsuitable because of unsatisfactory separations, or broad and asymmetrical peaks.

Fig. 2 shows calibration curves of the HFB derivatives. Peak height ratios of polyamine to internal standard, 1,5-diamino-3-azapentane, were plotted against the amount of each polyamine added to the reaction mixture. Linear relationships were obtained for up to 2 pmol of putrescine and cadaverine, and 1 pmol

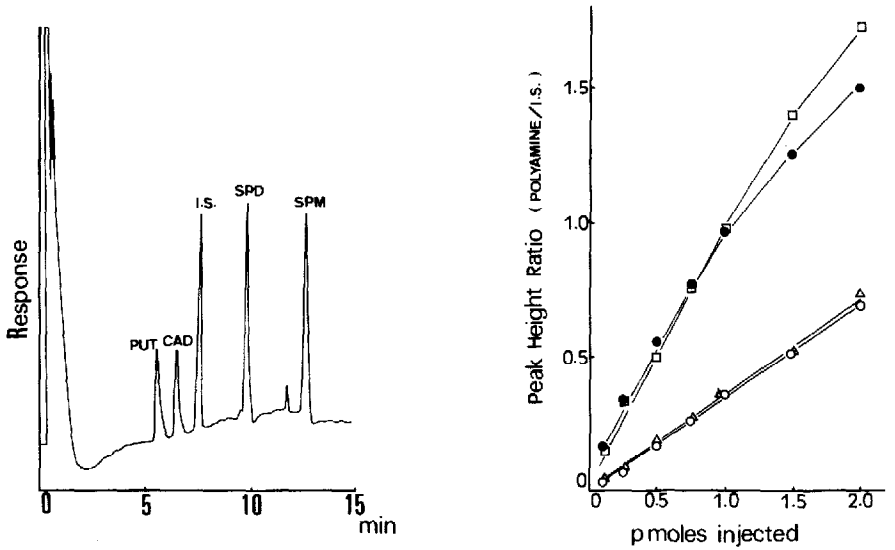

Fig. 1. Gas chromatogram of a standard mixture of polyamines. The polyamines were derivatized with HFBA as described in the text. Column: 3% OV-17 on 80—100 mesh Chromosorb W HP, 150 cm × 3 mm I.D. glass. Nitrogen gas flow-rate: 60 ml/min. Instrumental conditions: initial temperature 120°C, delay 2.5 min, 15°C/min, and final temperature 280°C. Detector: ^{63}Ni electron-capture detector, 300°C at a pulse interval of 50 μsec. Each peak represents 1 pmol of putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM). I.S.: 1,5-diamino-3-azapentane. The peak between SPD and SPM is a spurious peak eluted from the OV-17 column.

Fig. 2. Calibration curves for HFB polyamine derivatives. The analytical conditions of ECD—GLC are given in the legend of Fig. 1. (○), Putrescine; (△), cadaverine; (●), sperimidine; (□), spermine.

of spermidine and spermine. The detector response of HFB spermine declined sometimes because of adsorption of the derivative on the surface of the stationary phase or the glass column. The recovery of HFB spermine was improved by employing a column conditioner Silyl-8 (Pierce Chemical Co., Rockford, IL, U.S.A.) prior to the application of the samples into the GLC column. The detection limit of the method was 0.1 pmol for putrescine and cadaverine, 0.02 pmol for spermidine and spermine.

Generally, a major portion of the free spermidine and spermine of whole blood is found in the erythrocytes [15, 16]. Clinical attention has recently been focussed on the possible usefulness of the analysis of polyamines in this blood component, since in cancer patients abnormally high polyamine concentrations were detected more readily in the blood cells than in plasma [17—19]. In view of the planned medical application of our ECD—GLC technique, the method was applied to polyamine determinations in the erythrocyte fraction of human blood.

In the analysis of human blood, the separation of the polyamines from other compounds, especially amino acids, is a prerequisite, because HFBA readily reacts not only with polyamines but also with compounds such as phenols, alcohols, and amino acids, to form EC-sensitive derivatives. The usual clean-up method using a cation-exchange column [11, 14, 20] was unsuccessful

because of the incomplete removal of EC-sensitive materials in the sample solution. Pre-treatment of the acid extract with an activated Permutit was found to be a useful method. Permutit particles adsorbed polyamines over a wide pH range. The polyamines were easily eluted from the Permutit particles by shaking with a 40% solution of dimethylamine in methanol. Methylamine and trimethylamine solutions were less effective. The recovery of each polyamine during the batchwise Permutit treatment is shown in Table I. Major interfering substances could be removed by this simple procedure.

TABLE I

RECOVERY OF POLYAMINES FROM PERMUTIT WITH 40% DIMETHYLAMINE–METHANOL SOLUTION

	Recovery* (%)
Putrescine	96.9 ± 2.4
Spermidine	95.8 ± 3.5
Spermine	88.7 ± 4.0

*Mean ± S.E.M. (n = 12).

Fig. 3 is a gas chromatogram of a typical blood sample. The concentration of putrescine was considerably lower than that of spermidine and spermine; cadaverine was not detected in healthy subjects. Total recovery of the polyamines was examined using erythrocyte fractions. From the analysis of samples with and without polyamines (putrescine 20 nmol; spermidine 10 nmol; spermine 10 nmol) added to erythrocytes, the recovery of putrescine, spermidine and spermine was determined to be 73–81%, 72–98% and 59–72%, respectively.

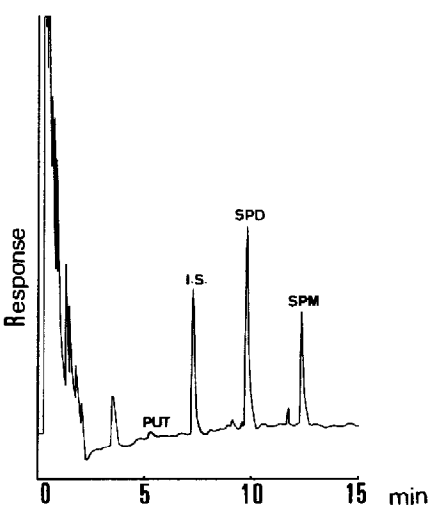

Fig. 3. Gas chromatogram of the polyamines isolated from human erythrocytes. The erythrocyte fraction was prepared from normal human blood. The polyamines were extracted, separated, and converted to HFB derivatives as descibred in the Experimental section. PUT, putrescine; SPD, spermidine; SPM, spermine. I.S., 1,5-diamino-3-azapentane.

The spermidine and spermine concentrations, and spermidine/spermine ratios in the erythrocytes from normal subjects (11 males, age 22—43 years) are listed in Table II. Alterations of the spermidine/spermine ratio in whole blood or erythrocytes were observed in patients with various diseases [21]. The spermidine/spermine ratio is therefore frequently used as an indicator for characterizing abnormalities. In the present investigation, the mean spermidine/spermine ratio in the erythrocytes of healthy subjects was 1.66. Despite the different methods employed for the extraction, pre-separation, and determination of the polyamines, this value was similar to those reported by other investigators [15, 16, 18, 22], indicating the applicability of the present ECD—GLC method to blood polyamine analysis.

TABLE II

SPERMIDINE AND SPERMINE CONCENTRATIONS IN ERYTHROCYTES FROM HEALTHY HUMAN VOLUNTEERS

Sample	nmol/ml packed cells		Spermidine/spermine ratio
	Spermidine	Spermine	
1 Y.T.	8.0	5.6	1.43
2 S.F.	8.4	3.8	2.21
3 T.K.	15.6	12.2	1.28
4 N.Y.	8.2	5.0	1.64
5 N.O.	14.4	11.3	1.27
6 C.T.	11.5	8.9	1.29
7 S.S.	15.5	7.0	2.21
8 T.N.	16.6	8.5	1.95
9 S.F.	18.6	8.6	2.16
10 K.O.	7.6	7.6	1.00
11 H.N.	13.9	7.7	1.81
Mean ± S.D.	12.6 ± 3.8	7.8 ± 2.4	1.66 ± 0.41

The high degree of sensitivity of our analytical method enabled the quantitative determination of polyamines in small blood samples. It should be noted, however, that special care is needed to avoid contamination with EC-sensitive materials from various sources during the experimental procedure. The rapid and accurate analysis of polyamines in extracellular fluids has significant clinical utility in the diagnosis and evaluation of patients with various diseases [23]. The assay procedure presented in this paper is thought to be suitable for routine assays of polyamines in biological fluids. The method has several advantages: a rapid pre-separation method, easy derivatization with HFBA, excellent sensitivity of EC detection, and a fast and reproducible gas chromatographic separation.

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