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# GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF URINARY ACETYLPOLYAMINES

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## SUMMARY

A gas chromatographic method was developed for the determination of monoacetylputrescine, monoacetylcadaverine, $N^1$-acetylspermidine and $N^8$-acetylspermidine in human urine. The amines were isolated from urine by silica gel column chromatography. 1,10-Diaminodecane was used as internal standard. The amines were reacted with ethyl chloroformate in aqueous medium to four ethyloxycarbonyl derivatives prior to application to gas chromatography using a flame ionization detector. Separation and determination of the derivatives were carried out on a Uniport HP column (1.0 m) impregnated with 0.5% SP-1000 under temperature-programmed conditions. The monoacetylpolyamines could be measured accurately at the nanomole level. The method was used for the determination of the monoacetylpolyamines in urine of healthy volunteers. The values obtained were in the range of the published data.

## INTRODUCTION

The importance and clinical significance of polyamine determinations in human patients for diagnosis and biochemical monitoring of the progression of neoplastic diseases for the evaluation of the efficacy of chemotherapy have been reviewed [1—3]. In most studies evaluating the relationship between cancer and urinary polyamine excretion, the urine was hydrolyzed with 6 *N* hydrochloric acid prior to analysis [1]. However, the polyamines are excreted in the urine both of normals and cancer patients predominantly as their monoacetylated derivatives [4—9] such as monoacetylputrescine, $N^1$-acetylspermidine and $N^8$-acetylspermidine. The increase of monoacetylpolyamine levels in the urine of cancer patients [7—10] is not in contradiction with the earlier observations obtained from the analysis of hydrolyzed urines of cancer patients. Abdel-Monem and co-workers [11, 12] have reported an attractive finding that the ratios of $N^1$-acetylspermidine to $N^8$-acetylspermidine in 24-h urine of

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cancer patients are higher than those of healthy volunteers, and have suggested that the ratio of the two isomeric monoacetylspermidines may provide a more reliable marker for cancer than the urinary concentrations of total polyamines obtained after hydrolysis. This proposal has recently been emphasized by the results obtained from analyses of urines of additional cancer patients [13], although an inconsistent result has been reported [14].

A number of methods are available for the determination of total polyamines in urine hydrolysates [15—22]. For the determination of monoacetylpolyamines in urine pre-separation by ion-exchange [22] or silica gel [13] column chromatography has been applied, followed by dansylation and subsequent thin-layer chromatography [23] or high-performance liquid chromatography (HPLC) [13]. Recently, an HPLC method using a reversed-phase column and post-column derivatization with *o*-phthalaldehyde has been developed [24]. This method excludes the determination of monoacetylputrescine in urine. More recently, two methods based on ion-exchange column chromatographic separations followed by post-column derivatization with *o*-phthalaldehyde have been developed [25, 26]. By the method of Mach et al. [25] it is not possible to separate the two isomeric monoacetylspermidines. Prussak and Russell [26] completely separated all monoacetylpolyamines including monoacetylcadaverine in urine.

To our knowledge, no method using gas chromatography (GC) or gas chromatography—mass spectrometry (GC—MS) exists which permits the separation of the urinary monoacetylpolyamines, especially of the two isomeric monoacetylspermidines. On the other hand, many GC and GC—MS methods suitable for the determination of urinary total polyamines have been reported [7,21,22,27—31].

In the present paper, a GC method for the determination of urinary monoacetylpolyamines and of monoacetylcadaverine is reported which has been developed on the basis of our previous investigations [21,32—34]. The method requires a simple procedure for isolation of monoacetylpolyamines from urine. Silica gel column chromatography was used prior to derivatization with ethyl chloroformate, which was followed by GC separation of the ethyloxycarbonyl (EOC) derivatives.

## MATERIALS AND METHODS

### *Chemicals*

The hydrochlorides of 1,3-diaminopropane, putrescine, cadaverine and spermidine were purchased from Nakarai Chemicals (Kyoto, Japan). 1,10-Diaminodecane was from Sigma (St. Louis, MO, U.S.A.). The monoacetylpolyamine hydrochlorides were prepared in our laboratory according to the method described by Tabor et al. [35].

Ethyl, *n*-propyl, *n*-butyl and isobutyl chloroformates were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Silica gel (Wakogel C-200) was obtained from Wako Junyaku Co. (Osaka, Japan). Before use, it was treated according to the procedure of Grettie et al. [36]. Sodium chloride and anhydrous sodium sulphate were purified as follows to remove contaminants which showed peaks on the gas chromatogram: the salts were dissolved in water and

the solutions extracted three times with an equal volume of chloroform. The resulting solutions were evaporated to dryness in vacuo in an all-glass apparatus. Sodium chloride was dried in an oven at 100°C and sodium sulphate on a clean hot-plate at ca. 200°C. All organic solvents and inorganic chemicals were of the highest purity commercially available. Deionized water was used after distillation in an all-glass apparatus.

*Sample preparation*

Twenty-four-hour urine samples from healthy volunteers ranging in age from 21 to 52 years were collected in glass bottles under toluene. No restrictions of food and fluid intake were imposed on the volunteers. After the 24-h collection was complete, the volume of urine was measured and an aliquot was transferred to a polyethylene bottle and stored frozen until analyzed.

A 5-ml aliquot of the urine sample was taken and mixed with 2.5 ml of the internal standard solution (25 nmol/ml), and then was adjusted to pH 9.0 with 1.0 *N* sodium hydroxide solution. The precipitate was removed by centrifugation. The clear supernatant was transferred to a 10-ml volumetric flask and was brought to volume with water (pH 9.0).

*Isolation of monoacetylpolyamines from urine*

The monoacetylpolyamines were isolated and concentrated from urine by using a silica gel column. The procedure was performed according to the procedure of Grettie et al. [36] with some modifications. The pre-treated silica gel (2.0 g) was suspended in 10 ml of 0.1 *N* hydrochloric acid solution and transferred to a glass column (9 mm I.D.) with a stop-cock, which had a glass-wool plug in the constriction. The column was washed with 20 ml of water. A 4-ml aliquot (corresponding to 2 ml of a 24-h urine collection) was applied to the column, which was then washed with 30 ml of water. The fraction eluted with 35 ml of 0.1 *N* hydrochloric acid solution was collected and was evaporated to dryness at 40°C under vacuum.

*Preparation of derivatives*

The residues of the acetylpolyamine fraction were dissolved in 2 ml of water. These solutions were transferred to reaction vials (50 mm × 21 mm O.D.; Mighty Vial, No. 3, Maruemu Co., Osaka, Japan) with PTFE-lined caps. After addition of 0.5 ml of 10% sodium hydroxide solution and 0.2 ml of ethyl chloroformate, the mixtures were shaken at a frequency of 300 $min^{-1}$ with a shaker for 10 min at room temperature. The resulting EOC derivatives were extracted three times with 2 ml of chloroform after saturation of the reaction mixtures with sodium chloride (1 g). The chloroform layers were collected, taking care to avoid aqueous droplets. The combined extracts were dried over anhydrous sodium sulphate (0.2 g) and evaporated to dryness at 65°C in a gentle stream of nitrogen. At this stage, the excess of reagent was also removed. The residues were dissolved in 50 $\mu$l of ethyl acetate; 4-$\mu$l aliquots of these solutions were analyzed by GC. The alkyloxycarbonyl derivatives other than EOC derivatives were prepared in the same way except that 0.1 ml of each reagent was used instead of ethyl chloroformate.

*Gas chromatography*

GC analyses were carried out on a Shimadzu 4CM gas chromatograph equipped with a flame ionization detector and a temperature programmer. Before packing, the glass column (1.0 m × 3 mm I.D.), the quartz wool which was placed in each end of the column, and the support (100—120 mesh Uniport HP; Gasukuro Kogyo Co., Tokyo, Japan) were silanized using 5% dimethyldichlorosilane in toluene [37]. The column packing, 0.5% SP-1000 on silanized Uniport HP, was prepared in our laboratory by using 1-butanol—chloroform (1:1, v/v) as a coating solvent according to the filtration method [37]. The packed column was conditioned at 280°C for at least 20 h with a nitrogen flow-rate of 30 ml/min. The operating conditions were as follows: nitrogen flow-rate, 40 ml/min; hydrogen flow-rate, 50 ml/min; air flow-rate, 0.8 l/min; injection and detector temperatures, 285°C; oven temperature, programmed to rise linearly at 6°C/min from 150°C to 280°C and held at 280°C for 5 min; chart speed, 0.5 cm/min; sensitivity, $10^2$ (× $10^6$ Ω); range, 4—8 (× 0.01 V).

*Gas chromatography—mass spectrometry*

The mass spectrometer coupled to a gas chromatograph was a Shimadzu LKB 9000, operated under the following conditions: trap current, 60 μA, ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source temperature, 290°C; separator temperature, 285°C. GC analyses were performed under the following conditions: column (1.0 m × 3 mm I.D.), packed with 0.5% SP-1000 on silanized Uniport HP; helium flow-rate, 25 ml/min; oven temperature, programmed from 150°C to 280°C at 6°C/min.

*Preparation of calibration curves and calculation*

Calibration curves for the polyamines and their monoacetyl derivatives in the range 5—25 nmol were constructed using 12.5 nmol of the internal standard. In a series of reaction vials, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the standard solution (25 nmol/ml) were placed, and to each solution 0.5 ml of the internal standard solution (25 nmol/ml) was added. The total volume was made up to 2 ml with water. These mixtures were treated in the same manner as the urine samples. The peak height ratios of the samples to the internal standard were calculated, and plotted against the known quantities of the amines. For the determination of the monoacetylpolyamines in urine, the peak height ratios obtained from urine samples were compared to those of one standard, usually containing 10 nmol of monoacetylcadaverine, $N^1$-acetylspermidine and $N^8$-acetylspermidine and 20 nmol of monoacetylputrescine. This comparison was done for correcting daily variations; the linearity of each calibration curve was found to be reproducible.

*Identification of chromatographic peaks from urine*

Chromatographic peaks from urine samples were identified by co-injecting authentic EOC derivatives and, in some representative samples, by GC—MS of the EOC derivatives obtained from 10 ml of urine. Moreover, the EOC derivatives obtained from some samples were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 4 h and analyzed by GC after re-derivatization in order to evaluate whether overlapping peaks were present at the positions of the EOC derivatives of monoacetylpolyamines.

*Recovery experiment*

To determine recovery of the overall procedure, four 24-h urine specimens were fortified with the standard solutions at the levels of 5, 7.5 and 10 nmol/ml urine. The overall recovery was calculated from triplicate analyses of these samples and of the corresponding urine specimens.

## RESULTS AND DISCUSSION

The reaction conditions for the preparation of the alkyloxycarbonyl derivatives of nine amines including monoacetyl-1,3-diaminopropane, monoacetylputrescine, monoacetylcadaverine, $N^1$-acetylspermidine and $N^8$-acetylspermidine as well as their non-conjugated forms were established on the basis of previous investigations [21,31—33]. At the initial stages of this work, we encountered the problem that the extraction of the alkyloxycarbonyl derivatives of monoacetyl-1,3-diaminopropane, monoacetylputrescine and monoacetylcadaverine from the aqueous reaction mixture was less efficient than that of the other derivatives when diethyl ether was used as solvent. However, this problem was solved by salting out the compounds with sodium chloride and by using chloroform instead of diethyl ether. Preparation of the derivatives could be performed within 20 min without special equipment, and several samples could be treated simultaneously. This derivatization method has, therefore, no obvious disadvantage over the dansylation procedure [13, 22].

The initial objective was to obtain a complete GC separation for each amine and our efforts were, therefore, directed towards testing the applicability of four alkyl chloroformates (ethyl, *n*-propyl, *n*-butyl and isobutyl), which are obtained from commercial sources. Each derivative was prepared from a standard solution containing all amines and was separated by GC using various columns in order to select the most suitable derivative and separation conditions. As a result, it was found that a 0.5% SP-1000 column (1.0 m) provided the desired separations with reasonable retention times. However, spermine could not be eluted from this column. Quantitatively measurable peaks were obtained of the EOC derivatives of all amines at the nanomole level.

A chromatogram showing separation of nine amines is reproduced in Fig. 1. A chromatographic run was completed within 20 min. In the case of urine analyses an additional 5 min were required for elution of other peaks. Formation of isobutyloxycarbonyl derivatives, which was developed by us [21] for the determination of free polyamines in urine hydrolysates and utilized by Bakowski et al. [38] for the analysis of plasma polyamines in the prognosis of response to chemotherapy of tumours, was not suitable for separation of spermidine, $N^1$-acetylspermidine and $N^8$-acetylspermidine.

The structures of all EOC derivatives were verified by GC—MS. Molecular ions with the expected *m/e* were observed for each derivative, suggesting that all primary and secondary amino groups were substituted by ethyloxycarbonyl groups, but the amide NH groups were not.

In order to examine the stability of the EOC derivatives, the amines were derivatized in the nanomole range, dissolved in ethyl acetate and stored at room temperature over a period of two weeks. No decomposition could be observed with any of the compounds, the peak heights remaining identical.

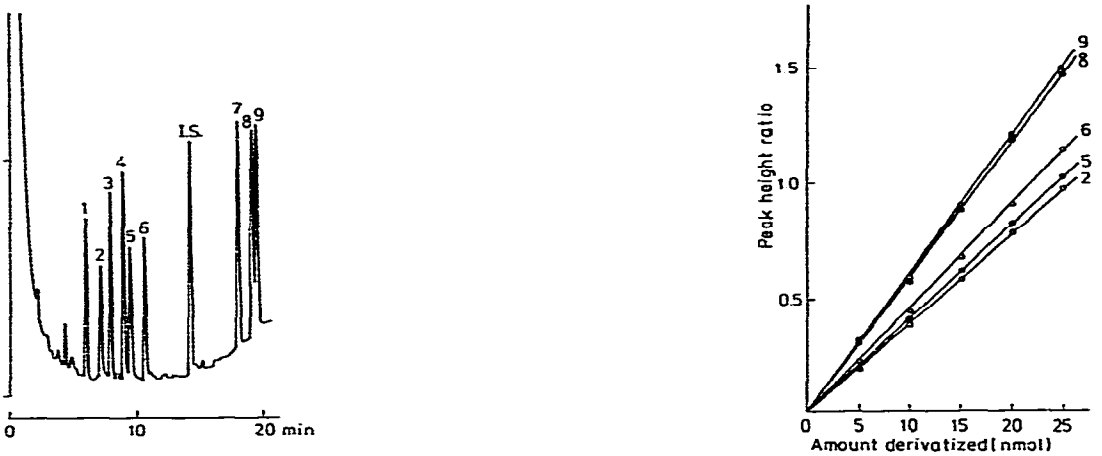


Fig. 1. GC separation of the EOC derivatives of a mixture of standard compounds. Each peak represents approximately 1.2 nmol. Peaks: 1=1,3-diaminopropane; 2=monoacetyl-1,3-diaminopropane; 3=putrescine; 4=cadaverine; 5=monoacetylputrescine; 6=monoacetylcadaverine; 7=spermidine; 8=$N^1$-acetylspermidine; 9=$N^8$-acetylspermidine; I.S.=internal standard (1,10-diaminodecane; this peak represents approximately 1.0 nmol). For details of the GC conditions see the Methods section.

Fig. 2. Calibration graphs for the monoacetylpolyamines. Internal standard: 1,10-diaminodecane, 12.5 nmol. Each line number corresponds to each peak number in Fig. 1.

A variety of compounds were evaluated for use as internal standard. 1,10-Diaminodecane was selected since it showed the same chromatographic behavior as the other amines on the silica gel columns and it did not co-chromatograph with other peaks derived from urine under the GC conditions used.

The calibration curves of the amines were constructed by ethyloxycarbonylating increasing amounts of the amines (5—25 nmol) and a fixed amount (12.5 nmol) of internal standard. Linear relationships were obtained for all amines. In Fig. 2, however, only the calibration curves of monoacetylpolyamines are shown. Each point in this figure represents the mean value obtained from six repetitive analyses of the same amounts of the various amines, and the relative standard deviation of the peak height ratios was 3.2% or less. The same is true for the free polyamines. This suggests that the reproducibility of both derivatization and GC analysis is adequate for quantitative determinations.

On the basis of these results, the applicability of the method in the determination of monoacetylpolyamines in urine was investigated. A clean-up procedure was needed to concentrate the compounds of interest to eliminate interfering urinary compounds. Several column chromatographic methods employing cation-exchange resin [23], silica gel [13, 36] and CM-cellulose [39] were evaluated. The silica gel column chromatography, which was originated by Grettie et al. [36], was found to be the most promising with respect to time requirement, simplicity and selectivity. Monoacetylpolyamines including monoacetylcadaverine were clearly separated from interfering urinary compounds, as is shown in Fig. 3A. Unfortunately, it turned out that a constituent which overlaps with putrescine could not be eliminated under our column chromato-

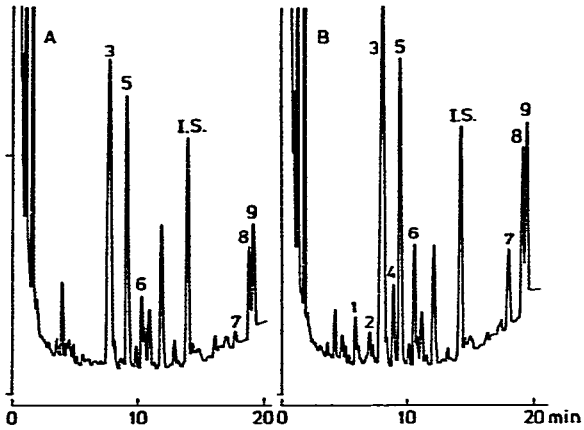


Fig. 3. Representative gas chromatograms obtained from a urine sample (A) and the same urine sample fortified with 5 nmol of each amine (B). Peaks: the same as in Fig. 1. I.S. = internal standard, 12.5 nmol of 1,10-diaminodecane. For details of the GC conditions see the Methods section.

graphic conditions. The peaks corresponding to monoacetylpolyamines were identified by analyzing urine samples fortified with standard solution (Fig. 3B) or by co-injecting authentic EOC derivatives. Further confirmation was carried out by GC—MS of some representative samples. The results showed that all peaks of interest were almost uniform.

Each quintuple of four different urine samples was treated as described in the Methods section in order to evaluate the overall reproducibility of peak height ratios. The results are summarized in Table I. They indicate that the reproducibility of the method is satisfactory. The overall recoveries of monoacetylpolyamines are summarized in Table II. It could be concluded that recovery was almost satisfactory. The overall recovery for cadaverine and spermidine was good. However, the concentrations of these amines were not determined in urine since their amounts were found to be small.

The 24-h excretion of monoacetylpolyamines in urine was estimated with the present method in fifteen normal individuals. The results are summarized in Table III. The concentrations of monoacetylputrescine, $N^1$-acetylspermidine

TABLE I

REPRODUCIBILITY OF PEAK HEIGHT RATIOS

Four different urine samples were treated as described in the text ($n$=5).

| Subject | Ac-Put* | | Ac-Cad | | $N^1$-Ac-Spd | | $N^8$-Ac-Spd | |
|---|---|---|---|---|---|---|---|---|
| | Mean** | S.D.(%) | Mean | S.D.(%) | Mean | S.D.(%) | Mean | S.D.(%) |
| A | 1.232 | 4.4 | 0.211 | 3.8 | 0.427 | 4.2 | 0.468 | 3.2 |
| B | 1.236 | 2.8 | 0.276 | 1.0 | 0.324 | 5.6 | 0.422 | 2.4 |
| C | 2.064 | 2.5 | 0.655 | 2.4 | 0.649 | 5.7 | 0.750 | 4.5 |
| D | 1.399 | 1.6 | 1.474 | 2.4 | 0.476 | 2.7 | 0.419 | 3.1 |

*Abbreviations: Ac-Put, monoacetylputrescine; Ac-Cad, monoacetylcadaverine; $N^1$-Ac-Spd, $N^1$-acetylspermidine; $N^8$-Ac-Spd, $N^8$-acetylspermidine.

**Peak height ratios relative to the internal standard.

TABLE II

OVERALL RECOVERY OF MONOACETYLPOLYAMINES

Polyamines and their monoacetyl derivatives were added to 24-h urine specimens and subsequently these samples were treated as described in the text. Three independent determinations were carried out for each urine specimen. Data are shown for those monoacetylpolyamines which are usually found in urine.

| Compound* | Recovery (%)** | | | | | |
|---|---|---|---|---|---|---|
| | Amount added (nmol/ml urine) | | | | | |
| | 5 | | 7.5 | | 10 | |
| | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range |
| Ac-Put | 84.0 ± 6.2 | 73.6—93.4 | 82.2 ± 8.0 | 75.9—93.4 | 86.6 ± 5.8 | 76.7—95.5 |
| Ac-Cad | 97.8 ± 6.0 | 91.6—104.7 | 92.5 ± 4.2 | 85.2—99.6 | 101.0 ± 5.9 | 92.5—108.6 |
| $N^1$-Ac-Spd | 102.0 ± 4.6 | 95.3—108.5 | 100.5 ± 5.4 | 86.9—106.1 | 101.6 ± 5.0 | 90.6—107.5 |
| $N^8$-Ac-Spd | 101.0 ± 3.9 | 93.4—105.1 | 100.5 ± 5.1 | 95.2—112.3 | 100.9 ± 5.5 | 93.2—108.4 |

*For abbreviations, see footnote to Table I.
**Calculated from the results of four different urine specimens.

TABLE III

24-h URINARY EXCRETION OF MONOACETYLPOLYAMINES IN NORMAL SUBJECTS

| Subject No. | Concentration (μmol per 24 h)* | | | | | |
|---|---|---|---|---|---|---|
| | Ac-Put** | Ac-Cad | $N^1$-Ac-Spd | $N^8$-Ac-Spd | $N^1$-Ac-Spd + $N^8$-Ac-Spd | Ratio*** |
| Male | | | | | | |
| 1 | 14.68 | 1.52 | 3.61 | 3.45 | 7.06 | 1.05 |
| 2 | 9.48 | 1.58 | 2.68 | 2.85 | 5.53 | 0.94 |
| 3 | 18.11 | 5.61 | 4.76 | 5.34 | 10.10 | 0.89 |
| 4 | 24.58 | 2.87 | 6.34 | 3.41 | 9.75 | 1.86 |
| 5 | 11.11 | 0.99 | 2.03 | 1.84 | 3.87 | 1.10 |
| 6 | 12.19 | 7.32 | 2.80 | 3.04 | 5.84 | 0.92 |
| 7 | 13.33 | 0.60 | 3.26 | 2.82 | 6.08 | 1.16 |
| Mean ± S.D. | 14.8 ± 5.1 | 2.9 ± 2.6 | 3.6 ± 1.5 | 3.3 ± 1.1 | 6.9 ± 2.3 | 1.1 ± 0.3 |
| Female | | | | | | |
| 1 | 11.82 | 2.69 | 2.59 | 3.48 | 6.07 | 0.74 |
| 2 | 10.29 | 8.91 | 2.68 | 1.96 | 4.64 | 1.37 |
| 3 | 15.55 | 10.41 | 2.79 | 3.49 | 6.28 | 0.80 |
| 4 | 7.83 | 0.55 | 3.04 | 2.57 | 5.61 | 1.18 |
| 5 | 8.12 | 0.89 | 2.18 | 1.82 | 4.00 | 1.20 |
| 6 | 14.67 | 1.93 | 3.36 | 3.09 | 6.45 | 1.09 |
| 7 | 7.98 | 13.94 | 4.76 | 3.00 | 7.76 | 1.59 |
| 8 | 11.58 | 9.44 | 3.66 | 2.37 | 6.03 | 1.54 |
| Mean ± S.D. | 11.0 ± 3.0 | 6.1 ± 5.2 | 3.1 ± 0.8 | 2.7 ± 0.6 | 5.9 ± 1.1 | 1.2 ± 0.3 |

*Mean values of duplicate determinations.
**For abbreviations, see footnote to Table I.
***Ratios of $N^1$-Ac-Spd to $N^8$-Ac-Spd.

and $N^8$-acetylspermidine obtained in this study are in the same range as those published previously [11,14,23]. The ratios of $N^1$-acetylspermidine to $N^8$-acetylspermidine are also in agreement with the reported values, the mean value being close to one. The concentrations of monoacetylcadaverine in some samples were considerably elevated compared with the results of Abdel-Monem et al. [11]. These samples were hydrolyzed with 6 *N* hydrochloric acid at 110°C for 4 h, and the hydrolysates were re-derivatized and analyzed. The results suggest that the peak corresponding to monoacetylcadaverine was uniform in our GC. This was further confirmed by GC—MS; the spectra obtained from the urine samples were identical with those obtained from the authentic EOC derivative of monoacetylcadaverine. It has been pointed out that cadaverine is presumably produced by bacteria in the lumen of the large intestine from dietary protein or lysine [5]. It is likely that the observed differences are due to differences in the intestinal bacterial flora and/or the diet taken by volunteers. However, it is not clear which organ is responsible for the formation of monoacetylcadaverine.

## CONCLUSSION

In the present work we have described a GC method for the determination of urinary monoacetylpolyamines. This is the first GC method which permits the separation of $N^1$-acetylspermidine and $N^8$-acetylspermidine. The method includes three steps: isolation of the monoacetylpolyamine fraction from urine, derivatization with ethyl chloroformate and determination by GC. The clean-up step using a silica gel column [36] is obligatory for the determination of urinary monoacetylpolyamines since urine contains many interfering substances. The preparation of the EOC derivatives can be carried out rapidly and simply, and no precaution is necessary in the handling and storage of these derivatives. A GC run takes 25 min.

The sensitivity of the method is modest compared with the method using fluorescence detection. However, the method is sufficiently applicable to the analysis of urinary monoacetylpolyamines. The reproducibility of the method for the measurement of monoacetylpolyamines in urine samples was reasonable, the relative standard deviations ranging from 1.0 to 5.7% (Table I). It was observed that the recoveries of monoacetylputrescine varied somewhat depending on the urine specimen, although individual specimens gave constant recoveries. This reflects the relatively large standard deviations of monoacetylputrescine (Table II). There are two serious limitations to the method: putrescine determination in urine is hampered by interfering peaks, and spermine can not be eluted from the GC column that is suitable for the analysis of other amines.

We believe that the method described here can be considered as an alternative routine method in biochemical and clinical research requiring the analysis of urinary monoacetylpolyamines.

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